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Sympatric occurrence of *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* ticks and *Rickettsia* and *Babesia* species in Slovakia

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ABSTRACT

Vojka nad Dunajom in the south-west of the Slovak Republic is a locality with sympatric occurrence of 3 species of ticks. This study investigated the spatial distribution of *Dermacentor reticulatus*, *Ixodes ricinus*, and *Haemaphysalis concinna* ticks in this area and determined the prevalence of *Babesia* and *Rickettsia* species in questing adults of these tick species considered as potential risk for humans and animals. Ticks were collected by blanket dragging over the vegetation from September 2011 to October 2012. All ticks were subjected to DNA extraction and individually assayed with PCR-based methods targeting the *gltA*, *sca4*, 23S rRNA genes of *Rickettsia* spp. and the 18S rRNA gene of *Babesia* spp.

D. reticulatus was the dominant species occurring in this area (67.7%, $n = 600$), followed by *I. ricinus* (31.8%, $n = 282$) and *H. concinna* (0.5%, $n = 4$) ticks. Rickettsial infection was determined in 10.8% ($n = 65$) and 11.7% ($n = 33$) of *D. reticulatus* and *I. ricinus* ticks, respectively. *Babesia* spp. infection was confirmed in 1.8% ($n = 11$) of *D. reticulatus* and 0.4% ($n = 1$) of *I. ricinus* ticks. DNA of 6 different pathogenic tick-borne species, *Rickettsia helvetica*, *Rickettsia monacensis*, *Rickettsia slovaca*, *Rickettsia raoultii*, *Babesia canis*, and *Babesia venatorum* were identified in this locality with sympatric occurrence of *I. ricinus*, *D. reticulatus*, and *H. concinna* ticks.

Introduction

Emergence of zoonotic vector-borne diseases increased over the last 15 years and is a significant burden on global economies and public health (Jones et al., 2008). The second medically most important group of vectors are the ticks after the mosquitoes. Tick-borne pathogens can cause diseases that result in productivity and economic losses in the livestock sector as well as causing diseases in humans. Hard ticks (Acari: Ixodidae) and soft ticks (Acari: Argasidae) are 2 families of ticks with medical importance. Hard ticks are obligate haematophagous ectoparasites and important vectors of viruses (e.g. the causative agents of tick-borne encephalitis, Colorado tick fever, Crimean-Congo haemorrhagic fever), bacteria (e.g. *Rickettsia* spp., *Coxiella burnetii*, *Anaplasma phagocytophilum*, *Ehrlichia* spp., *Borrelia burgdorferi* sensu lato, *Francisella tularensis*), and protozoa (e.g. *Babesia* spp.). From 896 known tick species approximately 10% are vectors of pathogens responsible for zoonoses (Jongejan and Uilenberg, 2004).

Ixodes ricinus is a small hard tick that transmits a large variety of pathogens of medical and veterinary importance. It is a widely distributed tick species in Europe and throughout the Slovak Republic (Černý, 1972). A shift of the tick altitudinal distribution limit and an extension of areas with potential risk of tick-borne diseases were observed by Lukáň et al. (2010) and Špitalská et al. (2014). In contrast to *I. ricinus*, *Dermacentor reticulatus* had a focal distribution in Slovakia in the past (Nosek, 1972). More recently, Bullová et al. (2009) showed that *D. reticulatus* ticks have extended their range, not only in the surroundings of its former habitats, but also at least 200 km further north and by 300 m into higher altitudes when compared to its former geographical distribution. Now, these arthropods can be found in Slovakia at sites between 90 and 520 m above sea level. *Dermacentor reticulatus* appears to be the second most important tick species especially in the eastern Slovak lowland. The occurrence of babesiosis is well known in this area for

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several years (Chandoga et al., 2002). *Babesia canis* was diagnosed in *D. reticulatus* ticks in south-western Slovakia in 2002, however at a low rate (Duh et al., 2006). In Europe, *D. reticulatus* ticks are the most important vectors of *Babesia canis*, the aetiological agent of canine babesiosis occurring in large parts of Europe (Lobetti, 1998). This tick species can transmit also *Francisella tularensis*, *Rickettsia slovaca*, *Rickettsia raoultii*, *Rickettsia sibirica*, and *C. burnetii* (Jongejan and Uilenberg, 2004; Anderson and Magnarelli, 2008).

Rickettsiae are Gram-negative obligate intracellular bacteria. More than 20 *Rickettsia* species or strains can cause tick-borne rickettsioses; zoonoses belonging to the oldest known vector-borne diseases (Parola et al., 2013). The presence of tick-borne rickettsiae has been reported from almost all European countries. The current view on the geographic distribution of *Rickettsia* species is available in Parola et al. (2013). In Slovakia, occurrence of rickettsiae has been continuously monitored mostly in localities of the southern part, and the presence of 6 rickettsial species/strains (*R. slovaca*, *R. raoultii*, *Rickettsia monacensis* strain IRS3 and IRS4, *Rickettsia helvetica*, and *Rickettsia conorii conorii*) transmitted by ticks have been confirmed. More data about isolation, serological, and molecular identification of these identified species were summarized by Sekeyová et al. (2013). The presence of rickettsial species (such as *Rickettsia africae*) usually transmitted by ticks, was identified in fleas in Slovakia. Sekeyová et al. (2012) provided the first evidence of its presence in *Ceratophyllus garei* fleas collected after a blood meal on reed warblers (*Acrocephalus scirpaceus*) crossing Slovakia during their spring migration.

