INCIDENCE OF VARIOUS TICK-BORNE MICROORGANISMS IN RODENTS AND TICKS OF CENTRAL SLOVAKIA

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Summary. – In this study, we detected *Rickettsia helvetica, Candidatus* Midichloria mitochondrii, *Anaplasma phagocytophilum, Ehrlichia muris, Candidatus* Neoehrlichia mikurensis, and *Bartonella* sp. infections in wild rodents and ticks collected from the vegetation of central Slovakia. The microorganisms were identified by PCR and sequencing. Yellow-necked mice (*Apodemus flavicollis*) were infected with *E. muris* and *Bartonella* sp., while ticks *Ixodes ricinus* collected from the vegetation were infected with *R. helvetica*, *Candidatus* M. mitochondrii, *Candidatus* N. mikurensis, *A. phagocytophilum,* and *E. muris.*

Key words: Rickettsia helvetica; Anaplasma phagocytophilum; Ehrlichia muris; Candidatus Neoehrlichia mikurensis; Bartonella sp.; central Slovakia

Introduction

Ticks are obligate hematophagous ectoparasites of terrestrial vertebrates including humans that are distributed worldwide from the Arctic to the tropical regions. In Europe, they are considered as the most important arthropods responsible for vector-borne diseases, such as rickettsiosis, Q fever, anaplasmosis, Lyme borreliosis, tularemia, and encephalitis. *Ixodes ricinus, Dermacentor marginatus, D. reticulatus, Haemaphysalis concinna, H. punctata,* and *H. inermis* (the family *Ixodidae*, the order *Acari*) are exophillic tick species occurring in Slovakia (Řeháček *et al*., 1991; Špitalská *et al*., 2002; Špitalská and Kocianová, 2003). They are monitored as carriers of *Coxiella burnetii* (the family *Coxiellaceae*, the order *Legionellales*) and spotted fever group (SFG) rickettsiae (the family *Rickettsiaceae*, the order *Rickettsiales*), (Řeháček *et al*., 1976, 1990). The first rickettsia found in Slovakia was *Rickettsia slovaca* isolated in 1968 from *D. marginatus* ticks collected in central Slovakia (Brezina *et al*., 1968). Later on, *Rickettsia* sp. IRS3 and *Rickettsia* sp. IRS4 were isolated from *I. ricinus* (Sekeyová *et al*., 2000). *R. raoultii* was recently detected in the ticks *D. marginatus* collected from vegetation, and *R. helvetica* in the roe deer (Boldiš *et al*., 2008; Štefanidesová *et al*., 2008). *A. phagocytophilum* (the family *Anaplasmataceae,* the order *Rickettsiales*) was identified in *I. ricinus* ticks from vegetation and Ehrlichia-like sp. "Schotti variant" referred to as novel bacteria *Candidatus* Neoehrlichia mikurensis (the family *Anaplasmataceae*, the order *Rickettsiales*) in *I. ricinus* nymph collected from a song thrush (*Turdus philomelos*) (Špitalská *et al*., 2006; Smetanová *et al*., 2006; Kawahara *et al.*, 2004; Derdáková *et al*., 2003; Špitalská and Kocianová, 2002).

The aim of the present study was to monitor the presence of different tick-borne microorganisms circulating in ticks and rodents of central Slovakia.

Materials and Methods

Area of study. Ticks and rodents were collected in previously characterized localities near the villages of Lutila, Malá Lehota, Horná Ves, and Hodruša Hámre in central Slovakia (Smetanová *et al*., 2007).

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Abbreviations: HT = hemocyte test; RFLP = restriction fragment length polymorphisms; RLO = rickettsia-like microorganisms; SFG = spotted fever group

Collection of samples. A total of 30 rodents (the family *Rodentia*) were live-trapped in October 2006. Next, 68 adult ticks *I. ricinus* were collected by blanket-dragging over the vegetation in June 2006. Additional 6 engorged larvae *I. ricinus* and 4 larvae *I. trianguliceps* were collected from yellow-necked mice.

Shell vial technique. The presence of microorganisms with rickettsial morphology in adult ticks *I. ricinus* was examined by microscopic hemocyte test (HT) (Burgdorfer, 1970). Isolation of rickettsiae from living ticks positive by HT was performed by the shell vial technique as previously described (Boldiš *et al*., 2008). One droplet of haemolymph was inoculated into one well (shell vial) containing monolayer of confluent L929 cells. After inoculation, the shell vials were centrifuged for 45 mins at 1000 x g and 25°C. Then the monolayer was incubated in a CO_2 incubator for 120 mins at 33°C. Finally, the cells were incubated in CO_2 incubator at 33°C for 7–10 days.

DNA extraction. The DNA from ticks was extracted by Rijpkema *et al*. (1996). The DNA from infected L929 cells was extracted using the Bactozol-Bacterial DNA Isolation Kit (Molecular Research Center) and from spleen of tested mice by DNeasy Tissue kit (Qiagen) according to the manufacturer's recommendations.

