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Abstract

Tick-borne pathogens (TBPs) pose a major threat to human health in Europe and the whole northern hemisphere. Despite a high prevalence of TBPs in *Ixodes ricinus* ticks, knowledge on the incidence of tick-borne diseases in humans infested by this tick species is limited. This study was conducted in the year 2019 on patients who presented themselves to the Pasteur Institute Novi Sad with tick infestations. Ticks (n = 31) feeding on human (n = 30) and blood samples from the same individuals were collected by physicians and a microfluidic real-time high-throughput PCR system was used to test the genomic DNA of the samples for the presence of 27 bacterial and eight parasitic microorganisms in Serbia. Except for one *Rhipicephalus sanguineus* s.l. adult male tick, all ticks infesting humans were morphologically identified as *I. ricinus*. A high proportion of ticks (74%, 23/31) were infected with at least one of the tested TB microorganisms, being *Rickettsia helvetica* (54%, 17/31) the most common pathogen, but *Borrelia afzelii* (9%, 3/31), *Anaplasma phagocytophilum* (6%, 2/31), *Borrelia miyamotoi* (6%, 2/31), and *Francisella* like-endosymbiont (6%, 2/31), *Borrelia valaisiana* (3%, 1/31), *Borrelia lusitaniae* (3%, 1/31), *Rickettsia felis* (3%, 1/31) and *Rickettsia aeschlimannii* (3%, 1/31) were also identified. Despite the high infection rate of TBPs in ticks, only two human blood samples (6%, 2/30) tested positive for the presence of TBPs, one patient (code H12, 67 years old female) was diagnosed with *Borrelia* spp. and the other patient was diagnosed (code H17, 71 years old female) with *R. felis* infection. The tick infesting patient H12 tested positive for *B. afzelii*, and *R. helvetica* and the tick infesting patient H17 tested positive for *R. felis*. Upon clinical examination, both patients were diagnosed with erythema migrans. No additional discomfort was reported by the patient and no additional pathology was observed by the physician. We concluded that humans bitten by *I. ricinus* in Serbia are exposed to a diverse array of TBPs with clinical impact in the Serbian cohort studied.

Keywords: tick-borne pathogens; *Rickettsia* spp.; *Borrelia* spp.; erythema migrans

1. Introduction

Ixodid ticks (Acari: Ixodida) are the most important vectors of a wide variety of pathogens that cause infectious diseases in humans and animals (de la Fuente et al., 2017). *Ixodes ricinus* is the most widespread hard tick species in Europe and feeds on a broad range of hosts, serving as reservoir and vector of many zoonotic pathogens, including bacteria, protozoa, viruses, and helminths (Rizzoli et al., 2014a). Lyme borreliosis, caused by some genospecies of the *Borrelia burgdorferi* sensu lato (s.l.) complex, is the most prevalent *I. ricinus*-borne disease in humans in Europe, with an estimated incidence of 85,000 cases per year (Pritt et al., 2016). The *B. burgdorferi* s.l. complex currently includes over 20 different genospecies, nine of which are circulating in Europe, including the human pathogens *B. burgdorferi* sensu stricto (s.s.), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia bavariensis*, and *Borrelia spielmanii*; and *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia bissettiiae*, and *Borrelia kurtenbachii*, for which the human pathogenicity remains uncertain (Clark et al., 2014; Raileanu et al., 2017).

Rickettsia spp. are fastidious obligate intracellular alpha-proteobacteria transmitted by hematophagous arthropods such as fleas, mites, ticks and lice (Fournier and Raoult, 2007). The genus *Rickettsia* comprises four large groups, namely the typhus group (TG), the spotted fever group (SFG), the transitional group (TRG) and the ancestral group (AG), of which the SFG and TG are the most important in terms of public health (Gillespie et al., 2008). Bacteria of SFG are transmitted mainly by hard ticks (Ixodidae), including *I. ricinus* which have been recognized as vectors and reservoirs of these pathogens (Parola et al., 2013). In Serbia, a previous study conducted by Milutinovic et al. (2008) described a high prevalence of *B. burgdorferi* s.l. (42.5%) in 287 host-seeking adult *I. ricinus* ticks collected from vegetation at 18 localities throughout nationwide. The presence of five *B. burgdorferi* s.l. genospecies, namely, *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. valaisiana* was identified by restriction fragment length polymorphism (RFLP) analysis. In addition, Radulovic et al. (2011) reported the presence of SFG rickettsiae members *Rickettsia helvetica* (7.7%) and *Rickettsia monacensis* (15.4%) in *I. ricinus* ticks, in a study aimed to investigate the presence of Rickettsiae in 131 questing ticks belonging to five different species (i.e., *Dermacentor marginatus*, *Dermacentor reticulatus*, *Haemaphysalis punctata*, *Haemaphysalis concinna*, and *I. ricinus*) collected from seven localities in Serbia.

