Monitoring Rickettsia spp. v podhorskej oblasti Slovenska

Monitoring of *Rickettsia monacensis* in the Mountain Area of Slovakia

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Súhrn
Ciele: Kliešte ako vektory rôznych infekcií sa v turisticky vyhľadávanej lokalite Slovenska – na Liptove, vyskytujú každoročne a ich výskyt má vo všeobecnosti zvyšujúci trend. Niekoľko rikétsií, ktoré spôsobujú u ľudí škvrtnité horúčky boli doteraz potvrdené vo vektoroch- kliešťoch z rôznych oblastí Slovenska. Preto je nevyhnutné nielen stanoviť riziko, ktoré tieto baktérie pre človeka predstavujú, ale aj identifikovať nové oblasti výskytu rikétsií.
Materiál a metodika: Kliešte, drobné cicavce, vtáky a diviaky z rekreačných oblasti Liptovskej kotliny v severnej časti Slovenska boli testované na prítomnosť Rickettsia spp.. Získané sekvencie boli následne ďalej analyzované.
Výsledky: Celkovo 8 z 55 ixodidových kliešťov (14,5 %) bolo testovaných s pozitívnym výskedkom na prítomnosť baktérie Rickettsia helvetica, jeden kliešť (1,8 %) bol infikovaný baktériou Rickettsia monacensis. Identifikácia tohto druhu bola založená na sekvenácii 23S-5S interného transkripčného medzerníka, a tiež génov gltA a ompA. Baktérie z rodu Rickettsia boli detekované sekvenáciou génu gltA aj u 7 hlodavcov (5,8 %) a 5 diviakov (5,9 %) z Liptovskej kotliny.
Záver: Naše výsledky potvrdili prítomnosť rikétsií spôsobujúcich škvrnité horúčky vo vektoroch (kliešťoch), a tiež v hostiteľoch (drobné cicavce, diviaky) zo severnej oblasti Slovenska. Dôkaz prítomnosti Rickettsia spp. vo vektoroch a rezervoároch naznačuje kolovanie patogéna v prírode a potrebu monitorovania situácie v tejto turisticky vyhľadávanej localite Slovenska.

Kľúčové slová: *Rickettsia monacensis*. *Rickettsia Helvetica*. *Ixodes ricinus.* Slovensko.

Abstract
Objectives: Ticks, as a vector of various infections occur every year in Liptov, a popular tourist location in Slovakia, and their occurrence generally has an increasing trend. As up to now several rickettsiae from the spotted fever group were confirmed in tick vectors from different areas of Slovakia. Therefore, identification of previously unknown areas with endemic occurrence of *Rickettsia* spp. is of great importance.
Material and methods: Questing ixodid ticks, small mammals, birds and wild boars from recreational areas of Liptov valley in northern Slovakia were tested for the presence of *Rickettsia* spp. and obtained sequences were further analyzed.
Results: Overall, 8 out of 55 ixodid ticks (14.5%) tested positive for *Rickettsia helvetica* and one male tick (1.8%) was infected with *Rickettsia monacensis*. The identification of this species was based on the sequencing of 23S-5S internal transcribed spacer (ITS), *gltA* and *ompA* genes. *Rickettsia* spp. was detected in 7 rodents (5.8%) and 5 wild boars (5.9%) originated from Liptov valley, using *gltA* gene primer set.
Conclusion: Our results confirm the presence of spotted fever group rickettsiae in tick vectors and hosts from northern part of Slovakia. Moreover, the evidence of the presence of Rickettsia spp. in vectors and in reservoirs indicate the circulation of the pathogen in nature and the need to monitor the situation in this popular location in Slovakia.

Keywords: *Rickettsia monacensis*. *Rickettsia Helvetica*. *Ixodes ricinus*. Slovakia.

1.Introduction

Several members from the order Rickettsiales may cause diseases of humans and animals. In Slovakia, five tick-borne species from the family Rickettsiaceae have already been isolated: *Rickettsia slovaca*, *R. raoultii, R. helvetica, Rickettsia* sp. "IRS3" and "IRS4" and *R. conorii* (*1,2,3,4*). Recently, *R. slovaca*, *R. raoultii* and *R. helvetica* were identified by the PCR amplification of 16S rRNA and *gltA* genes from blood samples of hospitalized patient from the rural area of southeastern Slovakia (*5*). Based on these observations and regarding the improvement of diagnostic methods, we can assume also the presence of other potentially pathogenic rickettsial species from the spotted fever group (SFG). In the present study, questing *Ixodes ricinus* ticks, tissue and blood samples of rodents, birds and wild boars originated from the highly frequented recreational areas of Liptov valley, in the northern part of the country were examined in order to investigate the role of ixodid ticks and vertebrate hosts in the circulation of rickettsiae from the SFG.

