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SHORT COMMUNICATION

Detection of *Rickettsia raoultii* in *Dermacentor reticulatus* and *Haemaphysalis inermis* ticks in Slovakia

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Abstract

Ticks are vector arthropods responsible for the transmission of several pathogenic agents that affect both human and animal health worldwide. In this study our objective was to analyse, using molecular tools, the bacterial community of *Dermacentor reticulatus* and *Haemaphysalis inermis* ticks collected in south-eastern Slovakia. Using real-time PCR, we identified the presence of *Rickettsia* spp. DNA at levels of 14/59 (23.72 %) and 29/173 (16.76 %) in *D. reticulatus* and *H. inermis*, respectively. In addition, using standard PCR and sequencing, we identified the presence of *Rickettsia raoultii* DNA in 13 ticks belonging to the two investigated species. *Rickettsia raoultii* blast results revealed an average identification percentage of 99.62 %. Following the results of this molecular study there is a possibility that *D. reticulatus* and *H. inermis* play a potential role in the transmission of *R. raoultii*. To prove the possibility of validity of this hypothesis, we suggest performing experimental models in future studies. Our results can serve as preliminary data for future transmission models.

Keywords Ticks · *Dermacentor reticulatus* · *Haemaphysalis inermis* · Slovakia

Introduction

Bacteria belonging to the genus *Rickettsia* are known as causative agents of various vector-borne zoonotic diseases. They are responsible for mild to severe diseases in humans (Parola et al. 2013; Eldin and Parola 2018).

The diversity of pathogens in ecosystems also depends on the species diversity of vectors and hosts and the diversity of habitats (de la Fuente et al. 2008). Many ticks in Europe, including *Dermacentor reticulatus* Fabricius, 1794 are known to act as carriers, reservoirs and/or vectors of different pathogenic *Rickettsia* species e.g., *Rickettsia slovaca* (Sekeyová et al. 1998; Garcia-Vozmediano et al. 2020) and *Rickettsia raoultii* (Boldiš et al. 2008; Földvári et al. 2013).

Revising the role of another tick species, *Haemaphysalis inermis* Birula, 1895 in the spread of rickettsiae continues to be challenging as few data have been previously published (Portillo et al. 2008; Špitalská et al. 2018). In a study carried out in Hungary, the authors detected the presence of *R. helvetica* in *H. inermis* and suggested that this tick species could be a potential vector of this pathogen (Hornok et al. 2010).

Recent epidemiological studies of *Rickettsia* in Slovakia were completed in suburban, natural and rural habitats (Minichová et al. 2017), or in an urban temperate forest (Chvostáč et al. 2018) in the western part of the country. A dry forest-steppe biotope is rarely referenced in literature except, in the past century, by Řeháček et al. (1976a, b). The authors indicated that: "The biotopes of the spotted fever group rickettsiae occurring in east Slovakia-namely, cultivated and meadow steppes with sparse forests-share some of the characteristics of the biotopes of *Rickettsia sibirica*. Thus, besides the new species of spotted fever group rickettsiae,

Highlights
- *Dermacentor reticulatus* and *Haemaphysalis inermis* ticks collected in south-eastern Slovakia are infected by *Rickettsia raoultii.*
- These preliminary data could be used in a future transmission model.

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the circulation of *R. sibirica* and perhaps of *Rickettsia conorii* in east Slovakia should not be excluded". However, none of them were ever confirmed in south-eastern Slovakia afterwards (Řeháček et al. 1976a, b).

Our objective was to investigate the possibility of detecting rickettsiae in *D. reticulatus* and *H. inermis,* two relatively abundant tick species in the steppe habitats of south-eastern Slovakia.

Materials and methods

Tick collection and sampling site

The tick collection was carried out on October 23, 2015 by flagging grass and shrubs using a white cotton flag passed over the vegetation at the level of three microhabitats (forest, ecotone and meadow) along a line of 100 m in the vegetation of the protected zone of Slovak Karst National Park in south-eastern Slovakia (Central Europe) near the village of Hrhov (200–220 m above sea level, 48°34.899 N, 20°46.743 E) (Fig. 1).

DNA extraction and molecular detection of bacteria in ticks using real time PCR

Half of the tick body without legs was selected for DNA extraction using EZ1 DNA tissue kit (Qiagen, Hilden, Germany) following a protocol previously elaborated in our laboratory (Diarra et al. 2017).

