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Molecular evidence and diversity of the spotted-fever group *Rickettsia* spp. in small mammals from natural, suburban and urban areas of Eastern Slovakia

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ABSTRACT

Rickettsiae of the spotted fever group are considered as emerging pathogens; ticks, fleas and mites are known to be their vectors. However, the prevalence and species diversity of rickettsiae in small mammals and the role of these hosts in the circulation of bacteria are much less studied. During 2014–2016, a total of 250 small mammals (*Apodemus agrarius*, *Apodemus flavicollis*, *Apodemus uralensis*, *Myodes glareolus*, *Crocidura leucodon*, *Crocidura suaveolens*, *Micromys minutus*, *Microtus arvalis*, *Microtus subterraneus*, *Neomys fodiens* and *Sorex minutus*) were captured in natural, suburban and urban habitats of eastern Slovakia. Ear biopsies of 245 individuals were examined for the presence of SFG rickettsiae by molecular methods. The overall prevalence of *Rickettsia* spp. in small mammals was 11%. The predominant species, *Rickettsia helvetica* was confirmed in the striped field mouse (*A. agrarius*), the yellow-necked mouse (*A. flavicollis*), the bank vole (*M. glareolus*) and the common vole (*M. arvalis*) in natural and suburban habitats, followed by *Rickettsia* sp. closely related to *R. felis* identified in *A. flavicollis* captured in a suburban habitat. Finally, *R. slovaca* was found in only one yellow-necked mouse (*A. flavicollis*) captured in a natural habitat, with the sympatric occurrence of *Dermacentor marginatus* and *Dermacentor reticulatus* ticks. We assume the presence of *R. slovaca* especially in sites with the occurrence of *Dermacentor* spp. All small mammals captured in the urban habitat tested were negative for the presence of rickettsiae. This study brings the first molecular evidence of *R. slovaca* in a rodent captured in Slovakia. *Rickettsia* sp. closely related to *R. felis* was first time detected in *A. flavicollis* in suburban site of Slovakia.

The highest species diversity of rickettsiae was observed in *A. flavicollis*, and the highest prevalence of bacteria was recorded in *M. glareolus*. The highest occurrence of rickettsiae-positive small mammals was recorded during the spring and autumn months, May, June and September, respectively. This may be related with the seasonal activity of the tick vectors. This study confirms the long-term persistence of *Rickettsia* spp. in small mammals in natural and suburban habitats of Slovakia.

Some rodent species that have a wider ecological valency may contribute to the maintenance, circulation and dissemination of rickettsiae within and out the natural foci more significantly than those species that have narrower relation to the certain type of habitat.

1. Introduction

The genus *Rickettsia* (Rickettsiaceae, Rickettsiales) includes Gram-negative obligate, intracellular, aerobic α-Proteobacteria invading hosts' eukaryotic cells. Species of the genus *Rickettsia* are divided into two groups: the spotted fever group (SFG) and the typhus group (TG) (Raoult et al., 2005). The main vectors of rickettsiae are blood-sucking arthropods, mainly ticks, but also fleas, mites and human body lice. The highest occurrence of rickettsiae in ticks was detected in early spring and autumn (Řeháček et al., 1972). Řeháček et al. (1972) suggested that rickettsiae are able to hibernate in ticks. The most important vectors and probably also the main reservoirs of rickettsiae in Southern, Western and Central Europe are *Dermacentor marginatus* and *Dermacentor reticulatus* ticks (Řeháček et al., 1972, 1977; Raoult et al., 2002).

Small rodents, hosting a wide spectrum of ectoparasites and arthropod vectors, are the most significant reservoirs of rickettsiae, many

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of which are responsible for serious zoonotic diseases (rickettsioses) of humans and animals (Řeháček et al., 1992; Burri et al., 2014; Han et al., 2015; Imhoff et al., 2015). Rickettsioses belong among the most frequent vector-borne infections in Europe (Fournier et al., 2012).

In Slovakia, six rickettsial species/strains of the SFG have thus far been identified by serological examination and/or different molecular methods. Among the identified species are *Rickettsia helvetica, Rickettsia slovaca*, *Rickettsia raoultii*, *Rickettsia monacensis* strain IRS3 and IRS4, *Rickettsia conorii* and *Rickettsia africae* (Brezina et al., 1969; Řehácek et al., 1975; Boldiš et al., 2008; Špitalská et al., 2012, 2014; Sekeyová et al., 2013). Recently, *Rickettsia* sp., closely related to *Rickettsia felis*, was detected in Slovakia (Špitalská et al., 2015). These species of rickettsiae are usually transmitted by the ticks, but *R. africae* and *R. felis* have also been identified in fleas (Sekeyová et al., 2013; Špitalská et al., 2015).

R. slovaca was isolated for the first time in 1968 from *D. marginatus* ticks in Central Slovakia (Brezina et al., 1969). After the first evidence of this agent in vector ticks, several patients with clinical signs similar to those associated with *R. slovaca* infection, confirmed in a French patient in 2003, have been reported (Parola et al., 2009; Cascio et al., 2013). Based on the most significant clinical symptoms in affected patients, such as eschar and cervical lymphadenopathies, several names have been proposed for diseases resulting from human infections, e.g. tick-borne lymphadenopathy (TIBOLA), *Dermacentor*-borne necrosis-erythrema-lymphadenopathy (DEBONEL) and/or scalp eschar and neck lymphadenopathy (SENLAT) (Lakos, 1997, 1999; Oteo et al., 2003; Angelakis et al., 2010). The peak incidence of human rickettsioses usually occurs from March to May and during September and November, except the dry summer season, and this is clearly connected with the seasonal activity of *Dermacentor* ticks (Estrada-Peña et al., 2004; Parola et al., 2009).