In the present study, we used a microfluidic-based high-throughput real-time PCR detection method to identify major tick-borne pathogens (TBPs) in human samples and the ticks collected on the same individuals in urban and sub-urban areas of Serbia.

2. Material and methods

2.1. Ethics statement

This study was approved by the ethical committee of Pasteur Institute Novi Sad (Ethical approval No. 03/2019) and conducted according to Declaration of Helsinki and The Patient Rights Law of the Republic of Serbia.

2.2. Sample collection, tick identification and DNA extraction

A study was conducted in the year 2019 to assess human exposure to TBP infection in individuals (n = 30) infested with ticks (n = 31) in Serbia. Ticks feeding on human and blood samples from the same individuals were collected by physicians and stored at -80°C until analysis. Besides, clinical examination was performed on each patient. For each patient, 2 ml of blood were collected and used for DNA extraction using the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions. All ticks collected from these patients were identified regarding species, sex, and life stage, based on morphological features according to standard taxonomic keys described by Estrada-Peña et al. (2004). For each tick collected on a patient, ticks were homogenized using Precellys 24 lyser/homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5500 rpm for 20 s using 2.8 mm stainless steel beads in 180 µL of Lysis buffer (T1 buffer) and 25 µL of Proteinase K from the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany). Then homogenates were incubated 3 hours at 56°C and DNA extraction was performed according to the manufacturer's instructions. Purified DNA was eluted into 50 µL elution buffer.

2.3. DNA pre-amplification

To allow better detection of pathogen DNAs, total DNAs were pre-amplified with the PreAmp Master Mix (Fluidigm, San Francisco, CA, USA) used according to the manufacturer's instructions. Primers (targeted all pathogens) were pooled combining equal volume of primers (200 nM final each), all primers pairs are listed in the Table 1. The reaction was performed in a final volume of 5 µL containing 1 µL Perfecta Preamp 5×, 1.25 µL pooled primers mix, 1.5 µL distilled water and 1.25 µL DNA. The thermocycling program consisted

of one cycle at 95°C for 2 min, 14 cycles at 95°C for 15 s and 4 min at 60°C. At the end of the cycling program the reactions were diluted 1:10 in Milli-Q ultrapure water. Pre-amplified DNAs were stored at -20°C until needed.

2.4. DNA Microfluidic real-time PCR

To detect major TBPs (25 bacteria species, 7 parasite species, 5 bacteria genera, 3 parasite genera), the BioMark™ real-time PCR system (Fluidigm, San Francisco, CA, USA) was used for high-throughput microfluidic real-time PCR amplification using the 48.48 dynamic arrays (Fluidigm, San Francisco, CA, USA). These chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble real-time PCR reactions in individual chambers before thermal cycling, resulting in 2,304 individual reactions. Briefly, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene expression master mix following manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). PCR cycling comprised 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 2-step amplification of 15 s at 95°C, and 1 min at 60°C. One negative water control was included per chip. To determine if factors present in the sample could inhibit the PCR, *Escherichia coli* strain EDL933 DNA was added to each sample as an internal inhibition control, and primers and probe specific for the *E. coli* eae gene were used. For more details regarding the development of this new high throughput tool based on real-time microfluidic PCRs (test of sensitivity, specificity, and controls used) please see Michelet et al. (2014).