After extraction, the DNA of each sample was screened with the CFX96 real-time PCR (Bio -Rad, Marnes-la-Coquette, France) and the LightCyclerR 480 Probes Master Mix (Roche Diagnostics, Indianapolis, USA) for the presence of the following bacterial microorganisms (*Rickettsia* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii* and *Anaplasma* spp.) using specific primers and probes listed in Diarra et al. (2020) and Ouarti et al. (2020).

The qPCR reaction mixture for each plate has been used according to the manufacturer's protocol. Negative controls were used in each qPCR and consisted of DNA extracted from uninfected *Rhipicephalus sanguineus* ticks from the laboratory colony of IHU - VITROME (Marseille, France). Positive controls included DNA extracted from a dilution of cultured strains of *Borrelia crocidurae*, *Bartonella henselae*, *Rickettsia montanensis*, *Ehrlichia canis* and *Coxiella burnetii* (Socolovschi et al. 2012b; Aouadi et al. 2017). Results were deemed positive if the cycle threshold (Ct) value obtained by CFX96 was lower than ≤ 35 (Ouarti et al. 2020).

Bacterial species identification using sequencing

All ticks tested positive for *Anaplasma* spp., *Borrelia* spp. and *Rickettsia* spp. in qPCR were subjected to amplification using standard PCR prior to sequencing for identification of the bacterial species (Dahmani et al. 2015b; Diarra et al. 2017). In standard PCR the primers used to amplify the *ompA* gene were (f-70 and r-701). For the sequencing we used the following primers (f-70, f-180 and r-701) which amplify (629 to 632 bp) of protein A (Table 1) (Socolovschi et al. 2012a).

Primers targeting the Anaplasmataceae *23S*, *Borrelia 16S* rRNA and *Rickettsia ompA* genes (Table 1), were used as described previously (Socolovschi et al. 2012a; Dahmani et al. 2015a; Ouarti et al. 2020).

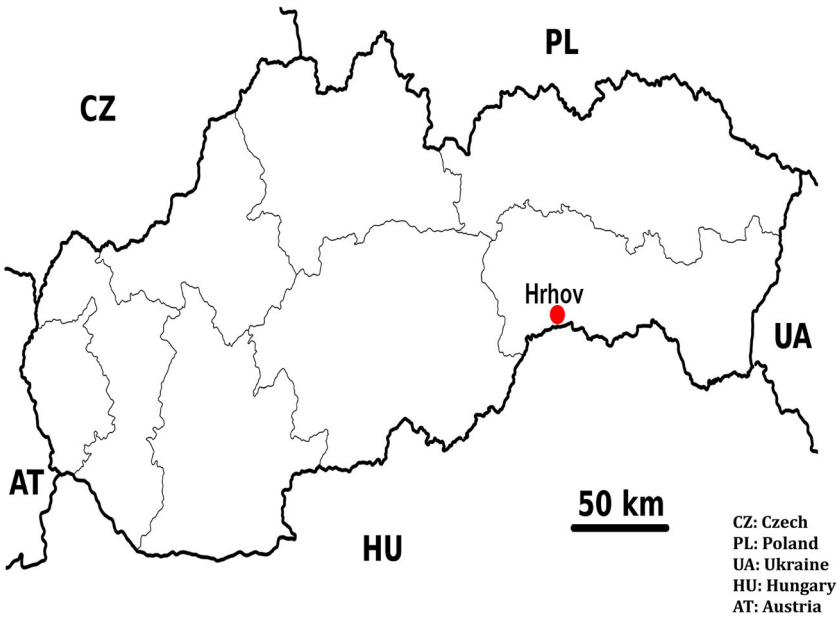

Fig. 1 Geographical position of the tick collection locality in the south-eastern region of Slovakia

Table 1 Standard PCR primers used for the detection of microorganisms in ticks

Bacterial species	Targeted sequences	Primers (5′–3′)	Reference
Anaplasmataceae	*23S* rRNA	f_ATAAGCTGCGGGGAATTGTC r_TGCAAAAGGTACGCTGTCAC	(Dahmani et al. 2015a)
Rickettsia spp.	*ompA*	f_70_ATGGCGAATATTTCTCCAAA A r_701_GTTCCGTTAATGGC AGCATCT f_180_GCAGCGATAATGCT GAGTA	(Socolovschi et al. 2012a)
Borrelia spp.	*16S* rRNA	f_GCTGGCAGTGCGTCTTAAGC r_GCTTCGGGTATCCTCAACTC	(Socolovschi et al. 2012a)

The purified DNA products were sequenced using a Big Dye Terminator kit and a genetic analyzer ABI PRISM 3130 (Applied BioSystems, Courtaboeuf, France). The sequences obtained were analyzed with the software ChromasPro, version 1.34 (Technelysium Pty, Ltd., Tewantin, Queensland, Australie) in order to compare them to the GenBank database using BLAST (http: //blast.ncbi.nlm. Nih.gov/Blast.cgi) (Diarra et al. 2020).