Up to now, studies were usually focused to study the presence of rickettsial microorganisms only in *I. ricinus* ticks or only in *D. reticulatus* ticks in different localities of Slovakia (Řeháček et al., 1976; Sekeyová et al., 2000; Boldiš et al., 2008; Špitalská et al., 2008a, 2012, 2014). The current study was conducted to investigate the spatial distribution of *D. reticulatus*, *I. ricinus*, and *Haemaphysalis concinna* ticks in surrounding areas of the lake close to the Vojka nad Dunajom village, as well as to determine the prevalence of *Babesia* and *Rickettsia* species in ticks collected from this area as a potential risk for humans and animals.

Materials and methods

Collection of ticks

Ticks were collected by blanket dragging over the vegetation in surrounding areas of the lake close to the Vojka nad Dunajom village, a locality with sympatric occurrence of different tick species, from September 2011 to October 2012. Ticks were identified to the species level, developmental stage, and sex and were maintained alive at 4 °C prior to examination.

Characteristics of collection area

Vojka nad Dunajom (47°57′ N, 17°24′ E) belongs to the orographic entity of Podunajská rovina. It is located 25 km south-east from Bratislava. The altitude is approximately 122 m above sea level. This area is characteristic of a lowland forest ecosystem with mainly willows and poplars. The climate is warm with an average annual temperature of 8–10 °C and an annual rainfall of 530–650 mm. The average monthly temperature in January is −1 to −3 °C and in July about 20 °C. The number of 'summer days' with a temperature of ≥25 °C is above 50. The winter is considered mild. From November to February it may snow. The conditions in the area are often windy. Characteristic for the area is the presence of different species of ticks, amphibians, migratory and nesting birds, and mammals (such as roe deer, wild boar, hare, pheasant, fox, voles, gopher, hamster, weasel, hedgehog) (Mazúr, 1980; www.slovak-republic.org/weather; http://www.rraister.sk/rastlinstvo-zivocisstvo).

DNA extraction

Total genomic DNA from ticks was extracted using alkaline hydrolysis (Derdáková et al., 2003). Each tick was washed with 70% ethanol and sterile water, crushed with sterile pipette tips, and treated with 0.7 M ammonium hydroxide (NH_4OH) for 30 min at 100 °C in sealed PCR tubes. Subsequently, NH_4OH was evaporated for approximately 20 min at 100 °C. Tubes with only NH_4OH were included as negative controls into each extraction to detect possible cross-contamination. DNA extractions were stored at −20 °C and later used as templates for the PCR amplification.

Molecular methods

PCR detection of *Rickettsia* spp. was performed using the genus-specific primer sets RpCS.877p–RpCS.1258n and D767f–D1390r (adapted from Regnery et al., 1991; Sekeyová et al., 2001). The primers amplify a partial region of the *gltA* and *sca4* genes with expected product lengths of 381 bp and 623 bp, respectively. Detection of *Babesia* spp. was performed using the genus-specific primers BJ1-BN2 to amplify a 450-bp fragment of the 18S rRNA gene (Casati et al., 2006). The BAB1–BAB3 primer set was used to amplify a specific fragment of 746 bp located between the 18S rRNA and the 28S rRNA genes of *B. canis* (Duarte et al., 2008). PCR amplifications were conducted using the DyNAzyme™ PCR Master Mix (Finnzymes, Finland) as recommended by the manufacturer on a thermocycler PTC-200 Peltier Thermal Cycler or Perkin-Elmer Applied Biosystems.

The DNA from uninfected ticks and nuclease-free water were used as negative controls in each reaction. DNA from *R. helvetica* and *R. slovaca* originated from ticks, *R. typhi* originated from previous collections by the Institute of Virology, *B. canis* originated from *Babesia*-positive dogs were used as positive controls.

R. slovaca in *Rickettsia*-positive ticks was identified by PCR-RFLP using the enzymatic digestion of the *sca4* gene by the *HaeIII* restriction endonuclease (Špitalská et al., 2008b).

PCR and PCR-RFLP products were analyzed by electrophoresis in a 1% agarose gel, stained with GelRed™ (Biotium, Hayward, California) and visualized with the UV transilluminator. *Rickettsia*-positive tick samples were screened for the presence of *R. helvetica* using a previously developed qPCR assay targeting a 65-bp fragment of the 23S rRNA gene using DyNAmo™ Probe qPCR on Bio-Rad CFX96™ Real-Time System as previously described by Boretti et al. (2009). All qPCR reactions were performed as duplicates, and each run included a negative template control, a positive control, and DNA standards containing 3×10^0–3×10^6 target copies with a sensitivity of 3 copies of the DNA.

Randomly selected amplicons of *gltA*, *sca4*, and 18S rRNA genes were purified using a QIAquick Spin PCR Purification Kit (Qiagen, Hilden, Germany) as described by the manufacturer. Sequencing was performed by Macrogen (http://www.macrogen.com). DNA sequences were compared with available databases in GenBank using the Basic Local Alignment Search Tool (BLAST) on http://blast.ncbi.nlm.nih.gov/. A phylogenetic analysis was further performed using the MEGA5 software (Tamura et al., 2011). The dendrograms were constructed by using the neighbour-joining method, and a distance matrix was produced on the basis of the Kimura's 2-parameter model. The confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates.