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MOLECULAR DETECTION OF *BABESIA* SPP. IN TICKS IN NORTHERN SERBIA

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Abstract - In order to evaluate the prevalence rate of *Babesia* spp. in ticks collected from vegetation at seven localities in northern Serbia, tick samples were subjected to molecular analysis. A total of 132 unfed adult ticks of five different species (*Dermacentor marginatus*, *Dermacentor reticulatus*, *Ixodes ricinus*, *Haemaphysalis concinna* and *Haemaphysalis punctata*), were examined by PCR for the presence of *Babesia* spp. Out of the analyzed ticks, 10.61% (14/132) were positive for babesial DNA. The presence of babesiae was found at the localities Pančevački Rit, Titov Gaj, Makiš, PKB and Kljajićevo. Prevalence in *D. reticulatus* ticks was 21.57% (11/51) and in *H. concinna* ticks, 8.57% (3/35). Sequencing and phylogenetic analysis showed a clustering of the obtained sequences with those of *B. canis* from the GenBank database. These results add to the knowledge of the distribution of babesial pathogens and their vectors in Serbia.

Key Words: Ticks, PCR, *Babesia* spp., Serbia

INTRODUCTION

Babesial parasites (and those of the closely related genus *Theileria*) are some of the most ubiquitous and widespread blood parasites in the world, after trypanosomes, and as such have considerable worldwide economic, medical, and veterinary impact (Homer et al., 2000). The babesiae are transmitted by ixodid ticks and are capable of infecting a wide variety of vertebrate hosts as reservoirs in maintaining the enzootic transmission cycle, while in susceptible species they bring about disease (Homer et al., 2000; Etkind et al, 1980; Spielman et al., 1981).

Many babesial species have been detected in domestic animals (*Babesia bovis*, *B. equi*, *B. bigemina*, *B. ovis*, *B. canis*, *B. felis*), while *B. microti* and *B. divergens* are the most common human pathogens. The first case of human babesiosis was documented in former Yugoslavia in 1957 (Škrabalo and Deanović, 1957). Babesial infections in humans generally follow regional distributions. Cases in the United States are caused primarily by *B. microti*, whereas in Europe they are usually caused by *B. divergens* (Quick et al., 1993; Thomford et al., 1994). Both animal and human babesiosis are recognized as emerging zoonoses.

Transovarial transmission is a characteristic of the *Babesia* genus and in addition to transstadial transmission, ensures persistence within the tick vector, sometimes over several tick generations (Chauvin et al., 2009).

At least six genera within the Ixodidae family serve as babesial vectors, experimental or natural (Homer et al., 2000). As regards Europe, *Ixodes ricinus* is the vector for *B. divergens*, *B. divergens*-like species, *B. microti*, *B. bovis* and recently described

Babesia sp. strain EU1 (Bonnet at al., 2007a; Bonnet et al., 2007b; Duh et al., 2001; Hilpertshauser et al., 2006; Milutinović et al., 2012). *Dermacentor reticulatus* transmits *Babesia canis* (Ionita et al., 2012), *Haemaphysalis punctata* serves as a vector for *B. major* and *B. motasi*, *Rhipicephalus sanguineus* for *B. gibsoni* and *B. canis*, *Hyalomma marginatum marginatum* transmits *B. caballi* (Milutinović et al., 2012), and finally *Ixodes hexagonus* is a suspected vector for a *B. microti*-like agent, also known as *Theileria annae* (Camacho et al., 2003).

The prevalence of *Babesia* spp. in different tick species in Europe is in the range of 0.10% to 0.90% in northern Poland (Cieniuch et al., 2009), 0.85% in northern Italy (Cassini et al., 2010), through 2.30% in southwest and 14.70% in eastern Slovakia (Kubelová et al., 2011), 2.70% in Luxembourg (Reye et al., 2010), 29.90% in Hungary (Földvári et al., 2007) up to 51.70% in Austria (Blaschitz et al., 2008). Moreover, Welc-Falęciak et al. (2008) pointed out that 3.40% and 5% of *I. ricinus* larvae and nymphs, respectively, as well as 12% of *D. reticulatus* larvae and 4% of nymphs, collected from rodents, have been DNA positive for *B. microti* in northeastern Poland.

