

1 **Humans infested with *Ixodes ricinus* are exposed to a diverse array of tick-borne**
2 **pathogens in Serbia**

3

4 Pavle Banović^{1,2,†}, Adrian Alberto Díaz-Sánchez^{3,†}, Clemence Galon⁴, Verica Simin⁵,
5 Dragana Mijatović^{1,6}, Dasiel Obregón^{7,8}, Sara Moutailler^{4,*}, Alejandro Cabezas-Cruz^{4,*}

6

7 ¹Ambulance for Lyme Borreliosis and Other Tick-Borne Diseases, Pasteur Institute Novi Sad,
8 Novi Sad 21000, Serbia.

9 ²University of Novi Sad, Medical faculty Novi Sad, Department of Microbiology with
10 Parasitology and Immunology. Hajduk Veljkova 1-3, Novi Sad, Serbia.

11 ³Clinical Laboratory, Department of Clinical Diagnostics and Services, and Center for
12 Clinical Studies, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.

13 ⁴UMR BIPAR, INRAE, ANSES, Ecole Nationale Vétérinaire d'Alfort, Université Paris-Est,
14 Maisons-Alfort, 94700, France.

15 ⁵Department for Microbiological & Other Diagnostics, Pasteur Institute Novi Sad, 21000
16 Novi Sad, Serbia.

17 ⁶Faculty of Medical Sciences, University of Pristina – Kosovska Mitrovica. 38220 Kosovska
18 Mitrovica, Serbia.

19 ⁷School of Environmental Sciences University of Guelph, Guelph, Ontario N1G 2W1,
20 Canada.

21 ⁸Center for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, São Paulo
22 13400-970 Brazil.

23 † **Equal contribution**

24 * **Corresponding author(s):** Alejandro Cabezas-Cruz (cabezasalejandrocruz@gmail.com)
25 and Sara Moutailler (sara.moutailler@anses.fr)

26 **Abstract**

27 Tick-borne pathogens (TBPs) pose a major threat to human health in Europe and the whole
28 northern hemisphere. Despite a high prevalence of TBPs in *Ixodes ricinus* ticks, knowledge
29 on the incidence of tick-borne diseases in humans infested by this tick species is limited. This
30 study was conducted in the year 2019 on patients who presented themselves to the Pasteur
31 Institute Novi Sad with tick infestations. Ticks ($n = 31$) feeding on human ($n = 30$) and blood
32 samples from the same individuals were collected by physicians and a microfluidic real-time
33 high-throughput PCR system was used to test the genomic DNA of the samples for the
34 presence of 27 bacterial and eight parasitic microorganisms in Serbia. Except for one
35 *Rhipicephalus sanguineus* s.l. adult male tick, all ticks infesting humans were
36 morphologically identified as *I. ricinus*. A high proportion of ticks (74%, 23/31) were infected
37 with at least one of the tested TB microorganisms, being *Rickettsia helvetica* (54%, 17/31) the
38 most common pathogen, but *Borrelia afzelii* (9%, 3/31), *Anaplasma phagocytophilum* (6%,
39 2/31), *Borrelia miyamotoi* (6%, 2/31), and *Francisella* like-endosymbiont (6%, 2/31),
40 *Borrelia valaisiana* (3%, 1/31), *Borrelia lusitaniae* (3%, 1/31), *Rickettsia felis* (3%, 1/31) and
41 *Rickettsia aeschlimannii* (3%, 1/31) were also identified. Despite the high infection rate of
42 TBPs in ticks, only two human blood samples (6%, 2/30) tested positive for the presence of
43 TBPs, one patient (code H12, 67 years old female) was diagnosed with *Borrelia* spp. and the
44 other patient was diagnosed (code H17, 71 years old female) with *R. felis* infection. The tick
45 infesting patient H12 tested positive for *B. afzelii*, and *R. helvetica* and the tick infesting
46 patient H17 tested positive for *R. felis*. Upon clinical examination, both patients were
47 diagnosed with erythema migrans. No additional discomfort was reported by the patient and
48 no additional pathology was observed by the physician. We concluded that humans bitten by
49 *I. ricinus* in Serbia are exposed to a diverse array of TBPs with clinical impact in the Serbian
50 cohort studied.