2.5. Validation of BioMark real-time PCR system results by PCR and DNA sequencing

In order to confirm the microfluidic real-time PCR results, all positive samples to infectious agents were subjected to conventional and nested PCR assays using different primers than those of the BioMark™ system (Table 1). Amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany) and assembled using the BioEdit software (Ibis Biosciences, Carlsbad). The final nucleotide sequences were analyzed to identify the sequenced microorganisms using the GenBank database through the National Center for Biotechnology Information (NCBI; Bethesda, MD) Basic Local Alignment Sequence Tool (BLAST) search engine (www.ncbi.nlm.nih.gov/blast). Nucleotide sequence data reported in the present study are available in the GenBank, EMBL and DDBJ databases under the accession numbers MT358275 to MT358279.

3. Results

3.1. Pathogen prevalence and coinfections

To assess the incidence of TBPs in humans infested by ticks, this study included a cohort of individuals (n = 30) infested by ticks (n = 31) from 13 municipalities of Serbia (Figure 1). Except for one *Rhipicephalus sanguineus* s.l. adult male tick, all ticks infesting humans were morphologically identified as *I. ricinus* (10 females, 0 males, 19 nymphs and 1 larva, Supplementary Table S1). The patients stated that, before removal by the patient or the physician, the ticks were attached for <24 hours (h) (45%, 14/31), between 24 h and 48 h (12%, 4/31) and >72 h (41%, 13/31). Most of the patients were older than 50 years old (45%, 14/31), followed by patients between 25 and 50 (29%, 9/31) years old and patients less than 25 years old (25%, 8/31) (Supplementary Table S1). Most patients were from the South Bačka district (70%, 22/31), and the remaining cases of tick infestation were spread in other districts of Serbia (Figure 1).

After the molecular diagnosis performed on ticks using a high-throughput microfluidic real-time PCR system, a total of 23 (74%, 23/31) ticks were infected with at least one of the tested pathogens. Out of 23 PCR-positive ticks, 11 (47%) harbored a single infection, 11 (47%) were co-infected by two pathogens, and one (4%) by four pathogens. Overall, 12 different pathogens were identified with variable prevalence, where *Rickettsia* spp. was the most common pathogen (67%, 21/31) detected in ticks, followed by *R. helvetica* (54%, 17/31), *Borrelia* spp. (29%, 9/31), *Anaplasma* spp. (9%, 3/31), *B. afzelii* (9%, 3/31), *A. phagocytophilum* (6%, 2/31), *B. miyamotoi* (6%, 2/31), and *Francisella* like-endosymbiont (6%, 2/31), whereas *B. valaisiana* (3%, 1/31), *B. lusitaniae* (3%, 1/31), *R. felis* (3%, 1/31) and *R. aeschlimannii* (3%, 1/31) were detected in one tick each. The occurrence of single and mixed infections of pathogens in ticks is summarized in Table 2. Despite the high frequency of infected ticks, only two human blood samples (6%, 2/30) tested positive for the presence of TBPs. The patients, identified in this study as H12 and H17, were diagnosed with *Borrelia* spp. and *R. felis* infection, respectively. The tick infesting patient H12 tested positive for *Borrelia* spp., *B. afzelii*, *Rickettsia* spp. and *R. helvetica* and the tick infesting patient H17 tested positive for *Rickettsia* spp. and *R. felis*.

3.2. Case descriptions

3.2.1 Case 1, patient code H12

On 12 June 2019, a 67-years-old female was presented at Pasteur Institute Novi Sad parasitized by a nymphal tick with a size of 2.5 mm and an approximate feeding time of more than 72 h. On 27 June 2019, examination of the tick bite lesion revealed a redness of 5 mm in diameter at the tick-bite site and no discomfort was reported by the patient. The diameter of the lesion was not adequate to be classified as migratory erythema. Therefore, the lesion was encircled with a felt-tip pen and the patient was advised to contact the physician if redness breach the circle. No antibiotic therapy was indicated. On 10 July 2019, the patient reports an erythema migrans of 5 cm diameter present at the tick-bite site. No additional pathology was observed. The patient was treated orally with doxycycline (Dovicin®, Galenika AD, Serbia) therapy at 100 mg twice daily for 14 days. After one week of treatment the migratory erythema was fading, and the patient was advised continue with doxycycline (Dovicin®, Galenika AD, Serbia) therapy until 14th day. In the last medical check-up, subsequent clinical examination revealed that the migratory erythema has completely disappeared.

