

Diversity of Coxiella-like and Francisella-like endosymbionts, and Rickettsia spp., Coxiella burnetii as pathogens in the tick populations of Slovakia, Central Europe

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Abstract

Ticks are important vectors of pathogens affecting humans and animals worldwide. They do not only carry pathogens but diverse commensal and symbiotic microorganisms are also present in ticks. A molecular screening for tick-borne pathogens and endosymbionts was carried out in *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis inermis* questing ticks collected in Slovakia. The presence of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella*-like and *Francisella*-like microorganisms was evaluated by PCR in 605 individuals and by randomly sequencing 66 samples. Four species of rickettsiae (*R. raoultii*, *R. slovacica*, *R. helvetica* and *R. monacensis*) were identified and reported with an overall prevalence range between 0.4 and 50.3% (± 8.0) depending on tick species, sex and locality. Partial sequencing of the *gltA* gene of 5 chosen samples in *H. inermis* showed 99% identity with *Candidatus Rickettsia hungarica*. The total prevalence of *C. burnetii* in ticks was $2.2 \pm 1.7\%$; bacteria were confirmed in *I. ricinus* and *D. reticulatus* ticks. The sequences from 2 *D. reticulatus* males and 1 *I. ricinus* female ticks were compared to GenBank submissions and a 99.8% match was obtained with the pathogenic *C. burnetii*. *Coxiella*-like endosymbionts were registered in all three species of ticks from all studied sites with an average prevalence of $32.7 \pm 3.7\%$. A phylogenetic analysis of this *Coxiella* sp. showed that it does not group with the pathogenic *C. burnetii*. The prevalence of *Francisella*-like microorganisms in questing ticks was $47.9 \pm 3.9\%$, however *H. inermis* ($n = 108$) were not infested. Obtained sequences were 98% identical with previously identified *Francisella*-like endosymbionts in *D. reticulatus* and *I. ricinus*. *Coxiella*-like and *Francisella*-like microorganisms were identified for the first time in Slovakia, they might be considered as a non-pathogenic endosymbiont of *I. ricinus*, *D. reticulatus* and *H. inermis*, and future investigations could aim to assess their role in these ticks. However, this work provided further data and broadened our knowledge on bacterial pathogens and endosymbionts present in ticks in Slovakia to help understanding co-infestations, combined treatments and public health issues linked to tick bites.

Keywords	tick; pathogen; endosymbiont; Slovakia
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4 pathogens in the tick populations of Slovakia, Central Europe
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62 35 ABSTRACT
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65 37 Ticks are important vectors of pathogens affecting humans and animals worldwide. They do not only
66 38 carry pathogens but diverse commensal and symbiotic microorganisms are also present in ticks. A
67 39 molecular screening for tick-borne pathogens and endosymbionts was carried out in *Ixodes ricinus*,
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69 41 of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella*-like and *Francisella*-like microorganisms was evaluated by
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81 53 were 98% identical with previously identified *Francisella*-like endosymbionts in *D. reticulatus* and *I.*
82 54 *ricinus*. *Coxiella*-like and *Francisella*-like microorganisms were identified for the first time in Slovakia,
83 55 they might be considered as a non-pathogenic endosymbiont of *I. ricinus*, *D. reticulatus* and *H. inermis*,
84 56 and future investigations could aim to assess their role in these ticks. However, this work provided
85 57 further data and broadened our knowledge on bacterial pathogens and endosymbionts present in ticks
86 58 in Slovakia to help understanding co-infestations, combined treatments and public health issues linked
87 59 to tick bites.
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101 61
102 61 Keywords: tick, pathogen, endosymbiont, Slovakia
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1. Introduction

Ticks are obligate blood sucking ectoparasites of vertebrate animals. Microbial communities hosted by ticks include tick-borne pathogens (viruses, bacteria, protozoa) and non-pathogenic microorganisms such as commensal and mutualistic microbes abundant in ticks (Andreotti et al., 2011; Carpi et al., 2011; Williams-Newkirk et al., 2014; Duron et al., 2015a, 2017). Diversity within microbial communities could be correlated to tick species, different tissues and organs, season, geographical regions, tick life stage, and feeding statuses (Carpi et al., 2011; Lalzar et al., 2012; Menchaca et al., 2013; Zhang et al., 2014; Egyed and Makrai, 2014; Budachetri et al., 2014; Qiu et al., 2014; Williams-Newkirk et al., 2014; Zolnik et al., 2016;).