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

52 **1. Introduction**

53 Ixodid ticks (Acari: Ixodida) are the most important vectors of a wide variety of pathogens
54 that cause infectious diseases in humans and animals (de la Fuente et al., 2017). *Ixodes ricinus*
55 is the most widespread hard tick species in Europe and feeds on a broad range of hosts,
56 serving as reservoir and vector of many zoonotic pathogens, including bacteria, protozoa,
57 viruses, and helminths (Rizzoli et al., 2014a). Lyme borreliosis, caused by some genospecies
58 of the *Borrelia burgdorferi* sensu lato (s.l.) complex, is the most prevalent *I. ricinus*-borne
59 disease in humans in Europe, with an estimated incidence of 85,000 cases per year (Pritt et al.,
60 2016). The *B. burgdorferi* s.l. complex currently includes over 20 different genospecies, nine
61 of which are circulating in Europe, including the human pathogens *B. burgdorferi* sensu
62 stricto (s.s.), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia bavariensis*, and *Borrelia spielmanii*;
63 and *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia bissettiiae*, and *Borrelia kurtenbachii*,
64 for which the human pathogenicity remains uncertain (Clark et al., 2014; Raileanu et al.,
65 2017).

66 *Rickettsia* spp. are fastidious obligate intracellular alpha-proteobacteria transmitted by
67 hematophagous arthropods such as fleas, mites, ticks and lice (Fournier and Raoult, 2007).
68 The genus *Rickettsia* comprises four large groups, namely the typhus group (TG), the spotted
69 fever group (SFG), the transitional group (TRG) and the ancestral group (AG), of which the
70 SFG and TG are the most important in terms of public health (Gillespie et al., 2008). Bacteria
71 of SFG are transmitted mainly by hard ticks (Ixodidae), including *I. ricinus* which have been
72 recognized as vectors and reservoirs of these pathogens (Parola et al., 2013). In Serbia, a
73 previous study conducted by Milutinovic et al. (2008) described a high prevalence of *B.*
74 *burgdorferi* s.l. (42.5%) in 287 host-seeking adult *I. ricinus* ticks collected from vegetation at
75 18 localities throughout nationwide. The presence of five *B. burgdorferi* s.l. genospecies,
76 namely, *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. valaisiana* was
77 identified by restriction fragment length polymorphism (RFLP) analysis. In addition,
78 Radulovic et al. (2011) reported the presence of SFG rickettsiae members *Rickettsia helvetica*
79 (7.7%) and *Rickettsia monacensis* (15.4%) in *I. ricinus* ticks, in a study aimed to investigate
80 the presence of Rickettsiae in 131 questing ticks belonging to five different species (i.e.,
81 *Dermacentor marginatus*, *Dermacentor reticulatus*, *Haemaphysalis punctata*, *Haemaphysalis*
82 *concinna*, and *I. ricinus*) collected from seven localities in Serbia.

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

86 **2. Material and methods**

87 **2.1. Ethics statement**

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

91 **2.2. Sample collection, tick identification and DNA extraction**

92 A study was conducted in the year 2019 to assess human exposure to TBP infection in
93 individuals ($n = 30$) infested with ticks ($n = 31$) in Serbia. Ticks feeding on human and blood
94 samples from the same individuals were collected by physicians and stored at -80°C until
95 analysis. Besides, clinical examination was performed on each patient. For each patient, 2 ml
96 of blood were collected and used for DNA extraction using the Nucleospin Tissue kit
97 (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions. All ticks
98 collected from these patients were identified regarding species, sex, and life stage, based on
99 morphological features according to standard taxonomic keys described by Estrada-Peña et al.
100 (2004). For each tick collected on a patient, ticks were homogenized using Precellys 24
101 lyser/homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5500 rpm for
102 20 s using 2.8 mm stainless steel beads in 180 μL of Lysis buffer (T1 buffer) and 25 μL of
103 Proteinase K from the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany). Then
104 homogenates were incubated 3 hours at 56°C and DNA extraction was performed according
105 to the manufacturer's instructions. Purified DNA was eluted into 50 μL elution buffer.

106 **2.3. DNA pre-amplification**

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

113 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
114 cycling program the reactions were diluted 1:10 in Milli-Q ultrapure water. Pre-amplified
115 DNAs were stored at -20°C until needed.