Several studies, based on PCR detection of babesial DNA have provided data about the prevalence of *Babesia* spp. in various mammalian hosts. The *Babesia* EU1 prevalence of up to 30% in roe deer (*Capreolus capreolus*) in Slovenia indicates suggests that this species is a possible reservoir (Duh et al., 2005). The prevalence of *B. microti* in rodents in northeastern Poland ranges from 11.90% to 35% (Welc-Falęciak et al., 2008). In Greece, 15% of analyzed goats and sheep were infected with *B. ovis* (Theodoropoulos et al., 2006). All of the eight clinically suspected dogs in Portugal, found to be infected with *B. canis* by blood-smear microscopy, were confirmed positive by PCR (Cardoso et al., 2008). Similarly, in Poland 90.80% of naturally infected dogs were PCR-positive for *B. canis canis* (Adaszek and Winiarczyk, 2008). Based on clinical, microscopic and molecular investigations, 5.90% of dogs analyzed were determined as being infected with babesiae in Slovenia (Duh et al., 2004). In Croatia, *B. canis canis* was detected in 69%, *B. gibsoni* in 21%, *B. canis vog*eli in 7% and *Theileria annae* in 3% of babesial positive asymptomatic dogs (Beck et al., 2009). In Hungary, molecular analysis of blood samples from dogs with clinical signs of babesiosis, revealed the presence of *Babesia* spp. DNA in 88.60% (Földvári et al., 2005). In Romania, 12 of 16 tested dogs, both clinically affected and normal, were positive for *Babesia* spp. (Ionita et al., 2012). A study in Albania revealed that 23% of clinically healthy dogs were positive for *B. canis* DNA, while IFAT showed a lower prevalence – 13% (Hamel et al., 2009).

Previous studies conducted in Serbia analyzed the presence of *B. canis* in pet dogs and ticks collected from these dogs, by microscopic method. Babesiae were detected in *R. sanguineus*, *Dermacentor marginatus* and *D. reticulatus* ticks with a prevalence of 66.10%, 18.70% and 46.40%, respectively (Pavlović et al, 2002; Pavlović et al., 2009).

As a step forward, the aim of this study was the molecular detection and characterization of *Babesia* spp. in unfed adult Ixodid ticks (Acari: Ixodidae) collected from several localities in northern Serbia, in order to provide a better understanding of the epidemiological and epizootiological situation in this part of the country.

MATERIALS AND METHODS

Ticks were collected in 2007 and 2009 from six localities (Padinska Skela PKB, Kovilovo, Jabučki Rit, Pančevački Rit, Makiš, Titov Gaj) situated in the Belgrade region and one locality (Kljajićevo) in the province of Vojvodina (Fig. 1). Samples were collected by flagging over the surface, which represented a mixture of forest-steppe, agro-ecosystems (Padinska Skela PKB, Kovilovo, Jabučki Rit, Pančevački Rit and Kljajićevo) and recreational sites (Makiš and Titov Gaj) with permanent anthropogenic influence.

A total of 132 adult ticks was chosen as a sample of convenience for further analysis. All ticks were morphologically identified to the species level by using existing standard taxonomic keys (Pomerancev, 1950), and split into different Eppendorf test tubes

Fig. 1. Map of North Serbia with tick collection sites (1 – Kljajićevo, 2 – PKB, 3 – Kovilovo, 4 – Jabučki Rit, 5 – Pančevački Rit, 6 – Makiš, 7 – Titov Gaj)

according to species and collection region. Each tick was washed in 70% alcohol, rinsed in sterile water and dried on sterile filter paper. Consequently, they were triturated individually in 500 µl of PBS, into sterile tubes in a laminar flow hood; a portion of the homogenized ticks in PBS was used to make pools according to species and collection region. Each pool contained 2-11 ticks, in a final volume of 200 µl. All samples were stored at -80°C until DNA extraction.