Ixodes ricinus, *Dermacentor reticulatus*, *Dermacentor marginatus*, *Haemaphysalis concinna*, *Haemaphysalis inermis* and *Haemaphysalis punctata* tick species are common and widespread in Slovakia. *Ixodes ricinus* ticks, considered as vectors and reservoir hosts, were collected from different localities in Slovakia, where it had been previously found to be infected with *Rickettsia helvetica* and *Rickettsia monacensis*, while *Dermacentor* spp. ticks were found infected with *Rickettsia slovaca* and *Rickettsia raoultii* (Špitalská et al., 2012, 2014, 2016; Minichová et al., 2017). Although these rickettsial species (Proteobacteria: Rickettsiales) are known to be pathogenic to humans they are usually linked to mild clinical symptoms (Uchiyama, 2012; Oteo and Portillo, 2012). Rickettsial species and the role of *Haemaphysalis* ticks as vectors in Slovakia have not been revealed to this day.

Coxiella burnetii (Proteobacteria: Legionellales) is the etiological agent of human Q fever, a zoonotic disease distributed worldwide and causing a disease with symptoms including fever, hepatitis, and respiratory complications (Raoult, 1993). Ticks play an important role in the circulation of *C. burnetii* in natural foci and are responsible for the dissemination of the infection among animals. The presence of *C. burnetii* was previously isolated from *I. ricinus*, *D. reticulatus*, *D. marginatus*, *H. concinna* and *H. inermis* ticks in Slovakia (Řeháček et al., 1991, Špitalská and Kocianová, 2003). *Coxiella*-like endosymbionts (CLEs), similar to *C. burnetii* are present in different tick species such as *Ornithodoros muesebecki*, *Rhipicephalus sanguineus*, *Haemaphysalis longicornis*, *Ixodes woodi*, *I. ricinus*, *Amblyomma americanum* (Zhong, 2012; Al-Deeb et al., 2016), without specific tissue location. The prevalence of CLEs varies among different species of ticks. As summarised by Zhong (2012) it is ranging from 5 to 100%. CLEs have not been studied in arthropods in Slovakia so far.

Francisella tularensis (Proteobacteria: Thiotrichales) is the etiological agent of the tularemia (Chu and Weyant, 2003). *Francisella tularensis* naturally occurs in vertebrates, invertebrates, and in contaminated soil, water, and vegetation (Mörner, 1992). The clinical presentation of tularemia varies depending upon the route of infection. The principal tick vectors include species of the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Ornithodoros* (Gordon et al., 1983). Many tick species are also hosts of *Francisella*-like endosymbionts (FLEs), bacteria closely related to *F. tularensis*

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105 (Dergousoff and Chilton, 2012). The pathogenic potential of FLEs remains unknown. FLEs appear to
106 replicate intracellularly, and they are transmitted transovarially. To date, there is no evidence of
107 horizontal transmission through tick bites (Ivanov et al., 2011). FLEs are widely distributed in Europe
108 and were identified in *D. reticulatus*, *Hyalomma marginatum*, *Hyalomma aegyptium* and *Rhipicephalus*
109 *sanguineus* sensu lato in Hungary, Portugal, France, Germany and Bulgaria (Sréter-Lancz et al., 2009;
110 Ivanov et al., 2011; Kreizinger et al., 2013; De Carvalho et al., 2011; Michelet et al., 2013; Gehringer et
111 al., 2013;). No data are known for the occurrence of FLEs in ticks of Slovakia.

112 No recent reports are available on the occurrence of rickettsial species in *Haemaphysalis* ticks,
113 *Coxiella*-like and *Francisella*-like endosymbionts in ticks, and simultaneous occurrence of pathogenic
114 *Rickettsia* species and *C. burnetii* with CLEs and FLEs in potential arthropod vectors in Slovakia. To
115 understand better the circulation in Slovakia of these pathogens and symbionts we collected questing
116 *D. reticulatus*, *I. ricinus* and *H. inermis* ticks.

2. Material and methods

2.1. Collection of ticks

120 A total of 605 questing ticks of following species *D. reticulatus*, *I. ricinus*, and *H. inermis* were
121 collected in March and April 2012, during year 2016 and in May 2017. Ticks were collected by dragging
122 a woollen flag over the lower vegetation and along the paths in mixed forests in four localities
123 Gabčíkovo, Zohor, Stará Lesná, and Hrhov. Gabčíkovo (47°54 N, 17°34.983 E) is situated in southwest
124 Slovakia, 110 m above sea level (asl), alluvial habitat near river Danube. Zohor (48°20.374 N, 16°56.791
125 E) is situated in west Slovakia, 144 m asl, with mixed deciduous forest of oak, hornbeam and hazel near
126 river Morava. Ticks were collected on the edge of forests near the Zohor, Láb and Vysoká pri Morave
127 villages. Stará Lesná (49°08.166 N, 20°18.575 E), High Tatras, 770 m a.s.l is located in north Slovakia,
128 with deciduous forest of birch, rowan and spruce. Ticks were collected across the woods along the
129 forest path, while the last site was a typical mixed forest with a predominance of beech, oak and
130 hornbeam. The last sampling site was located in the Slovak Karst National Park, near the village Hrhov
131 (200–220 m a.s.l., 48°34.899 N, 20°46.743 E). Ticks were collected on the edges of the forests and
132 pastures in this area.