2. Materials and Methods

2.1 Study area

The study was conducted in the Low Tatra National Park and Greater Fatra National Park which belong to the Outer Western Carpathians in the central Slovakia (48.96°N, 19.67°E and 49.07°N, 19.19°E respectively). These areas are mainly covered with forests and meadows. The climate is from mildly warm in the lowlands to cold in the higher altitudes. The average temperatures in the lowlands are of -2.5°C in January and 17°C in July. The average annual precipitation is 900 - 1000 mm and in the highest situated territories more than 1200 mm.

2.2 Materials

From October 2012 to April 2013, altogether 121 small mammals belonging to 10 species were trapped at two sites in Liptov valley. Animals were captured using the live traps and released after the ear biopsy. At the same period, tissue samples of 85 wild boars originated from Liptov valley were obtained

from the local Department of Veterinary Administration. During April 2013 bird nets were installed in the same area and 55 blood samples from several bird species were obtained. Additionally, 55 questing *I. ricinus* were collected from the vegetation at the same locations as where the small mammals, wild boars and birds were sampled.

2.3 Molecular techniques

Tissue samples of small mammals and wild boars were kept in 70% ethanol until DNA was isolated with commercial kit. Whole blood samples from birds were stored at 4°C and subsequently DNA isolation was performed. Ticks were stored in 70% ethanol and identified to species and sex. Genomic DNA was isolated using alkaline hydrolysis (6) and stored at -20°C until further analysis. Ticks and animals were screened for rickettsial DNA with single PCR amplification of the portion of citrat synthase gene (*gltA*) specific for *Rickettsia* spp. (*7*). Additionally, all positive samples were screened using the PCR assays amplifying the fragment of *ompA* gene encoding the outer membrane protein A and internal transcribed spacer 23S-5S ITS (*rrl-rrf* spacer) (*8,9*). Obtained gene fragments were sequenced. The complementary strands of sequenced products were manually assembled and compared with the GenBank entries by Blast N 2.2.13. Newly obtained sequences were deposited in GenBank database.

2.4 Phylogenetic analysis

As the ompA gene showed the greatest variability, in comparison with other two used gene fragments (ITS and gltA), we used this locus for the reconstruction of phylogenetic tree.

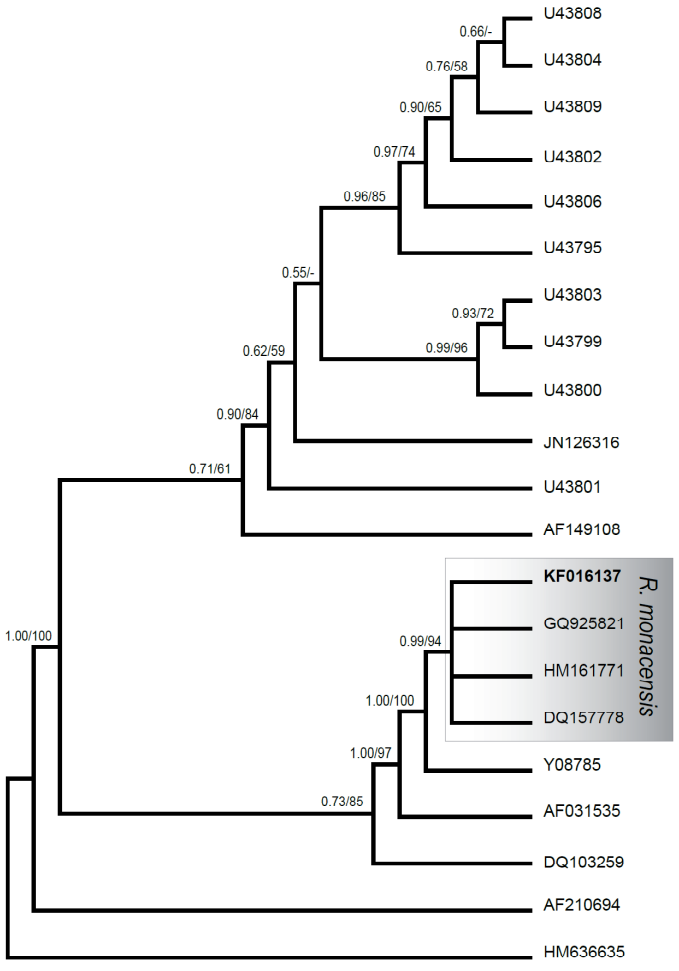
The translated *ompA* nucleotide dataset was aligned in BioEdit 7.0.5.3 (10) with default parameters using ClustalW algorithm and was edited manually. All unaligned or ambiguously aligned sites were deleted. Dataset was then translated back to nucleotides for inferring phylogeny. It contained 227 parsimony informative sites. We used two distinct methods to build a phylogenetic tree- Bayesian Inference (BI) and Maximum Parsimony (MP). BI tree was constructed in MrBayes 3.1.2 (11) software with GTR+I+Γ model with gamma distribution in six categories. The program ran for 5 million generations with sampling frequency 100. When MCMC chains converged a consensus tree was computed from 40 000 sampled trees. MP parsimony was computed in PAUP∗ 4.0b10 (12) using TBR algorithm with random sequence addition. Bootstrap tree was constructed to support branching of the BI tree.