116 **2.4. DNA Microfluidic real-time PCR**

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

133 **2.5. Validation of BioMark real-time PCR system results by PCR and DNA sequencing**

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

144 **3. Results**

145 **3.1. Pathogen prevalence and coinfections**

146 To assess the incidence of TBPs in humans infested by ticks, this study included a cohort of
147 individuals ($n = 30$) infested by ticks ($n = 31$) from 13 municipalities of Serbia (Figure 1).
148 Except for one *Rhipicephalus sanguineus* s.l. adult male tick, all ticks infesting humans were
149 morphologically identified as *I. ricinus* (10 females, 0 males, 19 nymphs and 1 larva,
150 Supplementary Table S1). The patients stated that, before removal by the patient or the
151 physician, the ticks were attached for <24 hours (h) (45%, 14/31), between 24 h and 48 h
152 (12%, 4/31) and >72 h (41%, 13/31). Most of the patients were older than 50 years old (45%,
153 14/31), followed by patients between 25 and 50 (29%, 9/31) years old and patients less than
154 25 years old (25%, 8/31) (Supplementary Table S1). Most patients were from the South
155 Bačka district (70%, 22/31), and the remaining cases of tick infestation were spread in other
156 districts of Serbia (Figure 1).

157 After the molecular diagnosis performed on ticks using a high-throughput microfluidic real-
158 time PCR system, a total of 23 (74%, 23/31) ticks were infected with at least one of the tested
159 pathogens. Out of 23 PCR-positive ticks, 11 (47%) harbored a single infection, 11 (47%) were
160 co-infected by two pathogens, and one (4%) by four pathogens. Overall, 12 different
161 pathogens were identified with variable prevalence, where *Rickettsia* spp. was the most
162 common pathogen (67%, 21/31) detected in ticks, followed by *R. helvetica* (54%, 17/31),
163 *Borrelia* spp. (29%, 9/31), *Anaplasma* spp. (9%, 3/31), *B. afzelii* (9%, 3/31), *A.*
164 *phagocytophilum* (6%, 2/31), *B. miyamotoi* (6%, 2/31), and *Francisella* like-endosymbiont
165 (6%, 2/31), whereas *B. valaisiana* (3%, 1/31), *B. lusitaniae* (3%, 1/31), *R. felis* (3%, 1/31) and
166 *R. aeschlimannii* (3%, 1/31) were detected in one tick each. The occurrence of single and
167 mixed infections of pathogens in ticks is summarized in Table 2. Despite the high frequency
168 of infected ticks, only two human blood samples (6%, 2/30) tested positive for the presence of
169 TBPs. The patients, identified in this study as H12 and H17, were diagnosed with *Borrelia*
170 spp. and *R. felis* infection, respectively. The tick infesting patient H12 tested positive for
171 *Borrelia* spp., *B. afzelii*, *Rickettsia* spp. and *R. helvetica* and the tick infesting patient H17
172 tested positive for *Rickettsia* spp. and *R. felis*.

173 **3.2. Case descriptions**

174 **3.2.1 Case 1, patient code H12**

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

188 **3.2.2. Case 2, patient code H17**

189 On 28 May 2019, a 71 years old female was presented at Pasteur Institute Novi Sad
190 parasitized by an adult female tick with a size of 3 mm and an approximate feeding time of
191 less than 24 h. On 4 June 2019, examination revealed a lesion at the tick-bite site that
192 appeared as an atypical migratory erythema. The lesion was red with a central darkening and
193 2 cm in diameter. The patient was diagnosed with atypical erythema migrans. In addition, the
194 patient reported itch at the tick-bite site. She denies the presence of nausea, fever, headache
195 and the rest of the general infectious syndrome symptoms since removal of the tick. No
196 additional pathology was observed. The patient was treated with doxycycline (Dovicin®,
197 Galenika AD, Serbia) therapy at 100 mg twice daily for 14 days. On 12 June, after eight days
198 of treatment the patient still complains about itching at tick-bite site, besides the lesion that
199 resembled to atypical migratory erythema was 2 cm in diameter and fading. The patient was
200 advised to continue with doxycycline therapy and to apply antihistamine for topical
201 usechloropyramine (Synopen®, Pliva AD, Croatia) two-three times daily until next
202 examination. Once finished the doxycycline treatment clinical examination revealed that the
203 lesion in the form of atypical migratory erythema disappeared, although a macula was present
204 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

236 occurrence of co-infections in *I. ricinus* tick species with TBPs have been frequently reported
237 in Europe in ticks collected from humans (Matei et al., 2017), domestic and wild animals
238 (Cabezas-Cruz et al., 2019; Ghafar et al., 2020), as well as in questing ticks (Klitgaard et al.,
239 2019). These findings confirm the capacity of this tick species to feed on a broad variety of
240 vertebrate species that can host multiple TBPs (Lommano et al., 2012). Multiple pathogen
241 infections in individual ticks may occur through several mechanisms, including superinfection
242 of ticks with prior transovarial infection, infection with multiple pathogens via co-feeding,
243 multiple infections after feeding on hosts positive for several TBPs, and successive infectious
244 blood meals (Raileanu et al., 2017). Tick-borne co-infections can have a huge impact on
245 public health due to the disease may enhance disease severity, or evolve with atypical
246 symptoms, resulting in diagnostic and treatment difficulties (Moutailler et al., 2016). For
247 instance, Krause et al. (1996) described that concurrent infection with Babesiosis and Lyme
248 disease, co-infected patients showed a greater number and severe influenza-like symptoms for
249 a longer duration than those with Lyme disease alone.