2.2. DNA extraction from ticks

135 Ticks were washed with sterile water, dried, transferred to individual tubes and crushed with
136 a sterile carbon steel surgical scalpel blade (Surgeon, JAI Surgicals Ltd., India). Total DNA was isolated
137 from ticks separately using the method of alkaline hydrolysis (Rijpkema et al., 1996). The concentration
138 and purity of DNA were measured by NanoPhotometer Pearl (Implen, Germany). DNA samples were
139 stored at -20 °C and later used as templates for the PCR amplifications.

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2.3. Molecular analysis

Ticks samples were screened by PCR-based methods for the presence of *Rickettsia* spp. and *C. burnetii* tick-borne pathogens, CLEs and FLEs tick endosymbionts. *Rickettsia* species were identified based on the amplification of the *gltA*, *ompA* and *sca4* genes, *C. burnetii* and FLEs based on the 16S rRNA, and CLEs based on the *GroEl* gene (Forsman et al., 1994; Roux et al., 1996; Sekeyová et al., 2001; Melničáková et al., 2003; Boretti et al., 2009; Duron et al., 2014). *Rickettsial* species were identified by species-specific real-time PCR, *Rickettsia helvetica* identification was based on the 23S rRNA gene, *Rickettsia slovaca* and *R. raoultii* identification were based on the *ompB* gene (Boretti et al., 2009; Jiang et al., 2012). PCR amplifications were performed on a TPersonal thermocycler (Biometra, Germany) or a Labcycler (SensoQuest, Germany). PCR products were analysed by electrophoresis in a 1% agarose gel stained with GelRed™ (Biotium, Hayward, California, USA) and visualized under a UV transilluminator. The real-time PCR assays were performed using a Bio-Rad CFX96™ Real-Time System. Negative and positive controls were included in each PCR-based assays.

2.4. DNA sequencing and phylogenetic analysis

In total, 66 randomly selected amplicons from *gltA*, *ompA*, 16S rRNA and *GroEl* genes were purified and both strands were sequenced by MacroGen Inc. (Amsterdam, The Netherlands). Obtained sequences were compared with available sequences listed in the GenBank nucleotide sequence database. The phylogenetic trees were produced according to the Neighbor-Joining method using bootstrap analyses with 1,000 replicates using MEGA 5 software (Felsenstein, 1985; Saitou and Nie, 1987; Tamura et al., 2011).

2.5. Statistical analysis

Statistical analyses to test for differences in the prevalence of microorganisms in questing ticks between tick species, tick sex and sites were carried out using Fisher's exact test with an online calculator (<http://www.socscistatistics.com>). A p value < 0.05 was considered significant. Ninety-five percent confidence intervals (CI) were calculated using an online calculator (<http://epitools.ausvet.com.au>).

3. Results

A total of 605 ticks of three species, 334 *D. reticulatus* (154 females and 180 males), 163 *I. ricinus* (93 females, 48 males and 22 nymphs), and 108 *H. inermis* (75 females, 33 males) were collected from vegetation of natural sites Zohor, Gabčíkovo, Stará Lesná and Hrhov, in Slovakia (Table 1).

