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Short communication

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. slovaca, 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 ± 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 ± 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 ± 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.

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

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

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

2. Material and methods

2.1. Collection of ticks

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

2.2. DNA extraction from ticks

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

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., 2013; 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 1000 replicates using MEGA 5 software (Felsenstein, 1985; Saitou and Nei, 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).

Rickettsia spp. DNA was confirmed in 215 (35.5 ± 3.8%) ticks of all