All DNA extractions were carried out using the QIAamp DNA Tissue kit (QIAGEN- Germany) according to the manufacturer's recommendations. DNA extracts were stored at -20°C until use in PCR amplification.

The detection of *Babesia* spp. DNA in the pools was performed by PCR amplification, using BABF and BABR primers targeting a 146 bp portion of the 18S rRNA gene sequence (Theodoropoulos et al., 2006). In the case of a positive pool, DNA from the original homogenates was extracted and samples were tested individually. Isolation of amplified DNA from the gel was performed by DNA Extraction Kit (Fermentas, Vilnus, Lithuania) and purification by QIAquick PCR Purification Spin Kit (Qiagen, Germany). PCR products were directly sequenced (Macrogen Inc., Seoul, South Korea). Sequencing revealed six sequences, which were processed using nucleotide Blast (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST) and Chromas v1.49, and were aligned using ClustalW. Two sequences were submitted at the GenBank database under accession numbers HM629446 and HM629447.

The Lasergene Ver.7.1 software (DNASTAR Inc., Madison, WI, USA) was used to align DNA sequences with the 18S rRNA sequences of various babesial species available in the GenBank: *B. divergens* (FJ944826), *B. divergens* (EF458228), *B. capreoli* (FJ944828), *B. ovis* (DQ287954), *B. ovis* (AY533146), *B. bigemina* (EF458199), *B. bigemina* (EF458204), *B. caballi* (AY534883), *B. canis* (L19079), *B. canis* (AY272047), *B. felis* (AY452707), *B. felis* (AY452697), *B. microti* (EF413181), *B. microti* (AB197940), *B. lengau* (GQ411416), *B. bovis* (L19077), *B. bovis* (EF458213), and *Plasmodium falciparum* (M19172) as an outgroup. Alignment was performed using ClustalW (Thompson et al., 1994). Phylogenetic distances were calculated using the neighbor-joining method. The phylogenetic tree was drawn using N-J plot software.

RESULTS

A total of 132 unfed adult ticks, of five species, namely, *D. marginatus* (2 males and 13 females), *D. reticulatus* (16 males and 35 females), *I. ricinus* (11 males and 18 females), *Haemaphysalis concinna* (18 males and 17 females) and *Haemaphysalis punctata* (1 male and 1 female), were chosen for analysis. The ticks were collected from seven localities in northern Serbia (Fig. 1, Table 1).

