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Research article

CANDIDATUS NEOEHRLICHIA SP. (FU98) AND *BORRELIA BURGDORFERI* SENSU LATO IN RED FOXES *(VULPES VULPES)* FROM SERBIA

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Human activities such as deforestation, urbanization, and environmental pollution lead to a reduction in the spatial boundary between wild animals, domestic animals and humans. These activities increase the risk for the emergence of pathogens from the sylvatic cycle in the population of domestic animals and humans. Foxes are recognized as potential reservoirs for a number of bacterial pathogens of medical and public health concern. The aim of the present study was to investigate the prevalence and spatial distribution of bacterial tick-borne pathogens from the Anaplasmataceae family, *Borrelia burgdorferi* sensu lato (s.l.), *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis*, *Bartonella* spp., in the red fox population from Serbia and to discuss the obtained results from the epidemiological point of view. Legally hunted red foxes (*Vulpes vulpes*) from 14 localities in Serbia were included in the study and spleen samples from 129 animals were tested with conventional PCR assays for the presence of bacterial tick-borne pathogens. DNA of *Candidatus* Neoehrlichia sp. (FU98), *Borrelia burgdorferi* sensu stricto, *Borrelia lusitaniae,* and *Borrelia garinii* was detected in 6 (4.7%), 1 (0.8%), 2 (1.6%) and 1 (0.8%) animals, respectively. Co-infection by *Candidatus* Neoehrlichia sp. (FU98) and *B. garinii* was detected in one animal. All samples were negative for other tested bacterial tick-borne pathogens. The results of the present study indicate the potential role of foxes in natural cycles of *Candidatus* Neoehrlichia sp. (FU98) and causative agents of Lyme borreliosis in the investigated areas. Further research is required to elucidate the role of foxes in the epidemiology of these and other tick-borne zoonotic pathogens in the Republic of Serbia.

Key words: Anaplasmataceae, *Borrelia* spp., *Candidatus* Neoehrlichia sp. (FU98), foxes, PCR, Serbia

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INTRODUCTION

The spatial boundary between wild canids, humans and domestic animals in the past decades has been greatly reduced due to degradation and fragmentation of the habitat caused by anthropogenic activities such as development of agriculture, deforestation, urbanization and environmental pollution [1]. These changes have led to the possibility of the emerging and re-emerging of diseases in human and domestic animals' population caused by pathogens that are maintained in wild canids [2]. The number of diseases originating in the wildlife are arthropod-borne and among arthropods, ticks are along with mosquitoes the most significant vectors of zoonotic diseases [3,4].

In recent years, researchers pointed out the possible role of red foxes (*Vulpes vulpes*) in the epizootiology of bacterial tick-borne diseases (TBDs) [5,6]. The presence of several tick-borne pathogens from the Anaplasmataceae family was detected among foxes in Europe: *Anaplasma phagocytophilum,* a causative agent of granulocytic anaplasmosis in humans and animals [7-9], *Anaplasma platys,* an etiologic agent of thrombocytic anaplasmosis in dogs [10], and *Ehrlichia canis* that causes monocytic ehrlichiosis in canids [11,12]. Recently, the role of foxes as potential reservoirs for the newly discovered member of the family Anaplasmataceae, *Candidatus* Neoehrlichia sp., (FU98) has been proposed [13]. Foxes are also recognized as reservoir hosts for the causative agents of Lyme borreliosis, spirochetes from the *Borrelia burgdorferi* sensu lato complex (s.l.) [14]. Molecular studies conducted in Europe, did not show the presence of *Rickettsia* spp. in red fox [11,15,16], although the presence of *Rickettsia* spp. in ticks collected from red foxes has been proved [17,18] and serological studies confirmed that foxes are exposed to those zoonotic pathogens [17]. The most commonly detected *Bartonella* species in European foxes is zoonotic *Bartonella rochalimae* [19–23]. A recently published serosurvey indicates that foxes are suitable hosts for *Francisella tularensis*, the causative agent of tularemia in humans and animals [24]. High seroprevalence rate (41.2%) of *Coxiella burnetii* was detected in red foxes in the United Kingdom [25]. Furthermore, foxes are hosts for a number of vectors of the above mentioned pathogens; ticks from four genera are found to parasitize red foxes in Europe (*Ixodes ricinus, I. canisuga, I. hexagonus, Dermacentor reticulatus*, *D. marginatus, Haemaphysalis concinna, H. punctata, H. inermis, H. erinacei, Rhipicephalus bursa, R. sanguineus, R. turanicus)* [26–29]. The results of previous studies and insufficient knowledge on the role of foxes in the eco-epidemiology of bacterial tick-borne pathogens justify further research. The red fox is one of three native canid species present in Serbia. The range of foxes covers the whole territory of the country and they are the most abundant medium-sized predators [30]. Abundant population, frequent infestation with various tick species and close contact with humans and domestic animals impose the need to explore the exact role of foxes in enzootic cycles of TBDs in a particular area. Based on the studies on ectoparasite fauna of red foxes in Serbia, the presence of nine hard tick species were reported, namely: *I. ricinus, I. hexagonus, I. canisuga, R. sanguineus, R. bursa, H. punctata, H. inermis, D. marginatus* and *D. reticulatus* [31,32]. However, research on the