PCR. The PCR for eubacteria was used for amplification of 470 bp part of the 16S rRNA gene using primers GA1B and 16S8FE (Bekker *et al*., 2002). The PCR for detection of ehrlichiae/anaplasmae amplified 298 bp part of the 16S rDNA using primers Ehr521 and Ehr790 (Kolbert, 1996). Positive samples were further analyzed by species-specific PCR for identification of *A. phagocytophilum* that amplified 382 bp fragment of the *groESL* gene. Primers HS43 and HS45 were used for the primary reaction, and GEHS1 and EHS6 for the nested reaction (Björsdorff *et al*., 2002). A primer pair IS58-62f and IS58-594r of the 16S rRNA gene was used for the specific identification of *Candidatus* N. mikurensis (Kawahara *et al*., 2004). Primers MSP4AP5 and MSP4AP3 used for study of genetic variants of *A. phagocytophilum* amplified 849 bp fragment of the *msp4* gene (de la Fuente *et al*., 2005a).

The PCRs for rickettsiae amplified 381 bp part of the *gtl*A gene using primers RpCS.877p and RpCS.1258n, 632 bp part of the *ompA* gene using RR190.70F, RR190.701R primers and a part of 623 bp of the *sca*4 gene using D767f and D1390r primers (Sekeyová *et al*., 2001; Roux *et al*., 1996; Regnery *et al*., 1991).

RFLP analysis. Enzymatic digestion for the identification of *R. slovaca* was performed by incubation of 17 µl of the PCR product of the *sca*4 gene with 2 µl of enzyme buffer and 1 µl (10 U) of *Hae*III restriction endonuclease (Takara Bio) for 3 hrs at 37°C (Špitalská *et al.*, 2008).

Sequence analysis. Amplicons were purified using a QIAquick Spin PCR Purification Kit (Qiagen) as described by the manufacturer. The sequencing was performed by Macrogen, Inc., Korea (www.macrogen.com). The obtained sequences were compared with those available in databases using the BLAST Program (www.ncbi.nlm.nih.gov/blast).

Results

Microorganisms detected in ticks collected from vegetation

Out of 68 ticks collected from vegetation 5 ticks were positive for rickettsiae detected by PCR (Table 1). PCR/RFLP analysis showed that this infection was not caused by *R. slovaca*. The sequencing of *gltA* fragments confirmed the infection with *R. helvetica*. The rickettsial bacterium *Candidatus* M. mitochondrii was detected in six *I. ricinus* females (identity from 97.8 to 100% with *Candidatus* M. mitochondrii, Acc. No. AJ566640) using fragments of the 16S rRNA of eubacteria.

Three ticks were infected with *A. phagocytophilum*. The detected sequences of *msp4* gene were identical (99.9%) with the pathogenic variant of *A. phagocytophilum*, Horse 31. *Candidatus* N. mikurensis infection was found in 2 ticks. Sequencing of these samples showed 99.5% identity with Ehrlichia-like sp. "Schotti variant" (Acc. No. AF 104680) and 98.8% identity with *Candidatus* N. mikurensis (Acc. No. AB196305). One female tick was infected with *E. muris* (Acc. No. EF660077). The obtained DNA sequence matched those already published for *E. muris* (Acc. Nos. AF312907, AB196302, AY58708, identity 99.3%, and M418451, identity 100%).

Table 1. Rickettsial infections of ticks (from the vegetation) and rodents

Ticks/rodents	Gender	Surveyed regions in central Slovakia				Σ
		Malá Lehota	Lutila	Horná Ves	Hodruša Hámre	
I. ricinus[1]	M	13/1/1/1/0/0/0	9/4/0/0/0/1/0			68/22/5/2/1/3/6
	F	17/6/1/1/1/0/1	29/11/3/0/0/2/5			
A. flavicollis[2]	M	5/1/0	2/0/0	2/0/0		19/1/1
	F	7/0/1	1/0/0	2/0/0		
A. sylvaticus	M	1/0/0				2/0/0
	F			1/0/0		
C. glareolus	M	5/0/0			1/0/0	9/0/0
	F	2/0/0		1/0/0		

M = male; F = female; [1]No. tested ticks/HT - *R. helvetica*- /*Candidatus* N. mikurensis- /*E. muri*- /*A. phagocytophilum*- /*Candidatus* M. mitochondria – positive; [2]No. tested rodents/*E. muris*- /*Bartonella* sp. – positive.

Rickettsia-like microorganisms (RLO) were detected by light microscopy in 27.3% of the infected L929 cell cultures (in 2 of 7 isolates of HT positive ticks from Malá Lehota and 4 of 15 isolates of HT positive ticks from Lutila), but PCR did not confirm the presence of *Rickettsia* sp. in the infected L929 cells. We tried to obtain primary isolates of potential rickettsiae circulated in central Slovakia, but without success.

Microorganisms detected in ticks collected from rodents

All nymphs developed from engorged *I. ricinus* and *I. trianguliceps* larvae collected from *A. flavicollis* mice were negative for rickettsiae, ehrlichiae, and anaplasmae.