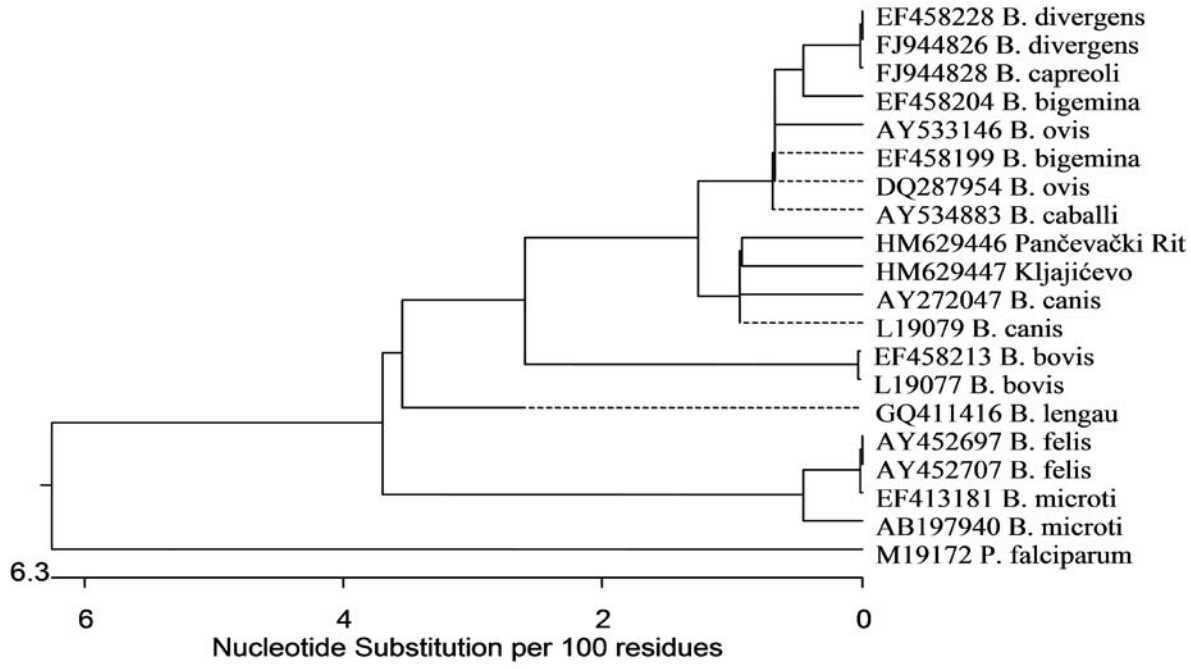

Fig. 2. Phylogenetic tree based on partial sequences of *Babesia* 18S rRNA gene obtained in this study and those deposited in GenBank (accession numbers are indicated next to each sequence). *Plasmodium falciparum* was used as a outgroup. Phylogenetic distances were calculated using the neighbour-joining method.

Out of 132 ticks, 10.61% (14/132) were positive for babesial DNA. Prevalence in *D. reticulatus* ticks was 21.57% (11/51) and in *H. concinna* 8.57% (3/35). The presence of babesiae was revealed at the Pančevački Rit, Titov Gaj, Makiš, PKB and Kljajićevo localities (Table 1).

Out of six sequences obtained, five showed similarities from 99.10% to 100% (Pančevački Rit, PKB, Titov Gaj and Makiš), while the sequence from Kljajićevo was unique with similarity from 98.20% to 99.10%. Genetic similarity analysis showed clustering of the obtained sequences in this study with those of *B. canis*, from the GenBank database (Fig. 2).

DISCUSSION

In most European countries, the occurrence of *B. canis* is in accordance with the geographical distribution of its vector, and it is one of the most important tick-transmitted infectious diseases of dogs in Europe (Ionita et al., 2012; Lobetti, 1998). Instead of sporadic findings of *B. canis* in other tick species (Cieniuch et al., 2009), *Dermacentor reticulatus* ticks are known to be vectors of this pathogen in Europe (Janisch, 1986; Ionita et al., 2012), and our study provides additional data about their potential role as vectors in the transmission cycles of *B. canis* in northern Serbia. In Serbia, this tick species, previously known as *Dermacentor pictus*, can be found more often in Vojvodina and the Belgrade area, while their distribution in the rest of the country is sporadic (Petrović, 1979; Milutinović, 1992; Tomanović, 2009). Expansion of this species has been recorded in Hungary and Germany (Földvári et al., 2007).

Relative abundance analysis revealed that the species *Dermacentor reticulatus* was absolutely dominant (38.64%) among the analyzed ticks in this part of Serbia, followed by *H. concinna* (26.52%), *I. ricinus* (21.97%), *D. marginatus* (11.36%) and *H. punctata*

Table 1. Prevalence of *Babesia* spp. in ticks from northern Serbia

Tick species Locality	*Dermacentor marginatus*	*Dermacentor reticulatus*	*Ixodes ricinus*	*Haemaphysalis concinna*	*Haemaphysalis punctata*
	No. positive / no. examined				
Jabučki Rit	0/1	0/1			
Pančevački Rit	0/10	1/2			
Titov Gaj	0/1	1/2	0/18		
Makiš		5/11			
PKB		4/17			
Kovilovo		0/12	0/1		
Kljajićevo	0/3	0/6	0/10	3/35	0/2
Total	0/15	11/51 (21.57%)	0/29	3/35 (8.57%)	0/2

(1.52%). The presence of *B. canis* DNA was detected in analyzed ticks from five out of the seven localities, on both agricultural and recreational sites.