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

261 **4.2. Clinical Cases**

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

268 exclusively is associated with erythema migrans (Balmelli and Piffaretti, 1995; Stanek et al.,
269 2011). To the authors' knowledge, this study is the first molecular evidence of *B. afzelii* in *I.*
270 *ricinus* ticks infesting humans in Serbia. Therefore, further studies are needed to determine
271 the prevalence, occurrence and risk assessment of the Lyme borreliosis to humans in Serbia.

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

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

294 **References**

- 295 Angelakis, E., Mediannikov, O., Parola, P., Raoult, D., 2016. *Rickettsia felis*: The Complex
 296 Journey of an Emergent Human Pathogen. Trends Parasitol. 32, 554-564.
 297 doi:[10.1016/j.pt.2016.04.009](https://doi.org/10.1016/j.pt.2016.04.009).
- 298 Balmelli, T., Piffaretti, J.C., 1995. Association between different clinical manifestations of
 299 Lyme disease and different species of *Borrelia burgdorferi* sensu lato. Res. Microbiol.
 300 146, 329-340. doi:[10.1016/0923-2508\(96\)81056-4](https://doi.org/10.1016/0923-2508(96)81056-4).
- 301 Cabezas-Cruz, A., Allain, E., Ahmad, A.S., Saeed, M.A., Rashid, I., Ashraf, K., Yousfi, L.,
 302 Shehzad, W., Indjein, L., Rodriguez-Valle, M., Estrada-Pena, A., Obregon, D., Jabbar,
 303 A., Moutailler, S., 2019. Low genetic diversity of *Ehrlichia canis* associated with high
 304 co-infection rates in *Rhipicephalus sanguineus* (s.l.). Parasit. Vectors 12, 12.
 305 doi:[10.1186/s13071-018-3194-9](https://doi.org/10.1186/s13071-018-3194-9).
- 306 Choi, Y.J., Lee, S.H., Park, K.H., Koh, Y.S., Lee, K.H., Baik, H.S., Choi, M.S., Kim, I.S.,
 307 Jang, W.J., 2005. Evaluation of PCR-based assay for diagnosis of spotted fever group
 308 rickettsiosis in human serum samples. Clin. Diagn. Lab. Immunol. 12, 759-763. doi:
 309 [10.1128/CDLI.12.6.759-763.2005](https://doi.org/10.1128/CDLI.12.6.759-763.2005).
- 310 Clark, K.L., Leydet, B.F., Threlkeld, C., 2014. Geographical and genospecies distribution of
 311 *Borrelia burgdorferi* sensu lato DNA detected in humans in the USA. J. Med.
 312 Microbiol. 63, 674-684. doi:[10.1099/jmm.0.073122-0](https://doi.org/10.1099/jmm.0.073122-0).
- 313 de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Pena,
 314 A., Johnson, N., Kocan, K.M., Mansfield, K.L., Nijhof, A.M., Papa, A., Rudenko, N.,
 315 Villar, M., Alberdi, P., Torina, A., Ayllon, N., Vancova, M., Golovchenko, M.,
 316 Grubhoffer, L., Caracappa, S., Fooks, A.R., Gortazar, C., Rego, R.O.M., 2017. Tick-
 317 Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for
 318 Tick-Borne Diseases. Front. Cell. Infect. Microbiol. 7, 114.
 319 doi:[10.3389/fcimb.2017.00114](https://doi.org/10.3389/fcimb.2017.00114).
- 320 Estrada-Peña, A., Bouattour, A., Camicas, J., Walker, A., 2004. Ticks of domestic animals in
 321 the Mediterranean region: A guide to identification of species. University of Zaragoza,
 322 Spain 131.
- 323 Fournier, P.-E., Raoult, D., 2007. Bacteriology, taxonomy, and phylogeny of *Rickettsia*.
 324 Infect. Dis. Therapy Ser. 43, 13-26.
- 325 Ghafar, A., Cabezas-Cruz, A., Galon, C., Obregon, D., Gasser, R.B., Moutailler, S., Jabbar,
 326 A., 2020. Bovine ticks harbour a diverse array of microorganisms in Pakistan. Parasit.
 327 Vectors 13, 1. doi:[10.1186/s13071-019-3862-4](https://doi.org/10.1186/s13071-019-3862-4).
- 328 Gillespie, J.J., Williams, K., Shukla, M., Snyder, E.E., Nordberg, E.K., Ceraul, S.M.,
 329 Dharmanolla, C., Rainey, D., Soneja, J., Shallom, J.M., Vishnubhat, N.D., Wattam,
 330 R., Purkayastha, A., Czar, M., Crasta, O., Setubal, J.C., Azad, A.F., Sobral, B.S.,
 331 2008. *Rickettsia* phylogenomics: unwinding the intricacies of obligate intracellular
 332 life. PloS one 3, e2018. doi:[10.1371/journal.pone.0002018](https://doi.org/10.1371/journal.pone.0002018).
- 333 Gondard, M., Delannoy, S., Pinarello, V., Aprelon, R., Devillers, E., Galon, C., Pradel, J.,
 334 Vayssier-Taussat, M., Albina, E., Moutailler, S., 2020. Upscaling surveillance of tick-
 335 borne pathogens in the French Caribbean islands. Pathogens 9, E176, doi:
 336 [10.3390/pathogens9030176](https://doi.org/10.3390/pathogens9030176).
- 337 Ionita, M., Silaghi, C., Mitrea, I.L., Edouard, S., Parola, P., Pfister, K., 2016. Molecular
 338 detection of *Rickettsia conorii* and other zoonotic spotted fever group rickettsiae in
 339 ticks, Romania. Ticks Tick Borne Dis. 7, 150-153. doi:[10.1016/j.ttbdis.2015.10.006](https://doi.org/10.1016/j.ttbdis.2015.10.006).
- 340 Klitgaard, K., Kjaer, L.J., Isbrand, A., Hansen, M.F., Bodker, R., 2019. Multiple infections in
 341 questing nymphs and adult female *Ixodes ricinus* ticks collected in a recreational

342 forest in Denmark. Ticks Tick Borne Dis. 10, 1060-1065.
343 doi:[10.1016/j.ttbdis.2019.05.016](https://doi.org/10.1016/j.ttbdis.2019.05.016).

344 Krause, P.J., Telford, S.R., 3rd, Spielman, A., Sikand, V., Ryan, R., Christianson, D., Burke,
345 G., Brassard, P., Pollack, R., Peck, J., Persing, D.H., 1996. Concurrent Lyme disease
346 and babesiosis. Evidence for increased severity and duration of illness. JAMA 275,
347 1657-1660. doi:[10.1001/jama.1996.03530450047031](https://doi.org/10.1001/jama.1996.03530450047031).

348 Legendre, K.P., Macaluso, K.R., 2017. *Rickettsia felis*: A Review of Transmission
349 Mechanisms of an Emerging Pathogen. Trop. Med. Infect. Dis. 2, 64.
350 doi:[10.3390/tropicalmed2040064](https://doi.org/10.3390/tropicalmed2040064).

351 Loh, S.-M., Gofton, A.W., Lo, N., Gillett, A., Ryan, U.M., Irwin, P.J., Oskam, C.L., 2016.
352 Novel *Borrelia* species detected in echidna ticks, *Bothriocroton concolor*, in Australia.
353 Parasit. Vectors 9, 339. doi: [10.1186/s13071-016-1627-x](https://doi.org/10.1186/s13071-016-1627-x).

354 Lommano, E., Bertaiola, L., Dupasquier, C., Gern, L., 2012. Infections and coinfections of
355 questing *Ixodes ricinus* ticks by emerging zoonotic pathogens in Western Switzerland.
356 Appl. Environ. Microbiol. 78, 4606-4612. doi:[10.1128/AEM.07961-11](https://doi.org/10.1128/AEM.07961-11).

357 Maina, A.N., Knobel, D.L., Jiang, J., Halliday, J., Feikin, D.R., Cleaveland, S., Ng'ang'a, Z.,
358 Junghae, M., Breiman, R.F., Richards, A.L., Njenga, M.K., 2012. *Rickettsia felis*
359 infection in febrile patients, western Kenya, 2007-2010. Emerg. Infect. Dis. 18, 328-
360 331. doi:[10.3201/eid1802.111372](https://doi.org/10.3201/eid1802.111372).