3.2.2. Case 2, patient code H17

On 28 May 2019, a 71 years old female was presented at Pasteur Institute Novi Sad parasitized by an adult female tick with a size of 3 mm and an approximate feeding time of less than 24 h. On 4 June 2019, examination revealed a lesion at the tick-bite site that appeared as an atypical migratory erythema. The lesion was red with a central darkening and 2 cm in diameter. The patient was diagnosed with atypical erythema migrans. In addition, the patient reported itch at the tick-bite site. She denies the presence of nausea, fever, headache and the rest of the general infectious syndrome symptoms since removal of the tick. No additional pathology was observed. The patient was treated with doxycycline (Dovicin®, Galenika AD, Serbia) therapy at 100 mg twice daily for 14 days. On 12 June, after eight days of treatment the patient still complains about itching at tick-bite site, besides the lesion that resembled to atypical migratory erythema was 2 cm in diameter and fading. The patient was advised to continue with doxycycline therapy and to apply antihistamine for topical usechloropyramine (Synopen®, Pliva AD, Croatia) two-three times daily until next examination. Once finished the doxycycline treatment clinical examination revealed that the lesion in the form of atypical migratory erythema disappeared, although a macula was present at tick-bite site.

205 ### 3.3. Sequence analysis

206 The sequencing analysis of the flagellin gene (*flaB*) nucleotide sequences confirmed the
207 presence of *B. burgdorferi* s.l. genospecies in ticks, including *B. afzelii*, *B. valaisiana*, and *B.*
208 *lusitaniae*, as well as the outer-membrane protein gene (*ompB*) nucleotide sequence
209 corroborated *R. felis* infection in patient H12. Table 3 shows details about the nucleotide
210 sequences obtained from genes of TBPs species detected infecting *I. ricinus* ticks and human
211 blood samples, including the highest percentages of identity with reference strain sequences
212 available in the GenBank database. Unfortunately, sequencing analysis could not be
213 performed for some of the pathogens detected in ticks due to low cycle threshold (Cq) values
214 of PCR-positive samples.

215 ## 4. Discussion

216 ### 4.1. Pathogen infection and co-infections

217 To our knowledge, this is the first comprehensive study to utilize a high-throughput
218 microfluidic real-time PCR system for large-scale screening of TBPs in ticks collected from
219 humans and human blood samples in Serbia. The microfluidic real-time PCR system herein
220 employed offers a unique ability to detect simultaneously a diverse array of TBPs, and
221 constitutes an alternative solution to use in large scale surveys instead of standard PCR assays
222 (Michelet et al., 2014). Likely, this result is due to the high sensitivity of this methodology
223 which combines pathogen DNA steps with specific quantitative amplification of target
224 pathogen DNA (Gondard et al., 2019). In the present study, except for one case of *R.*
225 *sanguineus* infestation, *I. ricinus* was the sole tick species identified parasitizing humans and
226 nymph life stage was the most prevalent. *I. ricinus* is the most widespread ixodid tick species
227 in Europe, which is known to transmit a wide number of infectious agents of veterinary and
228 public health concern (Rizzoli et al., 2014b), especially nymphs that represent the most high-
229 risk stage to public health due to their small size, their marked anthropophily, their
230 abundance, and capacity to remain attached to the hosts for long periods (Vassallo et al.,
231 2000).

232 Overall, a high percentage of ticks was test-positive for DNA of nine different pathogen
233 bacteria species belonging to four genera, including *Anaplasma*, *Borrelia*, *Francisella* and
234 *Rickettsia*. Interestingly, the obtained results showed that co-infections occurred in more than
235 half of the infected ticks, and that ticks could be infected with up to four pathogens. The

occurrence of co-infections in *I. ricinus* tick species with TBPs have been frequently reported in Europe in ticks collected from humans (Matei et al., 2017), domestic and wild animals (Cabezas-Cruz et al., 2019; Ghafar et al., 2020), as well as in questing ticks (Klitgaard et al., 2019). These findings confirm the capacity of this tick species to feed on a broad variety of vertebrate species that can host multiple TBPs (Lommano et al., 2012). Multiple pathogen infections in individual ticks may occur through several mechanisms, including superinfection of ticks with prior transovarial infection, infection with multiple pathogens via co-feeding, multiple infections after feeding on hosts positive for several TBPs, and successive infectious blood meals (Raileanu et al., 2017). Tick-borne co-infections can have a huge impact on public health due to the disease may enhance disease severity, or evolve with atypical symptoms, resulting in diagnostic and treatment difficulties (Moutailler et al., 2016). For instance, Krause et al. (1996) described that concurrent infection with Babesiosis and Lyme disease, co-infected patients showed a greater number and severe influenza-like symptoms for a longer duration than those with Lyme disease alone.

The present study describes the detection of several TBPs of zoonotic concern in *I. ricinus* ticks, including *A. phagocytophilum*, *B. afzelii*, *B. lusitaniae*, *B. miyamotoi*, *B. valaisiana*, *R. aeschlimannii*, *R. felis* and *R. helvetica.* Similarly, the occurrence of all these pathogens in *I. ricinus* ticks has been reported in former studies conducted in Serbia and neighboring countries including Bulgaria, Croatia, Hungary, and Romania, as well as in most of the Balkan Peninsula (Tomanovic et al., 2013; Ionita et al., 2016; Potkonjak et al., 2016; Szekeres et al., 2016). However, the results herein described are really interesting by itself, considering that this is the first molecular study reporting these pathogens in *I. ricinus* ticks collected feeding on humans, as well as the human infection with *Borrelia* spp. and *R. felis* in Serbia. Interestingly, despite the high infection prevalence found in ticks, there were only two patients that tested PCR-positive for TBPs infection.

4.2. Clinical Cases

Clinical examination of patient H12 revealed an erythema migrans and the PCR analysis was positive for *Borrelia* spp. infection. Although the *Borrelia* species could not be identified, since the *I. ricinus* nymph tick collected on patient H12 was tested positive for *B. afzelii* infection by PCR and sequencing analyses, based on these findings the patient was diagnosed with Lyme borreliosis. Among the four *B. burgdorferi* s.l. genospecies herein described, *B. afzelii* is the only one proven human pathogenic genospecies, which mainly, but not

exclusively is associated with erythema migrans (Balmelli and Piffaretti, 1995; Stanek et al., 2011). To the authors' knowledge, this study is the first molecular evidence of *B. afzelii* in *I. ricinus* ticks infesting humans in Serbia. Therefore, further studies are needed to determine the prevalence, occurrence and risk assessment of the Lyme borreliosis to humans in Serbia.

For the second patient, H17, the clinical examination revealed an atypical erythema migr ans, and both the patient and the tick collected from her were found to be positive for *R. felis* by PCR and sequencing analyses. Rickettsiosis in humans caused by *R. felis* is considered as an emerging disease variously referred to as flea-borne spotted fever (FBSF), cat flea typhus or cat flea spotted fever (Perez-Osorio et al., 2008). The clinical syndrome of FBSF includes several symptoms that range from non-specific flu-like illness (i.e., pyrexia, arthralgia, myalgia, headache, and fatigue) to severe multi-systemic disease accompanied by a maculopapular rash, due to widespread vasculitis (Richards et al., 2010; Maina et al., 2012; Angelakis et al., 2016). Currently, the transmission of *R. felis* via competent tick vectors is controversial (Legendre and Macaluso, 2017), however, the results herein incriminate *I. ricinus* as a vector of *R. felis* in humans.

In conclusion, the results of present study provide molecular evidence for the presence of 11 different tick-borne pathogens of zoonotic concern in ticks feeding on humans, as well as the human infection with *Borrelia* spp. and *R. felis* in Serbia. This study increases the awareness of public health officials and physicians about the potential risk of tick-borne diseases, which should be included in the differential diagnosis when dealing with a febrile patient with history of possible tick bite. In addition, the results obtained highlight evidence about the transmission of *R. felis* via tick vectors, although further experimental studies are necessary to confirm this statement. These findings expand current knowledge on the distribution of tick-borne pathogens in Serbia, which will contribute to a better understanding of the epidemiological and epizootiological situation resulting in the improvement of measures programs to diagnose, treat, and control of transmission to humans and animals.