The Belgrade area is known as a focus of canine babesiosis (Pavlović et al., 2002). Microscopic analysis of the midgut contents of ticks in this region showed the presence of *B. canis* in engorged *R. sanguineus*, *D. marginatus* and *D. reticulatus* ticks collected from dogs with clinical signs of babesiosis (Pavlović et al., 2009). The prevalence of *B. canis* in *D. reticulatus* ticks obtained in that study was 46.40%, while in our research prevalence was 21.57% for the same tick species. Although molecular methods are more sensitive than microscopic ones, the lower prevalence detected in our study is in harmony with the observation of Földvári et al. (2007) that ticks collected from symptomatic dogs brought to veterinary clinics show higher prevalence than unfed ticks. The prevalence of infected unfed ticks is lower, but represents more accurate data for epidemiology research and disease risk assessment.

However, the prevalence of infected unfed *D. reticulatus* ticks in our study is significantly higher than prevalences published by Rar et al. (2005a) – 3.60% and Rar et al. (2005b) – 4.20%, both in Siberia, and Kubelová et al. (2011) – 2.30-14.70% and Duh et al. (2006) – 1%, both in Slovakia. The finding of *Babesia* ssp. in *D. reticulatus* ticks poses a bigger threat to animal than to human health, since feeding rarely occurs on humans. Adult *D. reticulatus* ticks feed on larger wild and domestic mammals, and in Serbia, most often on dogs. This species also transmits agents of babesiosis to cattle, horses and sheep (Milutinović et al., 2012). A higher prevalence of infected ticks correlates to a more elevated disease risk and although the occurrence of dog babesiosis declined in the Belgrade area in the period 1997-2004 (Pavlović et al., 2009), data obtained in this study point out the need for constant population monitoring of this tick species.

Recent findings indicate that *D. reticulatus* ticks carrying *Francisella*-like endosymbiont (FLE) may have a higher vector potential for *B. canis*, but further research is necessary to confirm this (Tomanović et al., in press).

The Kljajićevo locality was the only one where babesial DNA-positive *H. concinna* ticks were found. To our knowledge, this was a unique finding of the presence of *B. canis* in this species. This and other data about the presence of various pathogens in this tick indicate its potential epidemiological and epizootiological significance in this area (Tomanović

et al., in press). Adult ticks of this species usually feed on large and medium large mammals, wild or domestic. Adults and nymphs can also be found on humans (Milutinović et al., 2012). The possible role of *H. concinna* ticks in maintenance of the enzootic cycle of *B. canis* requires further research.

Six different 146 bp-long babesial 18S rDNA sequences were obtained. Sequences from Pančevački Rit, Titov Gaj and one from PKB were identical, as well as sequences from Makiš and the PKB locality. The sequence from Kljajićevo was unique, showing 98.20% and 99.10% similarity with the previous two, respectively. The fact that the sequence from Kljajićevo was obtained from *H. concinna* as the first detection of *Babesia* spp. in this tick species, implicates further detailed molecular characterization.

Ixodes ricinus is one of the most widely distributed and abundant tick species in Serbia (Petrović, 1979; Milutinović, 1992; Milutinović and Radulović, 2002). Adults feed on large wild mammals and almost all domestic animals, as well as birds (Milutinović et al., 2012). Nymphs and adults can often be found on humans (Estrada-Peña and Jongejan, 1999; Hubálek et al., 2004). No *Babesia* DNA-positive *I. ricinus* ticks were detected during our study, probably due to the small number of analyzed ticks, taking into account the low prevalence rates (0.10-5%) of *Babesia* spp. in *I. ricinus* ticks obtained in several studies throughout in Europe (Cieniuch et al., 2009; Reye et al., 2010; Cassini et al., 2010; Welc-Falęciak et al., 2008). Moreover, the presence of *Babesia* spp. in *I. ricinus* ticks at these localities could not be excluded.

Our detection of *B. canis* in ticks from the Kljajićevo locality represents the most northern finding of this pathogen in Serbia. In Hungary, the presence of babesiae in regions where babesiosis have not been reported earlier as been noted (Földvári et al., 2007). In Romania, an increased prevalence of dog babesiosis has been registered in the last years (Ionita et al., 2012).