Results

A total of 232 adult ticks (63 *H. inermis* males and 110 females, 31 *D. reticulatus* males and 28 females) were collected from vegetation in the protected zone of the Slovak Karst National Park. The proportion of ticks examined are detailed in (Table 2).

Using qPCR, 14/59 (23.72 %) *D. reticulatus* ticks tested positive for *Rickettsia* spp., 2/173 (1.15 %) *H. inermis* ticks tested positive for Anaplasmataceae bacteria, 1/173 (0.57 %) for *Borrelia* spp., and 29/173 (16.76 %) for *Rickettsia* spp.

The 43 ticks positive for *Rickettsia* in qPCR were then subjected to standard PCR amplification.

A total of 13/43 (30.23 %) ticks were successfully sequenced. BLAST analysis of these 13 sequences obtained showed an identity percentage ranging from 97.98 to 100 % with the *R. raoultii* sequence. Details are given in Table 3. No *Borrelia* spp. or Anaplasmataceae sequences could be amplified.

Discussion

The investigated locality (Slovak karst near the village of Hrhov) is specific and unique due to the joint occurrence of seven tick species, *Ixodes ricinus* (Linnaeus, 1758), *I. trianguliceps* Birula, 1895, *I. frontalis* (Panzer, 1795), *Dermacentor marginatus* Sulzer, 1776, *D. reticulatus*, *Haemaphysalis concinna* C. L. Koch, 1844 and *H. inermis* (Černý 1972; Nosek 1972; Bona and Stanko 2013; Heglasová et al. 2020), which is an exceptional phenomenon in the conditions of Central Europe. *Haemaphysalis punctata* Canestrini & Fanzago, 1878 which was the dominant species in tick communities in the area of Slovak karst in the last century, has not been confirmed there in recent decades. The high concentration of potential vectors (seven tick species) and hosts (cattle, free-living ungulates, several species of rodents and shrews) in the studied locality makes the Slovak karst a unique model for studying the circulation of several species of pathogens in their natural habitat (Nosek 1971; Heglasová et al. 2020). Uniqueness of the site (hereby presented) is from the point of view of the common occurrence of several species of ticks (see above). In terms of uniqueness or differentiation of pathogens in ticks compared to other sites, confirmation of a variety of bacteria in *Haemaphysalis* spp. which live on a common site with both species the genus *Dermacentor* is exclusive. In Slovakia, the presence of *D. reticulatus* has been identified along the rivers in south-western and south-eastern Slovakia (Nosek 1972). Ticks of the genus *Haemaphysalis* (*H. inermis*, *H. concinna*, *H. punctata*) are known to have a more focal distribution (Černý 1972; Nosek 1973).

Table 2 The proportion of ticks examined according to microhabitats (forest: ecotone: meadow)

Species of ticks	Forest	Ecotone	Meadow	Total
Dermacentor reticulatus	2 (3.4 %)	20 (33.9 %)	37 (62.7 %)	59
Haemaphysalis inermis	27 (15.61 %)	121(69.94 %)	25 (14.45 %)	173

Table 3 BLAST analysis of *Rickettsia* obtained from tested ticks

Ticks species	Query cover (%)	Percent identity (%)	Accession number	Molecular identification by BLAST
Dermacentor reticulatus	100	97.98	HM161789.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	100	KX506737.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	99.84	HM161789.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	100	KX506737.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	99.68	HM161792.1	*Rickettsia raoultii*
Haemaphysalis inermis	99	99.84	CP003426.1	*Rickettsia raoultii*
Haemaphysalis inermis	99	98.63	CP003426.1	*Rickettsia raoultii*
Haemaphysalis inermis	100	99.41	CP003426.1	*Rickettsia raoultii*
Haemaphysalis inermis	99	99.84	HM161789.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	100	KX506737.1	*Rickettsia raoultii*
Dermacentor reticulatus	100	99.84	HM161789.1	*Rickettsia raoultii*
Haemaphysalis inermis	100	100	KX506737.1	*Rickettsia raoultii*
Dermacentor reticulatus	99	100	HM161792.1	*Rickettsia raoultii*

In previously published studies from the Slovak karst area, we confirmed several species of rickettsiae (*R. slovaca*, *R. helvetica*, *Rickettsia felis*, *Rickettsia* sp.) and *Borrelia miyamotoi* in small mammals (especially in rodents), from rodent-attached ticks, as well as from questing ticks sampled from vegetation (Radzijevskaja et al. 2015; Heglasová et al. 2018). There is a characteristically high concentration of deer in the area, as well as cattle grazing on the pastures and both vertebrate groups are hosts for adult ticks. These factors increase the significance of the study area from an epidemiological point of view (Černý 1972).