Microorganisms detected in rodents

The presence of *E. muris* and *Bartonella* sp. was found in 2 of the trapped rodents (6.7%) (Table 1). Male yellow-necked mouse was infected with *E. muris,* Acc. No. EF378623 (Smetanová *et al*., 2007). This isolate had 99.7% identity with sequences amplified from *I. ricinus* ticks collected in the same locality Acc. No. EF660077. The DNA obtained from the spleen of female yellow-necked mouse had 96.3% identity with *Bartonella* sp. (Acc. Nos. U64691, Z69039) and *B. clarridgeiae* (Acc. Nos. AB292603, AJ299444).

Discussion

The presented results confirmed the presence of *R. helvetica, E. muris, Candidatus* N. mikurensis, and *Candidatus* M. mitochondrii infections in *I. ricinus* ticks collected from vegetation and the presence of *Bartonella* sp. in the rodent *A. flavicollis* in central Slovakia.

I. ricinus is a vector and natural reservoir of *R. helvetica*. This bacterium was detected in *Ixodes* spp. in many European countries (Beati *et al*., 1994; Parola *et al*., 1998; Nilsson *et al*., 1999; Beninati *et al*., 2002; Christova *et al*., 2003; Nielsen *et al*., 2004; Hartelt *et al*., 2004; Sreter-Lancz *et al*., 2005) and our results confirmed its presence also in Slovakia. However, the role of wild animals in circulation of the *R. helvetica* is still not clear.

An intracellular rickettsial bacterium *Candidatus* M. mitochondrii, alpha-proteobacterial symbiont of *I. ricinus* females, characterized by Beninati *et al*. (2004) is restricted to the cytoplasm and mitochondria of the ovarian cells, what explained the fact that our findings were limited only to the female ticks. However, lower prevalence of *Candidatus* M. mitochondrii was observed also in male ticks (Sassera *et al*., 2006).

A. phagocytophilum is transmitted by *I. ricinus* ticks and its epizootiology includes domestic and wild ruminants. Up till now, a definitive role of rodents has not been determined (de la Fuente *et al*., 2005b). We detected an infection of *A. phagocytophilum* in the ticks and the high prevalence (51.9 ± 11.1%) of this bacterium was detected also in a roe and red deer (Štefanidesová *et al*., 2008). These findings suggested that central Slovakia could be a natural focus, where *A. phagocytophilum* circulation included *I. ricinus* ticks as a vector and the deer as a reservoir.

I. ricinus, *I. ovatus* and *I. persulcatus* ticks are the vectors of *Candidatus* N. mikurensis (Wielinga *et al*., 2006, Kawahara *et al*., 2004, Sphynov *et al*., 2004, 2006). In Slovakia, Ehrlichia-like sp. "Schotti variant" was detected in *I. ricinus* nymph collected from a song thrush (Špitalská *et al*., 2006). Our results showed that *Candidatus* N. mikurensis was found also in *I. ricinus* adult ticks collected from the vegetation. Up till now, a mammalian host for these bacteria has not been identified in Europe, but we expect that the rodents might play a role.

Primarily, *E. muris* was identified in Asia, the mice *Eothenomys kageus*, *A. speciosus, A. argenteus* were recognized as its reservoirs, and the ticks *H. flava*, *I. persulcatus* as its vectors (Kawahara *et al*., 1993, 1999; Ravyn *et al*., 1999; Alekseev *et al*., 2001; Rar *et al*., 2005; Eremeeva *et al*., 2006; Shpynov *et al*., 2006). Our previous report presented the first molecular evidence of the *E. muris* infection in yellow-necked mouse in Europe and consecutively, our present study confirmed *E. muris* as the infecting agent also in the ticks *I. ricinus* of central Europe (Smetanová *et al*., 2007).

Bartonella spp. was isolated from the rodents in UK and the ticks were recognized as its potential vectors (Birtles *et al*., 1994). On the other hand, we did not find any data about the occurrence of *Bartonella* sp. infection among the animals or ticks in Slovakia. It seemed that our detection of *Bartonella* sp. in yellow-necked mouse was the first random finding in our country.

Attempts to directly grow insect-associated intracellular bacteria in mammalian cell culture remain problematic. Low degree of success isolations could be caused by the absence of rickettsial bacteria in drop of hemolymph in time of isolation. HT of the ticks and isolation were not performed in the same time. The discrepancy in the obtained results of light microscopy of infected cells and PCR assay could be explained by presumable presence of morphologically identical, but genetically different microorganisms as RLO. Low number of rickettsia isolates from the collected ticks was confirmed also in other study (Boldiš and Kocianová, 2006).

We can conclude that central Slovakia is an area with the occurrence of various tick-borne microorganisms such as *R. helvetica, A. phagocytophilum, Candidatus* N. mikurensis, *E. muris,* and *Bartonella* sp. These microorganisms circulate in *I. ricinus* ticks or rodents, but ticks unlike rodents remain

active in the transmission of rickettsiae. In addition, the finding of Asian species *E. muris* and *Bartonella* sp. in Slovakia (central Europe) emphasizes the importance of our study.

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