Investigations of tick fauna and pathogen prevalence rates in ticks as vectors are essential for risk assessment and adequate prevention of tick-transmitted diseases. Although Fritz (2009) claimed that no canine *Babesia* spp. seemed to be zoonotic, cases of human babesiosis in France caused by *B. canis* (Marsaudon et al., 1995) indicate the possible crossing of species barriers and its zoonotic potential. The molecular approach in the detection of *Babesia* spp., as a sensitive and specific tool, has been used for the first time in Serbia in this study. The data on the presence of *B. canis* in *D. reticulatus* and *H. concinna* ticks in the Belgrade region and northern Serbia provide better insight into the transmission cycles and maintenance of babesiae in Serbia. Further studies need to be carried out to eliminate knowledge gaps in the ecology, epizootiology, and epidemiology of babesiosis in Serbia.

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REFERENCES

Adaszek, L., and *S. Winiarczyk* (2008). Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. *Vet. Parasitol.* **152**, 235-241.

Beck, R., Vojta, L., Mrljak, V., Marinculić, A., Beck, A., Živičnjak, T., and *S. M. Cacciò* (2009). Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int. J. Parasitol.* **39**, 843-848.

Blaschitz, M., Narodoslavsky-Gföller, M., Kanzler, M., Stanek, G., and *J. Walochnik* (2008). *Babesia* species occurring in Austrian *Ixodes ricinus* ticks. *Appl. Environ. Microb.* **74**, 4841-4846.

Bonnet, S., Jouglin, M., Malandrin, L., Becker, C., Agoulon, A., L'Hostis, M., and *A. Chauvin* (2007a). Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed on infected blood in a new skinfeeding technique. *Parasitology* **134**, 197-207.

Bonnet, S., Jouglin, M., L'Hostis, M., and *A. Chauvin* (2007b). *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. *Emerg. Infect. Dis.* **13**, 1208-1210.

Camacho, A. T., Pallas, E., Gestal, J. J., Guitián, F. J., Olmeda, A. S., Telford, S. R., and A. Spielman (2003). *Ixodes hexagonus* is the main candidate as vector of *Theileria annae* in northwest Spain. *Vet. Parasitol.* **112**, 57-63.

Cardoso, L., Costa, A., Tuna, J., Vieira, L., Eyal, O., Yisaschar-Mekuzas, Y., and *G. Baneth* (2008). *Babesia canis canis* and

Babesia canis vogeli infections in dogs from northern Portugal. *Vet. Parasitol.* **156**, 199-204.

Cassini, R., Bonoli, C., Montarsi, F., Tessarin, C., Marcer, F., and *R. Galuppi* (2010). Detection of *Babesia* EU1 in *Ixodes ricinus* ticks in northern Italy. *Vet. Parasitol.* **171**, 151-154.

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., and *L. Malandrin* (2009). Babesia and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet. Res.* **40**, 37.

Cieniuch, S., Stańczak, J., and *A. Ruczaj* (2009). The first detection of *Babesia* EU1 and *Babesia canis canis* in *Ixodes ricinus* ticks (Acari, Ixodidae) collected in urban and rural areas in Northern Poland. *Pol. J. Microbiol.* **58**, 231-236.

Duh, D., Petrovec, M., and *T. Avsic-Zupanc* (2001). Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). *J. Clin. Microbiol.* **39**, 3395-3397.

Duh, D., Tozon, N., Petrovec, M., Strašek, K., and *T. Avšič-Županc* (2004). Canine babesiosis in Slovenia: Molecular evidence of *Babesia canis canis* and *Babesia canis vogeli*. *Vet. Res.* **35**, 363-368.

Duh, D., Petrovec, M., Bidovec, A., and *T. Avsic-Zupanc* (2005). Cervids as *Babesiae* hosts, Slovenia. *Emerg. Infect. Dis.* **11**, 1121-1123.

Duh, D., Slovák, M., Saksida, A., Strašek, K., Petrovec, M. and *T. Avšič-Županc* (2006). Molecular detection of *Babesia canis* in *Dermacentor reticulatus* ticks collected in Slovakia. *Biologia, Bratislava* **61**, 231-233.

Estrada-Peña, A., and *F. Jongejan* (1999). Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. *Exp. Appl. Acarol.* **23**, 685-715.