361 Matei, I.A., Kalmar, Z., Lupse, M., D'Amico, G., Ionica, A.M., Dumitrache, M.O., Gherman,
362 C.M., Mihalca, A.D., 2017. The risk of exposure to rickettsial infections and human
363 granulocytic anaplasmosis associated with *Ixodes ricinus* tick bites in humans in
364 Romania: A multiannual study. Ticks Tick Borne Dis. 8, 375-378.
365 doi:[10.1016/j.ttbdis.2016.12.013](https://doi.org/10.1016/j.ttbdis.2016.12.013).

366 Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., Chirico, J.,
367 van der Wal, F.J., Sprong, H., Boye Pihl, T.P., Klitgaard, K., Bodker, R., Fach, P.,
368 Moutailler, S., 2014. High-throughput screening of tick-borne pathogens in Europe.
369 Front. Cell. Infect. Microbiol. 4, 103. doi:[10.3389/fcimb.2014.00103](https://doi.org/10.3389/fcimb.2014.00103).

370 Milutinovic, M., Masuzawa, T., Tomanovic, S., Radulovic, Z., Fukui, T., Okamoto, Y., 2008.
371 *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Francisella tularensis*
372 and their co-infections in host-seeking *Ixodes ricinus* ticks collected in Serbia. Exp.
373 Appl. Acarol. 45, 171-183. doi:[10.1007/s10493-008-9166-6](https://doi.org/10.1007/s10493-008-9166-6).

374 Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E.,
375 Cosson, J.F., Gasqui, P., Van, V.T., Mavingui, P., Vourc'h, G., Vayssier-Taussat, M.,
376 2016. Co-infection of Ticks: The Rule Rather Than the Exception. PLoS Negl. Trop.
377 Dis. 10, e0004539. doi:[10.1371/journal.pntd.0004539](https://doi.org/10.1371/journal.pntd.0004539).

378 Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T.,
379 Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.E., Raoult, D., 2013. Update on tick-
380 borne rickettsioses around the world: A geographic approach. Clin. Microbiol. Rev.
381 26, 657-702. doi:[10.1128/CMR.00032-13](https://doi.org/10.1128/CMR.00032-13).

382 Perez-Osorio, C.E., Zavala-Velazquez, J.E., Arias Leon, J.J., Zavala-Castro, J.E., 2008.
383 *Rickettsia felis* as emergent global threat for humans. Emerg. Infect. Dis. 14, 1019-
384 1023. doi:[10.3201/eid1407.071656](https://doi.org/10.3201/eid1407.071656).

385 Potkonjak, A., Kleinerman, G., Gutierrez, R., Savic, S., Vracar, V., Nachum-Biala, Y., Jurisic,
386 A., Rojas, A., Petrovic, A., Ivanovic, I., Harrus, S., Baneth, G., 2016. Occurrence of
387 *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* Ticks with First Identification of
388 *Borrelia miyamotoi* in Vojvodina, Serbia. Vector Borne Zoonotic Dis. 16, 631-635.
389 doi:[10.1089/vbz.2016.2008](https://doi.org/10.1089/vbz.2016.2008).

390 Pritt, B.S., Mead, P.S., Johnson, D.K.H., Neitzel, D.F., Respicio-Kingry, L.B., Davis, J.P.,
391 Schiffman, E., Sloan, L.M., Schriefer, M.E., Replogle, A.J., Paskewitz, S.M., Ray,

392 J.A., Bjork, J., Steward, C.R., Deedon, A., Lee, X., Kingry, L.C., Miller, T.K., Feist,
393 M.A., Theel, E.S., Patel, R., Irish, C.L., Petersen, J.M., 2016. Identification of a novel
394 pathogenic *Borrelia* species causing Lyme borreliosis with unusually high
395 spirochaetaemia: a descriptive study. *Lancet Infect. Dis.* 16, 556-564.

396 QGIS Development Team, 2020. QGIS Geographic Information System, v3.12 . Open Source
397 Geospatial Foundation Project. <https://qgis.org/>

398 Radulovic, Z., Chochlakis, D., Tomanovic, S., Milutinovic, M., Tselentis, Y., Psaroulaki, A.,
399 2011. First detection of spotted fever group Rickettsiae in ticks in Serbia. *Vector*
400 *Borne Zoonotic Dis.* 11, 111-115. doi:[10.1089/vbz.2009.0254](https://doi.org/10.1089/vbz.2009.0254).