role of foxes as potential vectors and reservoirs for tick-borne pathogens in this area has not been carried out so far. The aim of the present study was to investigate the presence of bacterial tick-borne pathogens in the fox population from Serbia and to discuss the obtained results from the epidemiological point of view.

MATERIALS AND METHODS

In total, 129 legally hunted red foxes from 14 localities in Serbia (Surčin, Obrenovac, Veliko Gradište, Velika Plana, Svilajnac, Negotin, Despotovac, Rekovac, Kraljevo, Vrnjačka Banja, Trstenik, Blace, Niš, Bela Palanka) were included in the study (Figure 1). Samples were collected over a period of seven years (2010-2016). Date of sampling,

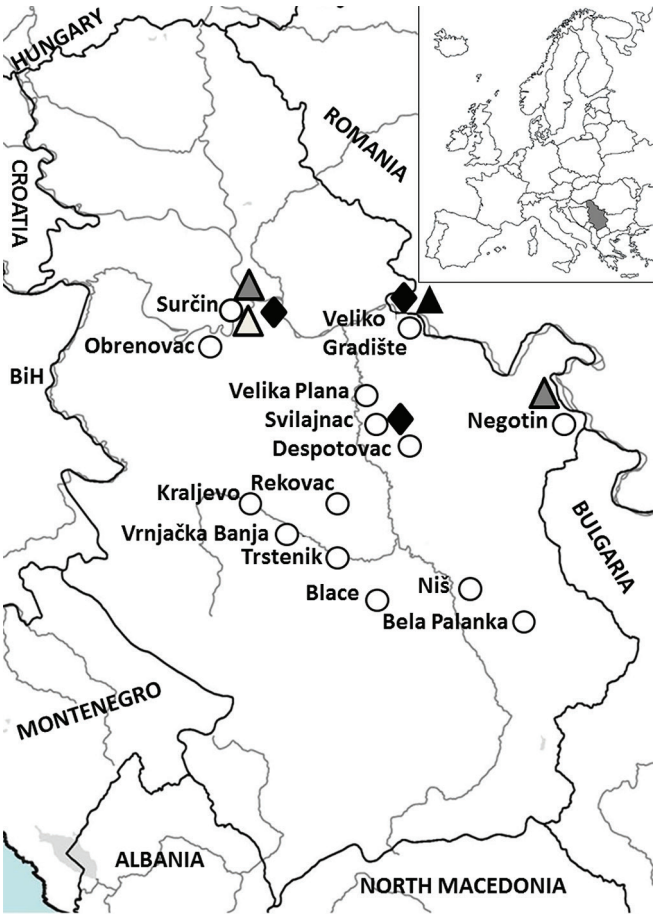

Figure 1. Geographical distribution of positive foxes for DNA of *Candidatus* Neoehrlichia sp. (FU98), *and Borrelia spp.*
- *Candidatus* Neoehrlichia sp. (FU98), (localities: Surčin, Veliko Gradište, and Svilajnac), - *Borrelia burgdorferi* sensu stricto, (locality Veliko Gradište), ▲ - *Borrelia lusitaniae,* (localities: Surčin and Negotin), △ - *Borrelia garinii* (locality Surčin).