Etkind, P., Piesman, J., Ruebush II, T. K., Spielman, A., and *D. D. Juranek* (1980). Methods for detecting *Babesia microti* infection in wild rodents. *J. Parasitol.* **66**, 107-110.

Földvári, G., Hell, E., and *R. Farkas* (2005). *Babesia canis canis* in dogs from Hungary: detection by PCR and sequencing. *Vet. Parasitol.* **127**, 221-226.

Földvári, G., Márialigeti, M., Solymosi, N., Lukács, Z., Majoros, G., Kósa, J. P., and *R. Farkas* (2007). Hard ticks infesting dogs in Hungary and their infection with *Babesia* and *Borrelia* Species. *Parasitol. Res.* **101**, 25-34.

Fritz, C. L. (2009). Emerging tick-borne diseases. *Vet. Clin. Small Anim.* **39**, 265-278.

Hamel, D., Silaghi, C., Knaus, M., Visser, M., Kusi, I., Rapti, D., Rehbein, S., and *K. Pfister* (2009). Detection of *Babesia canis* subspecies and other arthropod-borne diseases in dogs from Tirana, Albania. *Wien. Klin. Wochenschr.* **121**, 42-45.

Hilpertshauser, H., Deplazes, P., Schnyder, M., Gern, L., and *A. Mathis* (2006). *Babesia* spp. identified by PCR in ticks collected from domestic and wild ruminants in southern Switzerland. *Appl. Environ. Microb.* **72**, 6503-6507.

Homer, M. J., Aguilar-Delfin, I., Telford III, S. R., Krause, P. J., and *D. H. Persing* (2000). Babesiosis. *Clin. Microbiol. Rev.* **13**, 451-469.

Hubálek, Z., Halouzka, J., and *Z. Juřicová* (2004). Borreliae in *Ixodes ricinus* ticks feeding on humans. *Med. Vet. Entomol.* **18**, 228-231.

Ionita, M., Mitrea, I. L., Pfister, K., Hamel, D., Buzatu, C. M., and *C. Silaghi* (2012). Canine babesiosis in Romania due to *Babesia canis* and *Babesia vogeli*: a molecular approach. *Parasitol. Res.* **110**, 1659-1664.

Janisch, M. (1986). *Dermacentor pictus* tick species as the vector of *Babesia canis* in Hungary. *Magy. Állatorvosok.* **41**, 310-312 (in Hungarian with English abstract).

Kubelová, M., Tkadlec, E., Bednář, M., Roubalová, E., and *P. Široký* (2011). West-to-east differences of *Babesia canis canis* prevalence in *Dermacentor reticulatus* ticks in Slovakia. *Vet. Parasitol.* **180**, 191-196.

Lobetti, R. (1998). Canine babesiosis. *Compend. Cont. Educ. Pract. Vet*. **20**, 418-431.

Marsaudon, E., Camenen, J., Testou, D., Bourree, P., Samson, P. and *F. Luneau* (1995). *Babesia canis* human babesiosis causing a 40-day anuria. *Ann. Med. Interne* **146**, 451-452.

Milutinović, M. (1992). Ekološka istraživanja krpelja (Acarina, Ixodoidea, Ixodidae) Srbije. *Ph. D. Thesis*, Beograd, 82-98.

Milutinović, M. and *Radulović, Ž.* (2002). Ecological notes on ticks (Acari: Ixodidae) in Serbia (central regions). *Acta Vet.-Beograd* **52**, 49-58.

Milutinović, M., Radulović, Ž., Tomanović, S., and *Z. Petrović* (2012). *Krpelji (Acari: Ixodidae, Argasidae) Srbije*, 1 (ured. M. Anđelković), 214 - 236. Srpska akademija nauka i umetnosti, Beograd.

Pavlović, I., Milutinović, M., Petković, D., Terzin, D., and *V. Terzin* (2002). Epizootiological research of canine babesiosis in the Belgrade district. *J. Protozool. Res.* **12**, 10-15.