3. Results

3.1. Rickettsia spp. in ticks

In total, 9 *I. ricinus* ticks (16.4%) tested positive for the presence of *Rickettsia* spp. using *gltA* gene primer set. All of them were subjected for the sequencing. The presence of rickettsial DNA was confirmed in 7 (77.8%) sequenced samples. The rest of *gltA* PCR products could not be determined because of poor quality of the sequencing results. Six out of seven *gltA* nucleotide sequences obtained from *I. ricinus* ticks revealed 100% identity with *R. helvetica*. One representative *gltA* sequence of *R. helvetica* was embedded to GenBank database under the accession number KF016135. In one male tick, 100% identity with *R. monacensis* was confirmed and it was deposited in GenBank (KC996728). The portion of 23S-5S ITS was amplified in 7 *gltA* positive DNA samples from *I. ricinus* ticks. In 6 samples the presence of *R. helvetica* (~500 bp) was confirmed. One male *I. ricinus* tested positive for *R. monacensis* (~386 bp). The presence of approximately 530 bp long fragment of the *ompA* gene was confirmed in 5 *I. ricinus* DNA extracts (four were identical with *R. helvetica* and one with *R. monacensis*). Adult male *I. ricinus* tick provided *R. monacensis* positive results also with PCR assays for ITS (KF016136) and *ompA* (KF016137) genes. The Blast analysis showed the 99% identity of the sequence obtained in this study with *R. monacensis* WB5/Ir Pavullo strain (HM161771) from *Dermacentor marginatus* or Red3/Ir Camugnano strain (HM161774) from *Ixodes ricinus* from Italy or Serbia (GQ925821) as well as with the prototype *R. monacensis* Ir/Munich strain from Germany (AF201329). The *ompA* partial sequence from positive male tick was aligned with some of above mentioned representatives from the GenBank database (Figure 2). Similarly, sequencing of the *gltA* gene fragment confirmed the identity of *R. monacensis* isolated from the male *I. ricinus* tick from Slovakia. The multiple alignment of the nucleotide sequences with a length of 341 revealed the 100% identity with partial sequences from *I. ricinus* ticks from Romania (JX003686), Serbia (GQ925820), Italy (JQ669950), Hungary (EU853830) and the IrR/Munich strain from Germany (DQ100163). Partial sequence of an 23S-5S (rrl-rrf) internal transcribed spacer showed 99% identity with the sequence from the Swiss *I. ricinus* nymph - clone N27A09 (JQ670870) and *I. ricinus* ITS fragment did not reveal any variability within overlapping region.

3.2. Rickettsia spp. in hosts



Picture 1*R. monacensis obtained from tick (I. ricinus) from Liptov valle*

Altogether 121 small mammals were included into the study. In 7 (5.8%) samples *R. helvetica* was confirmed with *gltA* primer set. Additional amplification of ompA and ITS fragments provided only weak PCR signal and further sequencing failed. In the *gltA* PCR assay, 5 out of 85 (5.9%) wild boars tested positive for *R. helvetica*, but consecutive identification could not be determined because the amplification result was of poor quality. None bird sample tested positive for *Rickettsia* spp.