sex and origin of the animals were recorded. Spleen samples were collected from the necropsied animals and the cold chain was provided for transfer of the tissue samples to the laboratory. Prior to DNA extraction samples were stored at -80°C. DNA extraction was performed using the Gene Jet Genomic DNA Purification Kit (Fermentas, Thermo Scientific). A small portion of spleen tissue (up to 10 mg) was homogenized by micropestles (Eppendorf™), and extraction was carried out according to the manufacturer's instruction. The extracted DNA was placed at -20°C until PCR analyses.

PCR assay

In order to detect the DNA of tested pathogens, previously published conventional PCR protocols were used (Table 1).

The PCR reactions for detection of *B. burgdorferi* s.l., *Rickettsia* spp., *C. burnetii, F. tularensis, Bartonella* spp. and members of the family Anaplasmataceae were prepared using PCR Master Mix (2X) (Thermo Fisher Scientific Inc.). Amplification of targeted sequences was performed using Veriti Thermal Cycler device (Applied Biosystems). PCR products were electrophoresed on 2% agarose gel stained with Ethidium Bromide and visualized using BioDocAnalyze device (Biometra GmbH). Detection of *Candidatus* Neoehrlichia spp. and preparation of positive samples for sequencing was performed with GoTaq® G2 Polymerase (Promega, USA).

Sequencing and sequence analysis

The purification and bidirectional sequencing (Sanger) of obtained PCR products were performed by commercial companies (Macrogen, Amsterdam, the Netherlands and Microsynth, Austria). Sequences were compared with previously published nucleotide sequences available in the GenBank® database using the BLAST tool (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST), analysed using the FinchTV v1.5.0, software and aligned using Clustal W.

Representative sequences from this study have been deposited to the GenBank® database under the following accession numbers: MK043348 (16S rRNA), MK050781 (*groEL*) - *Candidatus* Neoehrlichia sp. (FU98), MK043031 - *Borrelia burgdorferi* sensu stricto (s.s.), MK043032 - *Borrelia garinii*, MK043041 - *Borrelia lusitaniae*.

Statistical analysis

Confidence intervals (95% CI) were calculated using the online calculator available at http://vassarstats.net/prop1.html.

Table 1. Primers used for detection of tick-borne bacterial pathogens in spleen samples of red foxes with PCR annealing temperatures

Pathogens	Target gene (size of the amplicons)	Primers sequence (5'–3')	Annealing °C	Reference
Anaplasmataceae	Anaplasmatic 16s rRNA gene fragment (~345 bp)	EHR16SD (GGTACCYACAGAAGAAGTCC) EHR16SR (TAGCACTCATCGTTTACAGC)	53	Parola et al. (2000)
Candidatus Neoehrlichia spp.	groEL (~806 bp)	NeoeGroELFw (CAGGTGAAGCACTAGATAAGTCCA) NeoeGroELRV (ACAGCAGCAACATGCAATCCA)	54	Hodžić et al. (2015)
	16S rRNA (~1053 bp)	16SCNM_for (GTGGCAGACGGGTGAGTAAT) 16SCNM_rev (TGCAGCACCTGTGTAAGGTC)	60	
Borrelia spp.	5S-23S rRNA intergenic spacer of *Borrelia* spp. (~250 bp)	RIS1 (CTGCGAGTTCGCGGGAGA) RIS2 (TCCTAGGCATTCACCATA) RIS3 (GGAGAGTAGGTTATTGCCAGG) RIS4 (GACTCTTATTACTTTGACC)	52	Masuzawa et al. (1996)
Rickettsia spp.	Citrate synthase (CS), (380 to 397 bp)	RpCS.877p (GGGGGCCTGCTCACGGCGG) RpCS.1258n (ATTGCAAAAAGTACAGTGAACA)	56	Regnery et al. (1991)
Coxiella burnetii	Superoxide dismutase (257 bp)	CB1 (ACTCAACGCACTGGAACCGC) CB2 (TAGCTGAAGCCAATTCGCC)	55	Spyridaki et al. (1998)
Francisella tularensis	17 kDa lipoprotein (428 bp)	TUL4-435 (GCTGTATCATCATTTAATAAACTGCTG) TUL4-863 (TTGGGAAGCTTGTATCATGGCACT)	55	Sjöstedt et al. (1997)
Bartonella spp.	16S-23S intergenic region (639 bp)	Urbarto1 (CTTCGTTTCTCTTTCTTCAA) Urbarto2 (CTTCTCTTCACAATTTCAAT)	45	Rolain et al. (2003)