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298 174 *Rickettsia* spp. DNA was confirmed in 215 (35.5±3.8%) ticks of all three species in all studied
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300 175 sites. DNA of *R. raoultii* and *R. slovaca* were identified in *D. reticulatus* ticks (78 females and 90 males),
301 176 *R. raoultii* was dominant species. DNA of *R. helvetica* and *R. monacensis* were found in *I. ricinus* ticks
302
303 177 (9 females, 6 males and 3 nymphs), *R. helvetica* was dominant species (Table 1). No significant
304 178 difference was found in the prevalence of *R. raoultii* (49.4±7.9% versus 49.4±7.3%) or *R. helvetica*
305 179 (9.7±6% versus 12.5±9.4%) between female and male ticks, respectively. No significant difference was
306 179 also observed for *R. helvetica* between adult and nymphal stages (9.7±6%, 12.5±9.4%). 12 positive
307 180 samples for *Rickettsia* in *H. inermis* were randomly selected for further sequencing. Partial sequencing
308 181 of *gltA* gene of 5 samples (MG821159) showed 99% identity with *Candidatus Rickettsia hungarica*
309 182 isolate Hu5-2007 (EU853834) identified in *H. inermis* collected in Hungary (Hornok et al., 2010).
310 183 Unfortunately, partial sequencing of the *gltA* gene of 7 samples (MG821160) did not match any
311 184 rickettsial species identified in this study. They showed 99% identity with uncultured *Bartonella* sp.
312 185 (KJ663731) previously identified in *R. sanguineus* collected in Sicily, Italy (Otranto et al., 2014).
313 186 Appendix 1 shows a phylogenetic tree constructed on the basis of the *gltA* sequences. Partial
314 187 sequencing of *ompA* gene of all randomly chosen 12 samples did not show any identity with known
315 188 rickettsial species and fragments of *sca4* gene were not successfully amplified.
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323 190 *Coxiella burnetii* DNA was found in 7 *D. reticulatus* (4 females and 3 males) and in 8 *I. ricinus* (3
324 191 females, 3 males and 2 nymphs) ticks. *H. inermis* ticks were *C. burnetii* negative (Table 1). However,
325 192 the overall prevalence of CLEs in this study was 32.7±3.7%. The highest prevalence was recorded in *H.*
326 193 *inermis* (58 females, 33 males) with significant difference between the prevalences in males and
327 194 females. However, the differences between prevalence in males and females of *D. reticulatus* (36
328 195 females, 56 males) and *I. ricinus* (13 females, 4 males and 6 nymphs) were not statistically significant.
329 196 The presence of CLEs in *I. ricinus* collected in Zohor and Stará Lesná was statistically significantly
330 197 different. Totally 33 randomly chosen *Coxiella* spp.-positive samples were analysed by sequencing of
331 198 the *groEL* gene fragments (Appendix 2). Sequences from 2 *D. reticulatus* males and 1 *I. ricinus* female
332 199 samples (MG860513) were 99% identical with sequences of *C. burnetii* (CP014557, CP020616,
333 200 LK937696). A phylogenetic analysis of 30 *Coxiella* spp. from this study showed that they do not group
334 201 with the pathogenic *C. burnetii*. Eighteen sequences, 7 from *H. inermis* females and 11 from *H. inermis*
335 202 males (MG860512) were 80% identical to *Coxiella* endosymbiont of *Rhipicephalus geigy* isolate Rgei1
336 203 (KP985514) identified in *R. geigy* from Benin (Duron et al., 2015a). Two sequences from *D. reticulatus*
337 204 females (MG860511) were 87% identical to *Coxiella* endosymbiont of *Ornithodoros sonrai* isolate
338 205 Oson1 (KP985474) identified in *O. sonrai* collected in Senegal (Duron et al., 2015a). Next two
339 206 sequences, from *D. reticulatus* female and *I. ricinus* male (MG860510) were 99% identical to
340 207 *Rickettsiella* endosymbiont of *Ixodes ventalloi* isolate ixoventa6 (KY678006) identified in *I. ventalloi* tick
341 208 tissues (Duron et al., 2017). Two sequences derived from 1 female and 1 male *I. ricinus* (MG860509)

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357 209 were 99% identical to *Rickettsiella* endosymbiont of *Ixodes arboricola* isolate ixoarbo827 (KY677998)
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359 210 identified in *I. arboricola* (Duron et al., 2017). And the last six sequences (MG860514) derived from 3
360 211 *I. ricinus* males, 2 *D. reticulatus* males and 1 *D. reticulatus* female were 99% identical to *Serratia*
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362 212 *proteamaculans* (CP000826).

363 213 Analysis of the 16S rRNA gene revealed that FLEs were present in 47.9±3.9% ticks. The
364 214 highest prevalence (79.9±4.3%) was found in *D. reticulatus* ticks (140 females, 127 males), while *H.*
365 215 *inermis* ticks were negative (Table 1). Statistically, significant differences were found between the
366 216 prevalence in *D. reticulatus* collected in Zohor and Gabčíkovo (86.6±4.0% versus 47.4±13.0%), the
367 217 prevalence in the sex of *D. reticulatus* (70.6±6.7% in males and 90.9±4.5% in females), and the
368 218 prevalence in *I. ricinus* ticks in Stará Lesná and Zohor (33.3±11.9 versus 2.9±3.3). Totally, 21 randomly
369 219 selected *Francisella sp.*-positive ticks (11 from females and 10 from males of *D. reticulatus*) showed
370 220 identical DNA sequences to each other (MG889594) and were 98% identical with FLEs from isolate FLE
371 221 D1 (JX561116) identified in *D. reticulatus* tick collected in France (Michelet et al., 2013) and with FLEs
372 222 of *I. ricinus* (JQ740890) previously identified in *I. ricinus* larvae collected from birds in Hungary (Hornok
373 223 et al., 2013) (see Appendix 3).

374 224 The simultaneous occurrence of endosymbionts (CLEs, FLEs) and pathogens *Rickettsia* spp., *C.*
375 225 *burnetii*) was recorded in 74 (41.1±7.2%) *D. reticulatus* males, 90 (58.4±7.9%) *D. reticulatus* females, 1
376 226 *I. ricinus* male, 3 *I. ricinus* females, and 2 *I. ricinus* nymphs. All *H. inermis* males and 42 females carried
377 227 DNA of *Rickettsia* spp. and CLEs.

