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Short communication

Molecular detection of emerging tick-borne pathogens in Vojvodina, Serbia

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ABSTRACT

Ticks play an important role in disease transmission globally due to their capability to serve as vectors for human and animal pathogens. The Republic of Serbia is an endemic area for a large number of tick-borne diseases. However, current knowledge on these diseases in Serbia is limited. The aim of this study was to investigate the presence of new emerging tick-borne pathogens in ticks collected from dogs and the vegetation from different parts of Vojvodina, Serbia.

A total of 187 ticks, including 124 *Rhipicephalus sanguineus*, 45 *Ixodes ricinus* and 18 *Dermacentor reticulatus* were collected from dogs. In addition, 26 questing *I. ricinus* ticks were collected from the vegetation, using the flagging method, from 4 different localities in Vojvodina, Serbia. DNA was extracted from each tick individually and samples were tested by either conventional or real-time PCR assays for the presence of *Rickettsia* spp.-DNA (*gltA* and *ompA* gene fragments), *Ehrlichia*/*Anaplasma* spp.-DNA (*16S rRNA* gene fragment) and *Hepatozoon* spp./*Babesia* spp.-DNA (*18S rRNA* gene fragment). In addition, all *I. ricinus* DNA samples were tested for *Bartonella* spp.-DNA (ITS locus) by real-time PCR.

In this study, the presence of novel emerging tick-borne pathogens including *Rickettsia raoultii*, *Rickettsia massiliae*, *Babesia venatorum*, *Babesia microti*, *Hepatozoon canis* and *Candidatus* Neoehrlichia mikurensis was identified for the first time in Serbia. Our findings also confirmed the presence of *Rickettsia monacensis*, *Babesia canis* and *Anaplasma phagocytophilum* in ticks from Serbia.

The findings of the current study highlight the great diversity of tick-borne pathogens of human and animal importance in Serbia. Physicians, public health workers and veterinarians should increase alertness to the presence of these tick-borne pathogens in this country.

1. Introduction

In the past three decades, many vector-borne pathogens have emerged, creating new challenges for public and animal health (Weaver and Reisen, 2010). Some are exotic pathogens that have been introduced into new regions, and others are endemic species that have greatly increased in incidence, not been detected before, or have started to infect local human populations (Kilpatrick and Randolph, 2012).

The spectrum of tick-borne diseases (TBDs) affecting humans and domestic animals has increased in recent years; many important zoonotic TBDs, such as anaplasmosis, babesiosis, ehrlichiosis, and Lyme borreliosis are increasingly gaining more attention from physicians and veterinarians. With the progress in molecular diagnosis, new species, strains, and genetic variants of microorganisms are being detected in ticks worldwide, and the list of potential tick-borne pathogens continues to increase (Dantas-Torres et al., 2012). TBDs show a complex epidemiological picture, strong seasonality and periods of pathogen persistence without manifestation of a clinical disease. Furthermore, a lower disease incidence, due to a low exposure rate or due to a small susceptible population, can contribute to delayed discovery of new emerging tick-borne pathogens as occurred in the cases of *Candidatus* Neoehrlichia mikurensis, *Borrelia miyamotoi*, and other *Rickettsia* species. These pathogens were

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discovered in ticks before the diseases in humans or animals (Tijsse Klasen et al., 2014). Recently, Vannier and Krause emphasized the importance of tick-borne and transfusion-transmitted pathogens such as *Babesia microti* and *Babesia venatorum* (Vannier and Krause, 2012). Also, Baneth et al. (2000) stated that canine hepatozoonosis is an emerging protozoal tick-borne infection of dogs (Baneth et al., 2000).