RESULTS

Sampling from 129 animals (73 males and 56 females) preceded analysis procedures. The majority of samples originated from three locations - Veliko Gradište (n=45 animals), Surčin (n=29) and Svilajnac (n=13) while remaining localities were represented with fewer samples.

DNA of the members of family Anaplasmataceae and *B. burgdorferi* s.l. was detected among analyzed spleen samples, while the presence of DNA of other tested bacterial pathogens (*Rickettsia* spp., *Bartonella* spp., *C. burnetii* and *F. tularensis*) was not confirmed. DNA of the members of *B. burgdorferi* s.l. complex was detected in 7 samples [(5.4%), 95% CI: 2.7-10.8%]. originated from 2/73 (2.7%) male and 5/56 (8.9%) female animals collected from 3 localities (Surčin, Obrenovac, Veliko Gradište). Sequencing of 5S-23S rRNA intergenic spacer region was successful for four samples and analysis of the obtained sequences confirmed the presence of three *B. burgdorferi* s.l. species, namely: *B. burgdorferi* s.s. (locality Veliko Gradište), *B. lusitaniae* (localities Surčin and Negotin) and *B. garinii* (locality Surčin), (Figure 1).

Molecular detection with primers specific for 16S rRNA gene fragment (~345 bp) of the family Anaplasmataceae showed positive bands in 21 animals, and these samples were preceded for sequencing for initial screening. Obtained sequences were compared with previously published sequences deposited at the GenBank®, and 8 sequences showed partial coverage with 93-99% similarity with *Candidatus* Neoehrlichia spp. sequences. These samples were further tested with primers specific for longer fragments of *groEL* (~806 bp) and 16S rRNA (~1053 bp) genes of *Candidatus* Neoehrlichia spp. Amplification was successful for 6/129 samples, [(4.7%), 95% CI: 2.2-9,8%] originating from 4/73 (5.5%) male and 2/53 (3.8%) female animals hunted at three out 14 localities: Surčin, Veliko Gradište, and Svilajnac (Figure 1). Positive samples were thereon sequenced. The sequences of *Candidatus* Neoehrlichia sp. (*groEL* and 16S rRNA), obtained in this study showed 100% similarity to the sequences of *Candidatus* Neoerlichia sp. (FU98) from red foxes from Austria (GenBank® accession numbers: KT833357, KT833358), from raccoon dogs (*Nyctereutes procyonoides*) from Poland (MG670107, MG670109) and from a badger (*Meles meles*) from Hungary (KX245423, KX231830). Also, researchers from Hungary have recently confirmed the presence of *Candidatus* Neoehrlichia sp. (FU98) in rural dogs [33]. The sequences were identical to the sequences that have been previously obtained from badgers in this country (KX245423, KX231830).

Despite relatively low prevalence of pathogens, co-infection of *Candidatus* Neoehrlichia sp. (FU98) and *B. garinii* was detected in one male fox (0.8%) hunted at the locality of Surčin.