378 228

379 229 4. Discussion

380 230 This study is the first survey focusing on the simultaneous occurrence of bacterial tick-borne
381 231 pathogens and endosymbionts in *D. reticulatus*, *I. ricinus* and *H. inermis* ticks in Slovakia. All three
382 232 species represent epidemiologically and epizootologically important genera. The list of known tick-
383 233 borne pathogens is still evolving and their presence in ticks and hosts in Slovakia have been previously
384 234 studied. Non-pathogenic microorganisms, commensal and mutualistic microbes are also abundant in
385 235 ticks, but their presence was not identified in ticks from Slovakia until our study. Results of PCR assays
386 236 and sequences analyses revealed that *Rickettsia* spp., *Coxiella burnetii*, CLEs and FLEs co-infect *D.*
387 237 *reticulatus*, *I. ricinus* and *H. inermis* collected in four localities in Slovakia. The prevalence range of
388 238 *Rickettsia* spp. in ticks in the present study was 0.4-50.3% according to tick species as in the previous
389 239 studies done in Slovakia (Špitalská et al., 2012, 2014, 2016; Švehlová et al., 2014; Minichová et al.,
390 240 2017). Species identification confirmed the presence of *R. helvetica* and *R. monacensis* in *I. ricinus* ticks,
391 241 and *R. raoultii*, *R. slovaca* in *D. reticulatus*. These rickettsial species were identified as well in previous
392 242 studies in Slovakia (Špitalská et al., 2012, 2014, 2016; Švehlová et al., 2014; Minichová et al., 2017). In
393 243 this study the occurrences of *R. raoultii* in *D. reticulatus* and *R. helvetica* in *I. ricinus* ticks were without

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416 244 statistical significance for localities, adult sex or between adults and nymphs. The absence of
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418 245 statistically significant differences could be explained by transovarial transmission and/or
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420 246 transstadially survival of these rickettsiae. However, the presence of rickettsial species in
421
422 247 *Haemaphysalis* spp. ticks have been determined only by the haemocyte test or PCR without the further
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424 248 identification and so the species present in this tick species in Slovakia was not known (Špitalská et al.,
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426 249 2002; Špitalská and Kocianová, 2003; Boldiš et al., 2008). The sequence data analysis of the *gltA* gene
427
428 250 suggested the presence of *Cand. R. hungarica*. The identification of the above species, in this study,
429
430 251 expands the range of rickettsial species circulating in Slovakia.

429 252 The prevalence of *C. burnetii* in ticks in the present study was 1.7-2.9% in *D. reticulatus* and 5%
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431 253 in *I. ricinus*. *Coxiella burnetii* was identified by PCR in questing ticks in Slovakia in 2003 (Špitalská and
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433 254 Kocianová, 2003). After more than one decade, Minichová et al. (2017) did not detect any questing
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435 255 ticks or rodents-feeding ticks. However, this pathogen was confirmed in 2.7% of ticks feeding on birds
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437 256 (Berthová et al., 2016), which is similar prevalence than in this study. *Coxiella* - like bacteria are diverse
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439 257 and widespread in ticks and distinct from *C. burnetii*. *Coxiella* - like bacteria can be transovarially and
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441 258 transstadially transmitted (Duron et al., 2015a, b; Machado-Ferreira et al., 2016). *Coxiella* - like
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443 259 bacteria are very common in ticks, but their presence has not been studied previously in Slovakia, thus
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445 260 information about prevalence and molecular identification in this study are new for this region. The
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447 261 total prevalence of CLEs was 32.7±3.7% and CLEs were found in all three species of ticks from all studied
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449 262 sites with the highest prevalence in *H. inermis* (84.3%). The occurrence of CLEs in *D. reticulatus* and *I.*
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451 263 *ricinus* ticks was without statistical significance between males and females. There was also no
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453 264 statistical difference between localities for the prevalence of CLEs in *D. reticulatus* (contrary to what
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455 265 we found in *I. ricinus*), which could be due to different habitats, the low number of tested ticks, and
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457 266 the presence of tested nymphs in one locality while being absent in the second one. The differences in
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459 267 CLEs occurrence are common and can be explained by many factors but it still indicates that it is the
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461 268 most widespread and biologically relevant tick symbiont (Bonnet et al., 2017). Molecular analysis
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463 269 showed that they do not group with the pathogenic *C. burnetii*, but they group with CLEs identified in
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465 270 different tick species (Appendix 2). Four samples in our study were similar also to *Rickettsiella*
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467 271 endosymbionts, a facultative mutualist genus in aphids with unknown effect in ticks, identified in
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469 272 *Ixodes* ticks by Duron et al. (2017), which is also the first identification in Slovakia.

461 273 FLEs have not been studied in Slovakia to this time. Genetic analysis of FLEs identified in our
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463 274 samples showed a close relationship with the FLEs of *Dermacentor* spp., *Hyalomma* spp., *Rhipicephalus*
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465 275 spp. and *Amblyomma* spp. previously identified and distinct from the FLEs of *Ornithodoros* spp.
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467 276 (Michelet et al., 2013). Effect of FLEs in tick is unknown. They probably are obligate symbionts (Duron
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469 277 et al., 2017).