Serbia is an endemic area for a large number of TBDs which comprise the most common vector-borne diseases in this region. In the last three decades *Borrelia* species were intensively studied, while some other pathogens were only sporadically investigated. It is known that five genospecies of the *Borrelia burgdorferi* s.l. complex (*Borrelia lusitaniae*, *Borrelia burgdorferi* s.s., *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana*) are present in ticks in Serbia (Tomanović et al., 2010). *Babesia canis* (Davitkov et al., 2015; Tomanović et al., 2013), *Babesia gibsoni* (Davitkov et al., 2015), *Anaplasma phagocytophilum* (Milutinović et al., 2008; Potkonjak et al., 2013), *Anaplasma ovis* (Tomanović et al., 2013), *Francisella tularensis* subsp. *tularensis* (Milutinović et al., 2008; Tomanović et al., 2010), *Coxiella burnetii* (Tomanović et al., 2010, 2013), *Rickettsia monacensis* and *Rickettsia helvetica* (Radulović et al., 2011; Tomanović et al., 2013) were also detected in ticks from Serbia.

The aim of this study was to investigate the presence of emerging and re-emerging tick-borne pathogens in ticks collected from dogs and the vegetation from different parts of Vojvodina, Serbia.

2. Materials and methods

2.1. Tick collection and identification

Questing ticks were collected from the vegetation using the "Flag-hour" method (Maupin et al., 1991). The study was performed in four localities in Vojvodina, Serbia: Andrevlje (N 45°10′268″ E 19°38′496″), Zmajevac (N 45°09′321″ E 19°46′508), Kać (N 45°17′667″ E 19°54′019″) and Subić (N 45°16′296″ E 19°54′571″). In addition, ticks were collected from infested dogs admitted to several veterinary clinics in Novi Sad (N 45°15′593″ E 19°49′592″), Vojvodina, Serbia. Tick species were identified according to the following morphological keys: Nosek and Sixl (1972), Estrada-Pena et al. (2004) and Walker et al. (2007). Ticks of the same species and life stages sampled on the same date and from the same dog or locality were pooled together in one vial and kept in 70% ethanol until further analyzed.

2.2. DNA extraction

After elimination of the ethanol from each vial, ticks were washed in 70% ethanol and then twice in phosphate-buffered saline (PBS). The ticks were cut symmetrically into two halves. One half of each tick was transferred to a plastic microtube and 0.5 mL of PBS was added, and the other half was stored frozen at −80 °C for further analysis. Each sample was manually homogenized with plastic sterilized pestles for 1 min, and then centrifuged for 10 s at 2000 × *g*. Then, the supernatant was collected and DNA was extracted with a DNA extraction kit (Illustra Tissue Mini Spin kit; GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

2.3. PCR amplification

Initial detection of *Rickettsia* species was performed by screening all the DNA samples by a real-time PCR assay targeting a 133-bp fragment of the citrate synthase gene (*glt*A). Only positive samples were further analyzed by targeting a 178- to 189-bp fragment of the outer membrane protein A gene (*omp*A) (Harrus et al., 2011; Kidd et al., 2008). The detection of *Babesia* and *Hepatozoon* species was performed by screening all DNA samples by a conventional PCR assay targeting a 400-bp fragment of 18S rRNA gene (Rojas et al., 2014; Tabar et al., 2008). The detection of *Ehrlichia* and *Anaplasma* species was performed by screening all DNA samples by a conventional PCR assay targeting a 345-bp fragment of 16S rRNA gene (Parola et al., 2000; Rojas et al., 2014). The detection of *Bartonella* species was performed by screening *Ixodes ricinus* DNA samples by a real-time PCR assay targeting a 190-bp fragment of the 16S–23S internal transcribed spacer (ITS) locus (Gutiérrez et al., 2013; Maggi and Breitschwerdt, 2005). Conventional PCR assays were carried out using the Biometra T-Personal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). Real-time PCR assays were carried out using the Rotor Gene 6000 cycler (Corbett Research, Sydney, Australia). For all pathogens, positive DNA, negative DNA, and a nontemplate DNA control were used in each run.