401 Raileanu, C., Moutailler, S., Pavel, I., Porea, D., Mihalca, A.D., Savuta, G., Vayssier-Taussat,
402 M., 2017. *Borrelia* Diversity and Co-infection with Other Tick Borne Pathogens in
403 Ticks. *Front. Cell. Infect. Microbiol.* 7, 36. doi:[10.3389/fcimb.2017.00036](https://doi.org/10.3389/fcimb.2017.00036).

404 Rar, V.A., Fomenko, N.V., Dobrotvorsky, A.K., Livanova, N.N., Rudakova, S.A., Fedorov,
405 E.G., Astanin, V.B., Morozova, O.V., 2005. Tick-borne pathogen detection, Western
406 Siberia, Russia. *Emerg. Infect. Dis.* 11, 1708-1715. doi: [10.3201/eid1111.041195](https://doi.org/10.3201/eid1111.041195).

407 Richards, A.L., Jiang, J., Omulo, S., Dare, R., Abdirahman, K., Ali, A., Sharif, S.K., Feikin,
408 D.R., Breiman, R.F., Njenga, M.K., 2010. Human Infection with *Rickettsia felis*,
409 Kenya. *Emerg. Infect. Dis.* 16, 1081-1086. doi:[10.3201/eid1607.091885](https://doi.org/10.3201/eid1607.091885).

410 Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubalek, Z., Foldvari, G., Plantard, O.,
411 Vayssier-Taussat, M., Bonnet, S., Spitalska, E., Kazimirova, M., 2014a. *Ixodes ricinus*
412 and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New
413 Hazards and Relevance for Public Health. *Front. Public Health* 2, 251.
414 doi:[10.3389/fpubh.2014.00251](https://doi.org/10.3389/fpubh.2014.00251).

415 Rizzoli, A., Silaghi, C., Obiegala, A., Rudolf, I., Hubálek, Z., Földvári, G., Plantard, O.,
416 Vayssier-Taussat, M., Bonnet, S., Špitalská, E., Kazimírová, M., 2014b. *Ixodes ricinus*
417 and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New
418 Hazards and Relevance for Public Health. *Front. Public Health* 2, 251.

419 Stanek, G., Fingerle, V., Hunfeld, K.P., Jaulhac, B., Kaiser, R., Krause, A., Kristoferitsch, W.,
420 O'Connell, S., Ornstein, K., Strle, F., Gray, J., 2011. Lyme borreliosis: clinical case
421 definitions for diagnosis and management in Europe. *Clin. Microbiol Infect.* 17, 69-
422 79. doi:[10.1111/j.1469-0691.2010.03175.x](https://doi.org/10.1111/j.1469-0691.2010.03175.x).

423 Szekeres, S., Docters van Leeuwen, A., Rigo, K., Jablonszky, M., Majoros, G., Sprong, H.,
424 Foldvari, G., 2016. Prevalence and diversity of human pathogenic rickettsiae in urban
425 versus rural habitats, Hungary. *Exp. Appl. Acarol.* 68, 223-226.

426 Tomanovic, S., Chochlakis, D., Radulovic, Z., Milutinovic, M., Cakic, S., Mihaljica, D.,
427 Tselentis, Y., Psaroulaki, A., 2013. Analysis of pathogen co-occurrence in host-
428 seeking adult hard ticks from Serbia. *Exp. Appl. Acarol.* 59, 367-376.
429 doi:[10.1007/s10493-012-9597-y](https://doi.org/10.1007/s10493-012-9597-y).

430 Vassallo, M., Paul, R.E.L., Pérez-Eid, C., 2000. Temporal Distribution of the Annual
431 Nymphal Stock of *Ixodes Ricinus* Ticks. *Exp. Appl. Acarol.* 24, 941-949.
432 doi:[10.1023/A:1010669003887](https://doi.org/10.1023/A:1010669003887).

433 **Tables**434 **Table 1.** Set of primers used for validation of microfluidic real-time PCR results

Pathogens	Target genes	Primers sequences (5' - 3')	Amplicon size	References	
<i>Borrelia</i> spp.	<i>flaB</i>	Outer primers			
		GCAGTTCARTCAGGTAACGG	645 pb		
		GCAATCATAGCCATTGCAGATTGT			
		Inner primers		Loh et al. (2016)	
		GCATCAACTGTRGTTGTAACATTAAC	407 pb		
		AGG ACATATTCAGATGCAGACAGAGGT			
<i>Anaplasma / Ehrlichia</i>	16S rRNA	Outer primers			
		GAACGAACGCTGGCGGCAAGC	686 pb		
		AGTA(T/C)CG(A/G)ACCAGATAGCCGC			
		Inner primers		Rar et al. (2005)	
		TGCATAGGAATCTACCTAGTAG	592 pb		
		AGTA(T/C)CG(A/G)ACCAGATAGCCGC			
<i>Rickettsia</i> spp.	<i>ompB</i>	Outer primers			
		GTCAGCGTACTTCTTCGATGC	475 pb		
		CCGTACTCCATCTTAGCATCAG			
		Inner primers		Choi et al. (2005)	
		CCAATGGCAGGACTTAGCTACT	267 pb		
		AGGCTGGCTGATACACGGAGTAA			