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278 Previous studies suggested that tick endosymbionts could have evolved from mammalian
279 pathogens or infective ancestors (Noda et al., 1997; Scoles, 2004; Machado-Ferreira et al., 2009; 2016;
280 Gerhart et al., 2016). The composition of microbial communities in tick is highly variable. Differences
281 in the internal bacterial flora among ticks of three species (*I. ricinus*, *D. reticulatus*, *H. concinna*) at the
282 same localities were confirmed by Egyed and Makrai (2014) too. Infestation of ticks can occur by
283 ingestion from the soil environment or through the blood (or skin) of the host. Variations in the
284 prevalence and the occurrence of tick symbionts can be linked to host preferences from the larval and
285 nymphal tick stages. For example, *H. inermis* larvae prefer lizards and feed on hosts very rapidly, only
286 1 - 2 hours (e.g. Nosek, 1973). By contrast, *D. reticulatus* larvae prefer several insectivores and rodent
287 species, feeding for several days on the hosts (Nosek, 1972; Földvari et al., 2016).

288 Our results showed the presence of pathogenic species of *Rickettsia* and *Coxiella burnetii* and
289 symbiotic *Coxiella*-like and *Francisella*-like microorganisms and their sympatric occurrence in *D.*
290 *reticulatus*, *I. ricinus* and *H. inermis* ticks. To know tick-borne bacteria, which could be affected by the
291 presence of another pathogens or symbionts is essential for monitoring and diagnosis of tick-borne
292 diseases in humans and animals.

293 294 **Conflict of interest**

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296 The authors declare that they have no conflict of interest.

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299
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807 473 Legends

809 474
810 475 **Table 1** Prevalence of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella*-like and *Francisella*-like
811 476 microorganisms in questing ticks collected at four sites in Slovakia [no. of infected/ no. of captured
812 477 (prevalence %±95% CI)]
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815 478 **Appendix 1** Phylogenetic tree inferred from comparison of the *Rickettsia gltA* partial sequences using
816 479 Neighbor-Joining method (Saitou, Nie, 1987). GeneBank accession numbers are included. Included
817 480 sequences without GeneBank accession numbers were previously published and not submitted to
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820 481 GenBank (Minichová et al. 2017). Bootstrap values of neighbor-joining (1,000 replicates) are shown.
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482 **Appendix 2** Phylogenetic tree inferred from comparison of the *Coxiella groEL* partial sequences using
483 Neighbor-Joining method (Saitou and Nie, 1987). GeneBank accession numbers are included.
484 Bootstrap values of neighbor-joining (1,000 replicates) are shown.

485 **Appendix 3** Neighbor-joining phylogenetic tree showing relationships of 16S rRNA gene sequences
486 obtained from *Francisella* species and *Francisella*-like endosymbionts (FLEs) with the novel *Francisella*-
487 like isolate from *Dermacentor reticulatus* ticks collected in Slovakia. Bootstrap values of neighbor-
488 joining (1,000 replicates) are shown.

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509 **Table 1**

	Rh	Rm	Rr	Rs	Rsp.	CB	CLEs	FLEs
Zohor								
DR males			65/130	0/130		2/130	42/130	105/130
			(50)			(1.5)	(32.3)	(80.8)
DR females			74/147	1/147		4/147	35/147	135/147
			(50.3)	(0.7)		(2.7)	(23.8)	(91.8)
DR			139/277	1/277		6/277	75/277	240/277
<i>Subtotal</i>			50.2±6.0	0.4±0.7		2.2±1.7	27.1±5.3	86.6±4.0
Gabčíkovo								
DR males			24/50	1/50		1/50	14/50	22/50
			(48)	(2)		(2)	(28)	(44)
DR females			2/7	1/7		0/7	1/7	5/7
			(28.6)	(20)			(14.3)	(71.4)
<i>Subtotal</i>			26/57	2/57		1/57	15/57	27/57
			45.6±12.9	3.5±5.0		1.8±3.4	26.3±11.4	47.4±13.0
Total DR					168/334	7/334	90/334	267/334
					50.3±5.4	2.1±1.5	26.9±4.7	79.9±4.3
Stará Lesná								
IR males	5/18	0/18				0/18	1/18	3/18
	(27.8)						(5.6)	(16.7)
IR females	0/20	0/20				1/20	6/20	11/20
						(5)	(30)	(55)
IR nymphs	1/22	2/22				2/22	4/22	6/22
	(4.5)	(9.1)				(9.1)	(18.2)	(27.3)
<i>Subtotal</i>	6/60	2/60				3/60	11/60	20/60
	10±7.6	3.3				5±5.5	18.3±9.8	33.3±11.9
Zohor								
IR males	1/30					3/30	1/30	1/30
	(3.3)					(10)	(3.3)	(3.3)
IR females	9/73					2/73	5/73	2/73
	(12.3)					(2.7)	(6.8)	(2.7)
<i>IR Subtotal</i>	10/103					5/103	6/103	3/103
	9.7±5.7					4.9±4.1	5.8±4.5	2.9±3.3