Pavlović, I., Petković. D., Terzin, V., Terzin, D., and *D. Vojinović* (2009). Babesiosis of dogs at Belgrade area in period 2004-2008. *XI Epizootiology Days with International Participation, Apatin, Banja Junaković, Abstr.* 166.

Petrović, Z. (1979). Fauna krpelja (Ixodidae) u Srbiji. *Arch. Biol. Sci.* **28**, 69-72.

Pomerancev, B. L. (1950). *Fauna SSSR. Paukoobraznie. Iksodovie kleščei (Ixodidae)*, **4,** 2-15. *Akadem Nauk SSSR*, Moskva-Leningrad.

Quick, R. E., Herwaldt, B. L., Thomford, J. W., Garnett, M. E., Eberhard, M. L., Wilson, M., Spach, D. H., Dickerson, J. W., Telford III, S. R., Steingart, K. R., Pollock, R., Persing, D. H., Kobayashi, J. M., Juranek, D. D., and *P. A. Conrad* (1993). Babesisosis in Washington State: a new species of *Babesia*? *Ann. Intern. Med.* **119**, 284-290.

Rar, V. A., Fomenko, N. V., Dobrotvorsky A. K., Livanova N. N., Rudakova, S. A., Fedorov, E. G., Astanin, V. B., and *O. V. Morozova* (2005a). Tick-borne pathogen detection, Western Siberia, Russia. *Emerg. Infect. Dis.* **11**, 1708-1715.

Rar, V. A., Maksimova, T. G., Zakharenko, L. P., Bolykhina, S. A., Dobrotvorsky, A. K., and *O. V. Morozova* (2005b). *Babesia* DNA detection in canine blood and *Dermacentor reticulatus* ticks in south-western Siberia, Russia. *Vector-Borne Zoonotic Dis.* **5**, 285-287.

Reye, A. L., Hübschen, J. M., Sausy, A., and *C. P. Muller* (2010). Prevalence and seasonality of tick-borne pathogens in questing *Ixodes ricinus* ticks from Luxembourg. *Appl. Environ. Microb.* **76**, 2923-2931.

Spielman, A., Etkind, P., Piesman, J., Ruebush II, T. K., Juranek, D. D., and *M. S. Jacobs* (1981). Reservoir hosts of human babesiosis on Nantucket Island. *Am. J. Trop. Med. Hyg.* **30**, 560-565.

Škrabalo, Z. and *Z. Deanović* (1957). Piroplasmosis in man: report of a case. *Doc. Med. Geogr. Trop.* **9**, 11-16.

Theodoropoulos, G., Gazouli, M., Ikonomopoulos, J. A., Kantzoura, V., and *A. Kominakis* (2006). Determination of prevalence and risk factors of infection with *Babesia* in small ruminants from Greece by polymerase chain reaction amplification. *Vet. Parasitol.* **135**, 99-104.

Thomford, J. W., Conrad, P. A., Telford III, S. R., Mathiesen, D., Bowman, B. H., Spielman, A., Eberhard, M. L., Herwaldt, B. L., Quick, R. E., and *D. H., Persing* (1994). Cultivation and phylogenetic characterization of a newly recognized human pathogenic protozoan. *J. Infect. Dis.* **169**, 1050-1056.

Thompson, J. D., Higgins, D. G. and *T. J. Gibson* (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position – specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Tomanović, S. (2009). Molekularna detekcija i karakterizacija bakterija *Borrelia burgdorferi*, *Anaplasma phagocytophilum* i *Francisella tularensis* u krpeljima *Ixodes ricinus* na području Srbije. *Ph. D. Thesis*, Beograd, 52.

Tomanović, S., Chochlakis, D., Radulović, Ž., Milutinović, M., Ćakić, S., Mihaljica, D., Tselentis, Y., and *A. Psaroulaki* (2012). Analysis of pathogen co-occurrence in host seeking adult hard ticks from Serbia. *Exp. Appl. Acarol.* In press.

Welc-Falęciak, R., Bajer, A., Behnke, J. M., and *E. Siński* (2008). Effects of host diversity and the community composition of hard ticks (Ixodidae) on *Babesia microti* infection. *International J. Med. Microbiol.* **298**, 235-242.