Here, we demonstrated the circulation of *R. raoultii* in the studied area. Although *R. raoultii* has been already detected in *D. reticulatus* and in patients bitten by *D. reticulatus* (Parola et al. 2009), the vector competence of *D. marginatus* and *H. inermis* to transmit *R. raoultii* cannot be confirmed, because the detection of this bacterium in the latter could be the consequence of a bacterial blood meal of these ticks. Therefore, additional epidemiological studies and experimental models will be needed.

Both *Dermacentor* and *Haemaphysalis* ticks from which confirmed positive cases of *Rickettsia* originate are typical species for forest-steppe zones (*D. marginatus*, *H. inermis*), alluvial forests and wet meadows, which are commonly seen biotopes in eastern Slovakia (*D. reticulatus*, *H. concinna*). The joint occurrence of steppe landscape and xerothermic tick species together with ticks that prefer humid habitats in the studied area is probably caused by the presence of ponds and canals in the karst area as well as by the high density of hosts (birds, small mammals, wild ungulates, cattle) for all life stages of ticks. *Dermacentor reticulatus* was described as harbouring more bacteria than *D. marginatus* (Zhang et al. 2019). *Rickettsia raoultii* is usually associated with *Dermacentor* spp. (Špitalská et al. 2012; Švehlová et al. 2014). Sequencing partial *ompB* genes revealed the presence of *R. raoultii* in the larvae and nymphs of *D. reticulatus* ticks in Germany (Schmuck et al. 2020). In recent decades, the spread of *D. reticulatus* to new territories has been confirmed, having been reported in, for example, in Poland (Kiewra and Czulowska 2013; Mierzejewska et al. 2016), Germany (Dautel et al. 2006) as well as other European countries (Földvári et al. 2016; Kjær et al. 2019; Capligina et al. 2020). One study carried out in Latvia detected the presence of *R. raoultii* in *D. reticulatus*, their results corroborate with our study. Experimental models will therefore be necessary to understand the role of *D. reticulatus* in the appearance of *R. raoultii* (Capligina et al. 2020).

The indication of *H. inermis* harbouring rickettsiae was noted (Řeháček et al. 1976a, b). However, the role of *Haemaphysalis* spp. as vectors of *Rickettsia* spp. is unknown (Minichová et al. 2017). There is little data on bacterial associations with *H. inermis*. *Rickettsia aeschlimannii* was detected in La Rioja, Spain (Portillo et al. 2008). *Rickettsia slovaca* (Ibarra et al. 2006) and *R. raoultii* are responsible for tick-

borne lymphadenopathy/ Dermacentor-borne necrosis erythema and lymphadenopathy/scalp eschar and neck lymphadenopathy (TIBOLA/-DEBONEL/SENLAT) (Oteo et al. 2004; Selmi et al. 2008; Parola et al. 2013). This illness commonly occurs in Slovakia (Sekeyová et al. 2013) and was recently identified as the causative agent of tick-borne lymphadenopathy in Belgium (Lernout et al. 2019). *Rickettsia helvetica* is considered less pathogenic than the two aforementioned rickettsial pathogens (Sprong et al. 2009; Boulanger et al. 2019).

Conclusions

This molecular study supports the data that *R. raoultii* could be a source of lymphadenopathy in Slovak populations. Further studies, including experimental models, will be needed to assess the role of *D. reticulatus* and *H. inermis* in the transmission of *R. raoultii*. Nevertheless, based on the positive detection of *R. raoultii*, we assume that *Haemaphysalis* and *Dermacentor* spp. should be considered as potential reservoirs of *R. raoultii* in Slovakia.

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Author Contributions ZS, PP and OM conceived and designed the research. BO, BE and ML conducted the experiments. MS, and ZS contributed analytical tools. BO and BE analysed the data. BO and ZS wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors have no conflicts of interest to disclose.

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