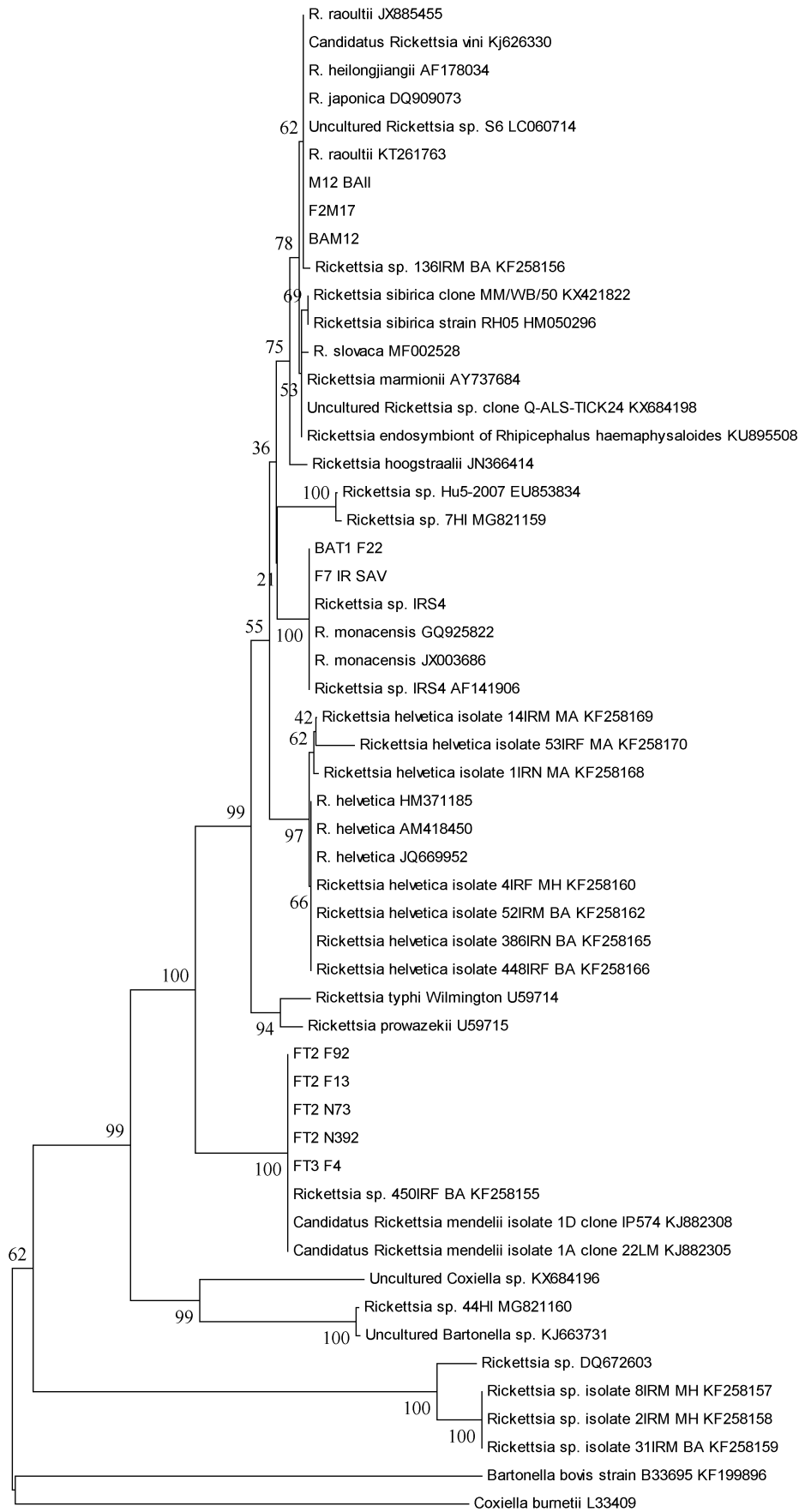
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Total IR	18/163	8/163	17/163
	11.0±4.8	4.9±3.3	10.4±4.7
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Hrhov			
HI males	8/33		33/33
	(24.2±14.6)		(100)
HI females	21/75		58/75
	(28±10.2)		(77.3)
Total HI	29/108		91/108
	26.9±8.4		84.3±6.9