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Tables

Table 1. Set of primers used for validation of microfluidic real-time PCR results

Pathogens	Target genes	Primers sequences (5´- 3´)	Amplicon size	References
Borrelia spp.	*flaB*	Outer primers		Loh et al. (2016)
		GCAGTTCARTCAGGTAACGG GCAATCATAGCCATTGCAGATTGT	645 pb	
		Inner primers		
		GCATCAACTGTRGTTGTAACATTAAC AGG ACATATTCAGATGCAGACAGAGGT	407 pb	
Anaplasma / *Ehrlichia*	*16S* rRNA	Outer primers		Rar et al. (2005)
		GAACGAACGCTGGCGGCAAGC AGTA(T/C)CG(A/G)ACCAGATAGCCGC	686 pb	
		Inner primers		
		TGCATAGGAATCTACCTAGTAG AGTA(T/C)CG(A/G)ACCAGATAGCCGC	592 pb	
Rickettsia spp.	*ompB*	Outer primers		Choi et al. (2005)
		GTCAGCGTTACTTCTTCGATGC CCGTACTCCATCTTAGCATCAG	475 pb	
		Inner primers		
		CCAATGGCAGGACTTAGCTACT AGGCTGGCTGATACACGGAGTAA	267 pb	

451 **Table 2.** Microfluidic real-time PCR frequency of vector-borne pathogens detected in ticks (*n*
452 = 31) from Serbia.

Vector-borne pathogen(s)	**Total**	**%**	**95 % IC[a]**
Total infected ticks (≥1 pathogen)	23	74	57–90
Rickettsia spp.	21	67	50–85
R. helvetica	17	54	36–73
R. felis	1	3	0–9
R. aeschlimannii	1	3	0–9
Borrelia spp.	9	29	12–45
B. afzelii	3	9	0–20
B. miyamotoi	2	6	0–15
B. lusitaniae	1	3	0–9
B. valaisiana	1	3	0–9
Anaplasma spp.	3	9	0–20
A. phagocytophilum	2	6	0–15
Francisella like-endosymbiont	2	6	0–15
Single infections	11	35	17–53
Rickettsia spp.	1	3	0–9
R. helvetica	9	29	12–45
R. felis	1	3	0–9
A. phagocytophilum	1	3	0–9
Mixed infections	12	38	20–56
Mixed infections with two pathogens	11	35	17–53
Borrelia spp. + *Rickettsia* spp.	4	12	0–25
R. helvetica + *B. afzelii*	2	6	0–15
R. helvetica + *B. miyamotoi*	1	3	0–9
R. helvetica + *B. valaisiana*	1	3	0–9
R. helvetica + *A. phagocytophilum*	1	3	0–9
R. helvetica + *Francisella* like-endosymbiont	1	3	0–9
R. aeschlimannii + *Francisella* like-endosymbiont	1	3	0–9
Mixed infections with four pathogens	1	3	0–9
B. lusitaniae + *B. miyamotoi* + *Rickettsia* spp. + *Anaplasma* spp.	1	3	0–9
Non-detected	7	22	6–38

453 [a] 95 % confidence interval, Yates continuity correction performed

Table 3. Sequence analysis of genes of TBPs species detected infecting *I. ricinus* ticks and human blood samples.

TBPs species (*number*)	Host species	Sequenced amplicon	% Query cover	% Identity	GenBank
B. afzelii (2)	*I. ricinus* (2N)*	*flaB*	100	98.55-99.84	CP018262
B. valaisiana (1)	*I. ricinus* (1F)*	*flaB*	100	100	CP009117
B. lusitaniae (1)	*I. ricinus* (1F)*	*flaB*	92	97.75	MK604288
R. felis (1)	Human (H17)**	*ompB*	100	100	CP000053

()* number, sex, and life stage of sampled tick, where N = Nymph, F = Female, M = Male.

()** patient code

Figures

Figure 1. Geographical location of the patients infested by ticks in this study conducted in Serbia. The samples distribution is presented by municipalities (red color), across the districts of Serbia. The Serbian and Kosovo shapefile for mapping at district and municipality levels is available at the GADM database of Global Administrative Areas (v3.6, April 2020, https://gadm.org/). The map was generated by using QGIS v3.12 (QGIS Development Team 2020).

Municipality	Samples (n)
Beočin	2
Bor	1
Čačak	1
Irig	1
Majdanpek	1
Novi Sad	10
Petrovaradin	6
Soko Banja	1
Sremski Karlovci	2
Titel	1
Bački Petrovac	1
Bajina Bašta	2
Sremska Mitrovica	2
Total	31