435

436

437

438

439

440

441

442

443

444

445

446

447

448

449

450

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
<i>Rickettsia</i> spp.	21	67	50–85
<i>R. helvetica</i>	17	54	36–73
<i>R. felis</i>	1	3	0–9
<i>R. aeschlimannii</i>	1	3	0–9
<i>Borrelia</i> spp.	9	29	12–45
<i>B. afzelii</i>	3	9	0–20
<i>B. miyamotoi</i>	2	6	0–15
<i>B. lusitaniae</i>	1	3	0–9
<i>B. valaisiana</i>	1	3	0–9
<i>Anaplasma</i> spp.	3	9	0–20
<i>A. phagocytophilum</i>	2	6	0–15
<i>Francisella</i> like-endosymbiont	2	6	0–15
Single infections	11	35	17–53
<i>Rickettsia</i> spp.	1	3	0–9
<i>R. helvetica</i>	9	29	12–45
<i>R. felis</i>	1	3	0–9
<i>A. phagocytophilum</i>	1	3	0–9
Mixed infections	12	38	20–56
Mixed infections with two pathogens	11	35	17–53
<i>Borrelia</i> spp. + <i>Rickettsia</i> spp.	4	12	0–25
<i>R. helvetica</i> + <i>B. afzelii</i>	2	6	0–15
<i>R. helvetica</i> + <i>B. miyamotoi</i>	1	3	0–9
<i>R. helvetica</i> + <i>B. valaisiana</i>	1	3	0–9
<i>R. helvetica</i> + <i>A. phagocytophilum</i>	1	3	0–9
<i>R. helvetica</i> + <i>Francisella</i> like-endosymbiont	1	3	0–9
<i>R. aeschlimannii</i> + <i>Francisella</i> like-endosymbiont	1	3	0–9
Mixed infections with four pathogens	1	3	0–9
<i>B. lusitaniae</i> + <i>B. miyamotoi</i> + <i>Rickettsia</i> spp. + <i>Anaplasma</i> spp.	1	3	0–9
Non-detected	7	22	6–38

453 ^a 95 % confidence interval, Yates continuity correction performed

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

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

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

457 (***) patient code

458

459

460

461

462

463

464

465

466

467

468

469

470

471

472

473

474

475

476

477

478

479

480

481

482

483

484 **Figures**

485 **Figure 1.** Geographical location of the patients infested by ticks in this study conducted in
486 Serbia. The samples distribution is presented by municipalities (red color), across the districts
487 of Serbia. The Serbian and Kosovo shapefile for mapping at district and municipality levels is
488 available at the GADM database of Global Administrative Areas (v3.6, April 2020,
489 <https://gadm.org/>). The map was generated by using QGIS v3.12 (QGIS Development Team
490 2020).

4800000

5000000

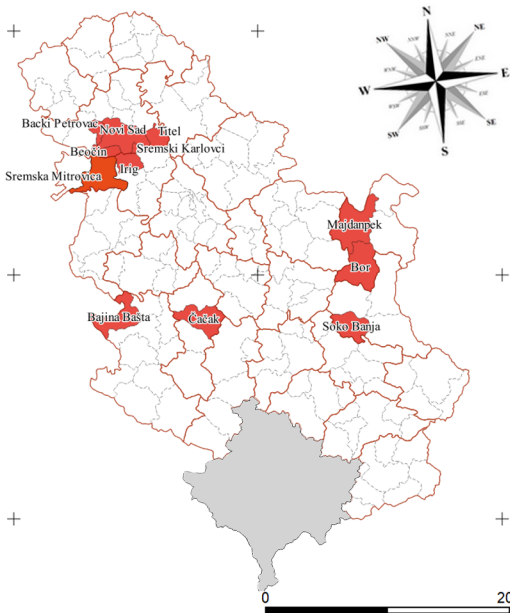
5200000

5400000

2600000

2600000

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



— District boundary
 - - - Municipality boundary

2200000

2200000

4800000

5000000

5200000

5400000