4. Discussion

Study of *Rickettsia* spp. has had a long tradition in Slovakia. Occurence of tick-borne species, namely *R. helvetica*, *R. slovaca*, *R. raoultii* and strains IRS3 and IRS4, have already been recorded mainly in southern sites of Slovakia (13).

R. helvetica was firstly detected in ixodid ticks by molecular methods (14) and subsequently confirmed in roe deer (15) and in hospitalized patients from southeastern parts of the country (16). This pathogen was firstly considered as nonpathogenic. In 1999 it was implicated in fatal perimyocarditis in several patients in Sweden (17). The infection is characterized as mild, self-limited illness associated with headache and myalgias and less frequently, with a rash and/or an eschar (18). *R. raoultii* and *R. slovaca* are transmitted by *Dermacentor marginatus* and *Dermacentor reticulatus*, respectively. Infection with *R. raoultii* seems to be less pathogenic in humans than with *R. slovaca* (19).

In 2000, two rickettsial strains, namely IRS3 and IRS4 were confirmed in *I. ricinus* collected in north eastern and south western Slovakia (1). After phylogenetic analysis based on gltA and 16S rDNA genes they were considered as quite different strains, than those previously known spotted fever rickettsiae, but closely related to *R. helvetica*. In 2002 Simser et al. (20) isolated and characterized *R. monacensis* for the first time in *I. ricinus* tick from the city park in Germany and this isolate showed high level of sequence similarity to IRS3 and IRS4. However, in our study *R. monacensis* and *R. helvetica* were identified in *I. ricinus* ticks from northern Slovakia for the first time based on the multi-locus (*gltA*, *ompA* and internal transcribed spacer) sequence typing. Moreover, the *ompA* sequences of *R. monacensis* were nearly identical to sequence obtained from human patient with the febrile illness in Spain (DQ157778) and with the sequence from Italian patient, which was 100% identical with isolate N72 from *I. ricinus* tick (FJ919650) (*21,22*). Phylogenetic analysis based on *ompA* gene fragment confirmed the similarity of our *R. monacensis* isolate with isolates obtained from ticks from mediterranean region (Serbia (GQ925821) and Italy (HM161771)). Just on the basis of these results, it is interesting to estimate, how these rickettsia species occured in the mountain area of central Europe. Migratory birds and wild animals inhabiting this region, but especially *I. ricinus* ticks feeding on them are considered as possible way of explanation of this migration, but there is still a lack of studies confirming this theory. As mentioned, three patients with MSF caused by *R. monacecsis* have been confirmed until now and they came from mediterranean region (21,22). *R. monacensis* seqences from ticks similar or identical with seqences obtained from human patients were confirmed in Switzerland at sites with mixed deciduous forests (23). As in our study, in Poland, nucleotide sequences of the *R. monacensis* gltA gene fragment obtained from *I. ricinus* showed 100% similarity to those of the IR/Munich strain (DQ 100163). Thertefore the bacteria may have been extended from Poland, as the presence of *R. monacensis* in ticks have been confirmed there before (24).

I.ricinus is the most widespread tick species in Slovakia and furthermore, as the vector and reservoir of *R. monacensis* plays an important role in its spreading into new areas. Due to climate and structural landscape changes *I. ricinus* ticks are also spreading to the central parts of the country and further north, where reach suitable conditions and create a new habitats. Studied region represents a part of the Outer Western Carpathians and it consists of mountain and wooded parts, which have been changed to recreational areas and game parks in recent years. A growing number of tourists and locals are visiting these places for the leisure activities, therefore we consider obtained results as of the great importance in the terms of public health. Our results are demonstrating that even in the mountain areas of central Europe there is a potential risk for exposure to *R. monacensis* strains, which can cause human infections similar to Mediterranean spotted fever (MSF).

Conclusions

According to our knowledge, this is the first molecular evidence of *R. monacensis* in questing ixodid ticks from Slovakia with the *ompA* nucleotide sequences nearly identical to those obtained previously from humans. In addition, the study was carried out in areas without previous confirmation of the presence of rickettsial agents. Further attention is needed to find out how the rickettsioses are emerging and spreading in mountain regions of central Europe.

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Biographical Sketch

Ms. Mária Nováková is a post-doc at the Institute of Parasitology of the Slovak Academy of Sciences in Košice. Her major research interests focus on the ecology and epidemiology of tick-borne diseases.

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