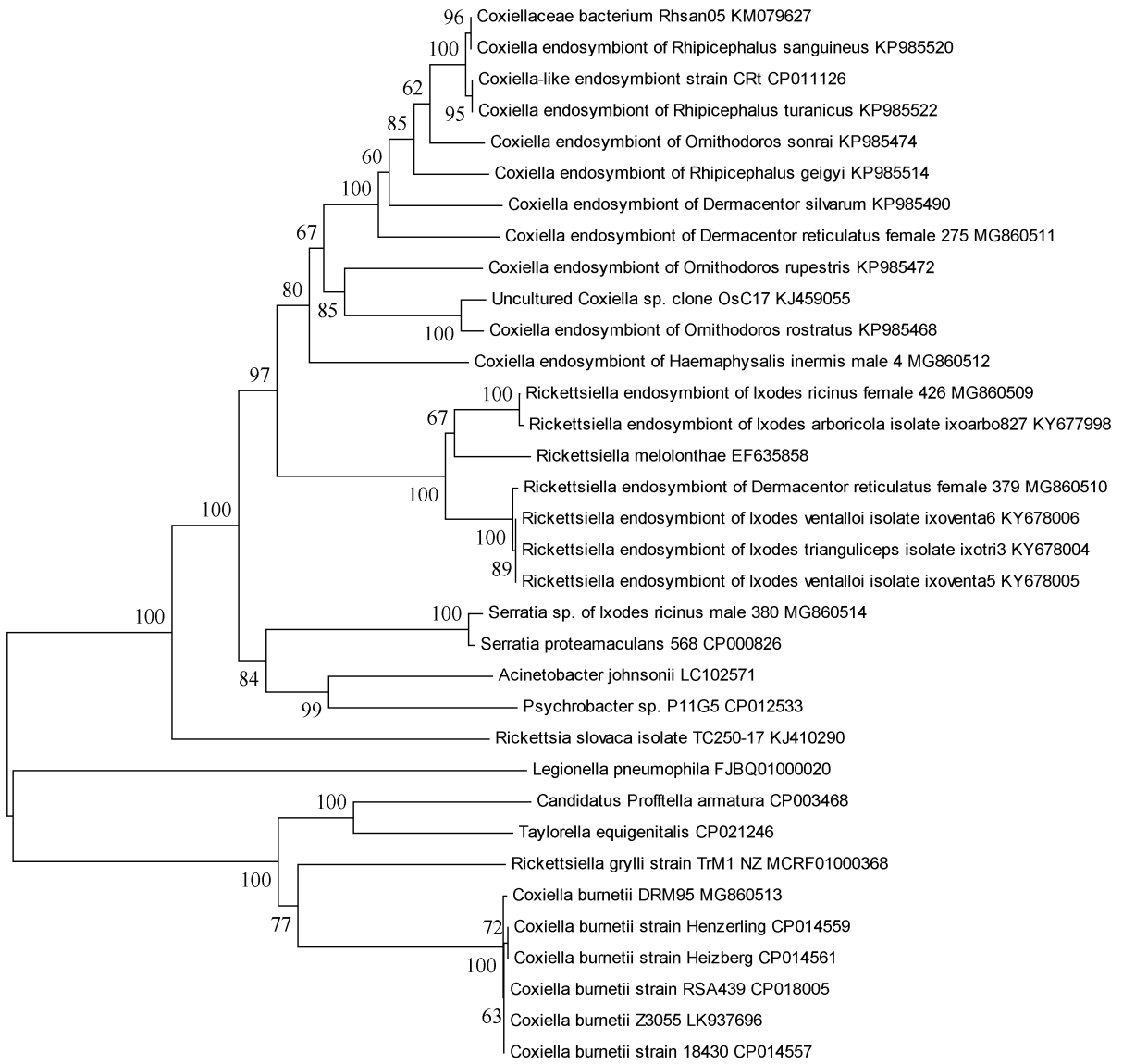
510 Rh - *Rickettsia helvetica*, Rm - *Rickettsia monacensis*, Rr - *Rickettsia raoultii*, Rs - *Rickettsia slovaca*,

511 Rsp - *Rickettsia* species, CB - *Coxiella burnetii*, CLEs - *Coxiella*-like endosymbionts, FLEs - *Francisella*-

512 like endosymbionts, DR - *Dermacentor reticulatus*, IR - *Ixodes ricinus*, HI - *Haemaphysalis inermis*



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89 Francisella tularensis strain 3523 AY243028
92 Francisella hispaniensis FSC454 CP018093
84 Francisella tularensis subsp. holarctica AJ698866
83 Wolbachia persica strain ATCC VR-331 M21292
68 Francisella endosymbiont of Hyalomma asiaticum isolate XJ-S3 KX852466
96 Francisella endosymbiont of Amblyomma geoemydae isolate AGMI6 KT382876
Francisella-like endosymbiont of Dermacentor reticulatus strain HS249 JQ942365
Francisella-like endosymbiont of Dermacentor reticulatus strain FDrH EU234535
95 Francisella-like endosymbiont strain FLE04 HQ705173
Francisella-like endosymbiont of Dermacentor reticulatus isolate FLE D1 JX561116
Francisella endosymbiont of Dermacentor reticulatus female 93 MG889594
Francisella tularensis subsp. tularensis strain NR-21737 CP017155
100 Francisella cf. novicida 3523 CP002558

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