DISCUSSION

Presented study is the first one that investigates red foxes as hosts of bacterial tick-borne pathogens in Serbia. Based on the obtained results, the potential role of foxes in natural cycles of *Candidatus* Neoehrlichia sp. (FU98) and *B. burgdorferi* s.l. complex in the investigated area was discussed. Two species are currently proposed to be classified in the Anaplasmataceae family: *Neoehrlichia mikurensis* (Nm) and *Candidatus* Neoehrlichia lotoris (*C*Nl). Nm is a recently cultured [34] emerging tick-borne zoonotic pathogen, identified for the first time in ticks from the Netherlands [35] and small mammals from China [36]. The first human case was detected in Sweden in 2010 [37]. *I. ricinus* is considered as the main vector in Europe and rodents are suggested as reservoirs [38]. Another agent closely related to Nm was detected for the first time in raccoons (*Procyon lotor*) in North America [39] and then successfully isolated from tick cells lines. Based on phylogenetic analysis of three genes (*16S rRNA*, *groEL*, *gltA*) it was clear that the new agent is distinct from Nm and it was named *C*Nl [40]. Experimental infection of raccoons with *C*Nl was successfully demonstrated while infection of laboratory rodents failed [41]. It is believed that the ticks of the genus *Ixodes* are the main vectors.

A newly discovered sequence type FU98, phylogenetically closely related to *C*Nl, but clearly distinct from Nm previously isolated from various hosts in Europe, was detected for the first time in Austrian foxes [23,42]. It has been also confirmed in one fox from the Czech Republic [43]. The present study brings evidence toward a wider distribution of this potentially new bacterial species in foxes in Europe, while further research is needed to investigate the exact role of foxes in the epizootiology of *Candidatus* Neoehrlichia sp. (FU98). Due to its genetic similarity to *C*Nl isolated from raccoons in North America, the hypothesis that a newly discovered sequence type was introduced to Europe with raccoons and spilled over to foxes should be validated. Recently, *Candidatus* Neoehrlichia sp. (FU98) was detected in a raccoon dog from Poland [44] and in a badger from Hungary [45]. The latest finding of Hornok et al., (2018) reports the presence of a strain similar to *Candidatus* Neoehrlichia sp. (FU98) in dogs from Hungary. The aforementioned findings raise the possibility that more mammalian species are susceptible hosts for this new strain type and make the epizootiological situation more complex. Presence of free-ranging raccoons, raccoon dogs and badgers has been reported on the territory of the Republic of Serbia [46,47], however studies concerning the role of the mentioned animals in the epidemiology of tick-borne bacterial pathogens in Serbia have not so far been carried out. Future research concerning *Candidatus* Neoehrlichia sp. (FU98) should be directed toward the detection of the presence and determination of the pathogenic potential in different hosts and to more integrated epidemiological studies in order to evaluate previous findings from the standpoint of human and veterinary medicine.

Borrelia burgdorferi s.l. circulates in nature through enzootic cycles that involve different vertebrate hosts and ticks as vectors, with *I. ricinus* recognized as the main vector in Europe [48]. Data on the prevalence of *B. burgdorferi* s.l. in foxes in Europe are

scarce, although it has been previously shown that foxes may serve as competent reservoirs for *B. burgdorferi* s.l. [14,49]. German researchers detected DNA of *B. burgdorferi* s.l. in the skin of foxes in two separate studies, with prevalences of 7% and 24%, respectively [14,50]. In Romania, the prevalence of *B. burgdorferi* s.l. in analyzed heart tissue of foxes was 1.42% [51]. Previous serosurveys in Serbia have shown the exposure of dogs to *B. burgdorferi* s.l. [52,53], while data on the diversity of *B. burgdorferi* s.l. species in *I. ricinus* ticks collected from the vegetation across the country, indicate the presence of the following five species: *B. burgdorferi* s.s., *Borrelia afzelii, B.lusitaniae, B.garinii* and *Borrelia valaisiana* [54,55]. Prevalence of *B.burgdorferi* s.l. detected in foxes in our study (5.4%) is in the range of previously published data for Europe, however it is the first record of *Borrelia* spirochetes in spleen samples of red foxes. Among analyzed samples, the presence of three *Borrelia* species was confirmed: *B. burgdorferi* s.s., *B. lusitaniae* and *B. garinii*. These pathogens were confirmed for the first time in foxes from Serbia. *B. burgdorferi* s.s. and *B. garinii* are proven causative agents of Lyme borreliosis while pathogenicity of *B. lusitanae* is still unclear [56]. Further studies are needed to elucidate the exact role of foxes in the epidemiology of zoonotic *Borrelia* spp., detected in the present study. Detected co-infection with *Candidatus* Neoehrlichia sp. (FU98) and *B. garinii* in one fox represents a basis for assuming the possibility for overlapping of natural cycles of these two pathogens. Previous research has shown that questing *I. ricinus* ticks from Serbia are co-infected with several tick-borne bacterial pathogens [54,55] although the co-infection with *Candidatus* Neoehrlichia sp. (FU98) and *B. garinii* has not been proven in ticks so far. The significance of this finding from the epidemiological aspect remains to be defined in future studies.