In Slovakia, the *Ixodes ricinus* tick is considered the main vector and natural reservoir of *R. helvetica* (Špitalská et al., 2008, 2014; Sekeyová et al., 2013).

Rickettsia felis is primarily associated with an arthropod reservoir and vector, the cat flea *Ctenocephalides felis*. It causes infection in humans, known as flea-borne spotted fever or cat-flea typhus (Abdad et al., 2011). In Slovakia, *Rickettsia* sp. closely related to *R. felis* was detected for the first time in a *Ctenophthalmus solutus* flea removed from a striped field mouse (*A. agrarius*) (Špitalská et al., 2015).

In Slovakia, research on rickettsiae has a long tradition (Sekeyová et al., 2013), but the role of rodents in their circulation has been much less studied by molecular methods so far. Recently, only a few studies referring to the presence of *R. helvetica* and *R. monacensis* in *Apodemus flavicollis* and/or *M. arvalis* (Minichová et al., 2017), and *Rickettsia* spp. in *M. arvalis* (Smetanová et al., 2006) have been published.

In the works of Jablonskaja (1978), *R. slovaca* was found in three dominant rodent species, *A. agrarius*, *A. flavicollis* and *M. arvalis*, in Eastern Slovakia; however, these results were only based on serological analysis and cross-immunity studies, without further molecular evidence. Řeháček et al. (1972), have also confirmed the presence of antibodies against *R. slovaca* by serological analysis in small mammals from Slovakia.

Until now, mostly experimental studies of rickettsial infection of *A. flavicollis*, *Clethrionomys glareolus*, *M. arvalis* and *Mus musculus*, susceptibility and antibody formation were conducted in Slovakia (Řehácek et al., 1976, 1992).

In order to update information on the occurrence and diversity of rickettsiae, in the present study we focused on investigation of SFG *Rickettsia* spp. in the wild small mammals from natural, suburban and rural environments in eastern part of the country.

2. Material and methods

2.1. Sampling sites

Rodents and rodent-attached ticks were collected at three study sites in Eastern Slovakia during the years 2014–2016. The first study site, a Botanical garden with deciduous forest vegetation is located in the city of Košice (208 m a.s.l.; 48°446·84″N; 21°14′16·14″ E).

It represents an urban habitat with a significant anthropogenic impact. The second site, Čermeľ, is characterized by the presence of mixed forest vegetation, with a predominance of

beech, hornbeam and spruce (208–600 m a.s.l.; 48°45′46·67″N; 21°8′8·17″E), and it represents a suburban habitat. The third site, with mixed forest vegetation and a predominance of beech, oak and hornbeam, is located in the Slovak Karst National Park, near the Hrhov village (200–220 m a.s.l., 48°34.899 N, 20°46.743 E), and it represents a natural habitat.

2.2. Rodents sampling

In total, 100 Swedish bridge metal traps filled with sunflower seeds were set at each site, 5 m away from each other, for one night, and these were checked next morning. Captured small mammals were transported to the laboratory and euthanized in accordance with the licences of the Ministry of Environment of the Slovak Republic No. 4874/2011–2.2 and 4559/2015–2.3. Subsequently, they were examined for the presence of ectoparasites, which were removed, collected and stored in 70% ethanol.

Each individual was identified into species, weighed with an accuracy of ± 0.5 g, measured, euthanized and dissected and the sexual condition of each animal was ascertained. Classification of mammals into categories subadults (immature) and adults (mature) was used according to Pelikán (1965) with respect to the sexual condition of individuals. The removed ticks and other ectoparasites were determined with the use of an identification key (Filippova, 1977), by light microscopy. An ear biopsy was obtained from 245 animals and stored at −20 °C until further molecular analysis.

2.3. Molecular analysis

Genomic DNA was extracted from each ear-tissue sample after incubation with a Proteinase K solution at 56 °C overnight, using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The presence of *Rickettsia* spp. in the genomic DNA samples was evaluated by PCR (Polymerase Chain Reaction) amplification of the 381-bp long fragment of the citrate synthase gene (*gltA*), using the genus-specific primers RpCS.877p and RpCS.1258n (Regnery et al., 1991).

PCR amplifications were performed in a total of 25 µl of reaction mixture containing 14.9 µl of nuclease-free water, 2.5 µl of 10 × PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.125 U of Taq DNA polymerase (Qiagen), 0.5 µl of 10 mM dNTP Mix, 0.5 µl of 10 µM primers and 5 µl of DNA template. Previously sequenced DNA of *Rickettsia* spp. isolated from infected rodents was used as a positive control and sterile water as a negative control in each reaction.

PCR products were analysed by electrophoresis in a 1.5% agarose gels stained with GoldView Nucleic Acid Stain (Beijing SBS Genetech, Beijing, China) and visualized with a UV transilluminator. After PCR analyses, several positive samples with amplified *gltA* gene fragments were purified for the sequencing. The aim was to choose the representatives from each location, and from different rodent species and sex. The PCR products selected for further phylogenetic analyses were purified using Isolate II PCR and Gel Kit (Bioline) and sequenced (at the University of Veterinary Medicine and Pharmacy in Košice, Slovakia) in both directions using the same primers as for the PCR amplification.