2.4. DNA sequencing

All positive PCR products were purified using a PCR purification kit (Exo-SAP; New England BioLabs, Inc., Ipswich, MA) and subsequently sequenced by the BigDye Terminator cycle sequencing chemistry from an Applied Biosystems ABI 3700DNA analyzer and evaluated by the ABI's data collection and sequence analysis software (ABI, Carlsbad, CA). The obtained sequences were analyzed initially by BLAST through the NCBI's Mega-BLAST algorithm. Further analyses were done with the MEGA alignment software (version 5.05; The Biodesign Institute).

3. Results

3.1. Identification of tick species

A total of 213 ticks were collected: 187 engorged adult ticks from dogs and 26 questing ticks (12 nymphs and 14 adult stages) from the vegetation. Three tick species were identified on dogs: 124 *Rhipicephalus sanguineus* (Latreille 1806), 45 *I. ricinus* (Linnaeus 1758) and 18 *Dermacentor reticulatus* (Fabricius 1794). All ticks collected from the vegetation were identified as *I. ricinus*.

3.2. Rickettsia spp.

The rickettsial *glt*A gene fragment was detected in 26 of the 71 (36.6%) *I. ricinus*, in 3 of the 18 (16.6%) *D. reticulatus*, and in one of the 124 (0.8%) *R. sanguineus* ticks. Rickettsial *omp*A DNA was amplified in 16 of the 26 *glt*A-positive *I. ricinus* ticks. All of these sequences were identical to *R. monacensis* (100% sequence identity; GenBank accession number LN794217.1). *R. monacensis omp*A DNA was detected in five questing *I. ricinus* ticks collected from the vegetation in two geographical localities (Andrevlje and Zmajevac) and in 11 *I. ricinus* ticks collected from dogs. The total prevalence of *omp*A-confirmed *R. monacensis* in *I. ricinus* ticks was 22.5%. One *D. reticulatus* tick (collected from a dog), contained detectable rickettsial *omp*A DNA, which was identical to *Rickettsia raoultii* (100% sequence identity; GenBank accession number JX648103.1; prevalence of 5.6%). One *R. sanguineus* tick (collected from a dog), contained detectable rickettsial *omp*A DNA, which was identical to *Rickettsia massiliae* (100% sequence identity; GenBank accession number CP003319.1; prevalence of 0.8%).

3.3. Hepatozoon spp. and Babesia spp.

The piroplasmid 18S rRNA gene fragment was detected in 6 of the 18 (33.3%) *D. reticulatus* ticks. All these sequences were identical to *B. canis* (4 of the 6 sequences were 100% identical to GenBank accession number KP745630.1 and KP216422.1; the remaining 2 sequences were 99% identical to GenBank accession

Table 1
Summary of the pathogens' DNA detected in ticks from Vojvodina, Serbia, according to the tick species, host and origin of detection.

Pathogen	Tick species	Number of ticks total/positive	% of positive	Tick host or origin
R. monacensis	*I. ricinus*	71/16	22.5	Dog, vegetation
R. raoultii	*D. reticulatus*	18/1	5.6	Dog
R. massiliae	*R. sanquineus*	124/1	0.8	Dog
B. canis	*D. reticulatus*	18/6	33.3	Dog
B. venatorum	*I. ricinus*	71/2	2.8	Dog, vegetation
B. microti	*I. ricinus*	71/1	1.4	Vegetation
H. canis	*I. ricinus*	71/6	8.4	Dog
Candidatus N. mikurensis	*I. ricinus*	71/3	4.2	Vegetation
A. phagocytophilum	*I. ricinus*	71/1	1.4	Dog
Bartonella spp.	*I. ricinus*	71/0	0	Dog, vegetation

number KP745630.1 and KP216422.1). Piroplasmid 18S rRNA gene fragment was also detected in 9 of the 71 (12.6%) *I. ricinus* ticks. Six of these sequences were identical to *Hepatozoon canis* (100% sequence identity; GenBank accession number KP216462.1). They were found in *I. ricinus* ticks collected from dogs with the total prevalence of 8.4%. Moreover, two sequences of the piroplasmid 18S rRNA gene fragment obtained from one questing *I. ricinus* collected from vegetation (Subić) and from one *I. ricinus* collected from a dog, were identical to *B. venatorum* (both sequences were 100% identical to GenBank accession number LC005776.1). The total prevalence of *B. venatorum* DNA in *I. ricinus* ticks was 2.8%. One 18S rRNA gene fragment, detected in one questing *I. ricinus* collected from the vegetation in Kać, was identical to *B. microti* (100% sequence identity; GenBank accession number KP688578.1). The total prevalence of *B. microti* in *I. ricinus* ticks was 1.4%.

3.4. *Ehrlichia and Anaplasma spp.*

Ehrlichial 16S rRNA gene fragment was detected in 4 of the 71 (5.6%) *I. ricinus* ticks. Three of these sequences were identical to *Candidatus* Neoehrlichia mikurensis (100% sequence identity; GenBank accession number KF155504.1). This species was detected in three questing *I. ricinus* ticks collected from the vegetation in two geographical localities (Andrevlje and Zmajevac) and their total prevalence was 4.2%. One sequence of ehrlichial 16S rRNA gene fragment was identical to *A. phagocytophilum* (100% sequence identity; GenBank accession number KR021166.1). It was detected in one *I. ricinus* tick collected from a dog and its total prevalence was 1.4%.

3.5. *Bartonella spp.*

None of the tested *I. ricinus* ticks was positive for *Bartonella* spp.-DNA.

All results are shown in Table 1.

4. Discussion

Although Serbia is known as an endemic area for several tick-borne rickettsiae, the information in this regard is still limited. Radulović et al. (2011) previously identified the presence of *R. helvetica* and *R. monacensis* in *I. ricinus* ticks, with infection rates of 7.7% and 15.4%, respectively (Radulović et al., 2011). In a more recent study, Tomanović et al. (2013) confirmed the presence of these 2 *Rickettsia* spp. in *I. ricinus* (Tomanović et al., 2013). In this study, we confirmed the presence of *R. monacensis* in *I. ricinus*. Furthermore, we detected the presence of *R. raoultii* and *R. massiliae* in Serbia for the first time. Since its emergence, *R. raoultii* has been found to be associated with *Dermacentor* ticks throughout Europe (Špitalská et al., 2012) and in some parts of Asia, including Mongolia (Speck et al., 2012) and China (Tian et al., 2012). The prevalence of *R. raoultii* in Slovakia was 8.1–8.6% and 22.3–27% in *Dermacentor marginatus* and *D. reticulatus*, respectively (Špitalská et al., 2012). Matsumoto et al. (2009) showed that *D. reticulatus* ticks in the Bialowieza area, Poland, host *R. raoultii* (Matsumoto et al., 2009). Nijhof et al. (2007) detected *R. raoultii* in 14% *D. reticulatus* collected from dogs in the Netherlands (Nijhof et al., 2007). *D. reticulatus* is primarily a tick of dogs and carnivores (Estrada-Pena et al., 2004), but although it is known that *Rickettsia conorii* (Mannelli et al., 2003; Solano-Gallego et al., 2006) and *Rickettsia rickettsii* (Elchos and Goddard, 2003; Paddock et al., 2002) infect dogs, it is not clear whether *R. raoultii* can naturally infect dogs. Renvoisé et al. (2012) found 7 *R. massiliae*-positive *R. sanguineus* ticks collected from a household in southern France (Renvoisé et al., 2012). Márquez et al. (2008) detected specific rickettsial DNA in 90 pools (18%) of *R. sanguineus* tested ticks from southwestern Spain. Sequence analysis of amplicons revealed that *R. sanguineus* ticks were infected exclusively with *R. massiliae* (Márquez et al., 2008). *R. massiliae* has now been detected in several countries of southern Europe, the Mediterranean basin, Africa, Argentina and eastern USA. It is still not known if dogs are naturally infected with this *Rickettsia* species (Eremeeva et al., 2006).

In Serbia, *B. canis* (Davitkov et al., 2015; Tomanović et al., 2013) and *B. gibsoni* (Davitkov et al., 2015) were previously identified in ticks and dogs' blood. In this study we confirmed the presence of *B. canis* in *D. reticulatus* ticks from dogs. We also detected *B. venatorum* DNA for the first time in Serbia and *B. microti* DNA in one questing *I. ricinus* tick. In Europe, *Babesia* spp. such as *B. microti*, *B. divergens*, *B. odocoilei*-like, and the newly described *B. venatorum* (formerly EU1 *Babesia* organism) are known to be prevalent in *I. ricinus* (Cieniuch et al., 2009; Duh et al., 2001, 2005; Foppa et al., 2002; Hartelt et al., 2004; Siński et al., 2006; Skotarczak et al., 2002; Wielinga et al., 2009). Stensvold et al. found that 8% of *I. ricinus* collected from Danish domestic dogs were positive for *Babesia* spp. (*B. microti*, 82%; *B. venatorum*, 18%) (Stensvold et al., 2015). In this study, we identified *H. canis* DNA in six *I. ricinus* ticks collected from dogs from Serbia for the first time. This pathogen has been reported in southern Europe, the Middle East, Africa, and the Far East, South-, Central- and North America (Baneth, 2011). It has also been reported in foxes and jackals in nearby Hungary (Farkas et al., 2014) and in foxes from Bosnia and Herzegovina (Hodžić et al., 2015). The main vector for this protozoon is the tick *R. sanguineus* (Baneth et al., 2000). Interestingly, *H. canis* was detected in this study in *I. ricinus* collected from dogs, but not in *R. sanguineus* ticks. The study by Giannelli et al. (2013) indicated that *H. canis* sporogony does not occur in *I. ricinus*, but in *R. sanguineus*, suggesting that *I. ricinus* is not a biological vector of *H. canis* (Giannelli et al., 2013). As such, it is probable that the tick acquired this organism by a blood meal from an infected dog.

We report the presence of *Candidatus* N. mikurensis in Serbia for the first time. In Europe, this pathogen is transmitted by *I. ricinus* and Silaghi et al. reported that overall prevalence rates of *Candidatus* N. mikurensis in *I. ricinus* or *Ixodes persulcatus* ticks collected from the vegetation range from below 1% to over 20% (Silaghi et al., 2015). In Serbia, *A. phagocytophilum* was previously identified in ticks (Milutinović et al., 2008; Potkonjak et al., 2013). In this study

we confirmed the presence of *A. phagocytophilum* in *I. ricinus* ticks collected from a dog in this country.

Bartonella spp.-DNA was not detected in *I. ricinus* ticks in this study. This finding is in agreement with a previous report from Serbia (Tomanović et al., 2013). Evidence of *I. ricinus* ticks infected with *Bartonella* spp. have been collected worldwide (Dietrich et al., 2010; Halos et al., 2005; Podsiadly et al., 2007; Sytykiewicz et al., 2012). Moreover, Cotte et al. (2008) showed experimental evidence of the competent vectorial role of *I. ricinus* for *Bartonella henselae* (Cotte et al., 2008). Nevertheless, our results could suggest that *I. ricinus* does not represent an active vector for bartonellae in Serbia.

5. Conclusion

In this study, we have identified, for the first time in Serbia, the presence of emerging zoonotic tick-borne pathogens including *R. raoultii*, *R. massiliae*, *B. venatorum*, *B. microti* and *Candidatus* N. mikurensis, as well as the non-zoonotic canine pathogen *H. canis*. The findings of the current study highlight the great diversity of tick-borne pathogens of human and animal importance in Serbia. Physicians, public health workers and veterinarians should increase alertness to the presence of these tick-borne pathogens in this country. In order to determine the "true" prevalence of these novel emerging tick-borne pathogens and their public health significance in Serbia, larger scale studies including all geographic regions, over several years, should be carried out.

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