CONCLUSION

Candidatus Neoehrlichia sp. (FU98) and *B. burgdorferi* s.l. were detected for the first time in the spleen of foxes from Serbia. Obtained results indicate to the potential role of foxes as a reservoir for *Candidatus* Neoehrlichia sp. (FU98) and causative agents of Lyme borreliosis. Further research is needed to reveal the importance of foxes in the eco-epidemiology of these and other tick-borne pathogens in Serbia.

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Authors' contributions

RS carried out the molecular genetic studies, participated in the sequence analysis and drafted the manuscript, SJ carried out the molecular genetic studies, AH carried out part of the molecular genetic studies, participated in the sequence analysis, AP

provided valuable samples for the study, DĆ provided valuable samples for the study and critically revised the manuscript, DM and GV conducted statistical analysis, SR and GD critically revised the manuscript, ST initiated and supervised the study, coordinated the activities and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CANDIDATUS NEOEHRLICHIA SP. (FU98) I *BORRELIA BURGDORFERI* SENSU LATO KOD CRVENIH LISICA (*VULPES VULPES*) IZ SRBIJE

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Aktivnosti ljudi kao što su krčenje šuma, urbanizacija i zagađenje životne sredine, dovode do smanjenja prostorne granice između divljih, domaćih životinja i ljudi. Ove aktivnosti povećavaju rizik za prelivanje patogena iz silvatičnog ciklusa u populacije domaćih životinja i ljudi. Lisice su prepoznate kao potencijalni rezervoari za brojne bakterijske patogene koji su važni sa aspekta humane i veterinarske medicine.

Cilj ove studije bio je da se utvrdi prisustvo i istraži prevalencija i prostorna distribucija bakterijskih patogena koji se prenose krpeljima u populaciji crvenih lisica iz Srbije, kao i da se analiziraju dobijeni rezultati sa epidemiološkog stanovišta. Analizirani su patogeni iz porodice Anaplasmataceae, *Borrelia burgdorferi* sensu lato (s.l.), *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis*, *Bartonella* spp. Materijal za studiju su bile legalno odstreljene crvene lisice (*Vulpes vulpes*) poreklom sa 14 lokaliteta iz Srbije. Uzorci tkiva slezine od 129 životinja su testirani metodom konvencionalnog PCR-a na prisustvo bakterijskih patogena koji se prenose krpeljima.

Kod analiziranih životinja dokazano je prisustvo DNK sledećih bakterija: *Candidatus* Neoehrlichia sp. (FU98) kod šest životinja (4,7%), *B. burgdorferi* sensu stricto kod jedne (0,8%), *B. lusitaniae* kod dve (1,6%) i *B. garinii* kod jedne životinje (0,8%). Koinfekcija sa *Candidatus* Neoehrlichia sp. (FU98) i *B. garinii* potvrđena je kod jedne životinje. Svi uzorci su bili negativni na druge testirane krpeljima prenosive bakterijske patogene.

Rezultati ove studije ukazuju na potencijalnu ulogu lisica u prirodnim ciklusima kruženja *Candidatus* Neoehrlichia sp. (FU98) i uzročnika lajmske borelioze na istraživanom području. Potrebna su dalja istraživanja kako bi se rasvetlila uloga lisica u epidemiologiji ovih i drugih zoonotskih patogena koje prenose krpelji u Republici Srbiji.