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Rickettsia slovaca and *Rickettsia raoultii* in *Dermacentor marginatus* and *Dermacentor reticulatus* ticks from Slovak Republic

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Abstract Rickettsiae, obligate intracellular Gram-negative bacteria, responsible for mild to severe diseases in humans are associated with arthropod vectors. *Dermacentor marginatus* and *Dermacentor reticulatus* are known vectors of *Rickettsia slovaca* and *Rickettsia raoultii* distributed across Europe. A total of 794 *D. marginatus*, *D. reticulatus* and *Ixodes ricinus* adult ticks were collected from the vegetation, removed from horses, sheep, goats and dogs in Slovakia. The DNA of *Rickettsia* sp. was found in 229 ticks by PCR amplifying parts of *gltA*, *ompA* and *sca4* genes. Next analyses of *Rickettsia*-positive samples by PCR–RFLP and/or sequencing showed *D. reticulatus* ticks were more infected with *R. raoultii* and *D. marginatus* were more infected with *R. slovaca*. The prevalence of *R. raoultii* was 8.1–8.6% and 22.3–27% in *D. marginatus* and *D. reticulatus*, respectively. The prevalence of *R. slovaca* was 20.6–24.3% in *D. marginatus* and 1.7–3.4% in *D. reticulatus*. Intracellular growth of *R. raoultii* isolate from *D. marginatus* tick was evaluated by rOmpA-based quantitative SybrGreen PCR assay. The highest point of multiplication was recorded on the 7th and 8th day postinfection in Vero and L929 cells, respectively. *R. raoultii* was transmitted during feeding of *R. raoultii*-positive ticks to guinea pigs and subsequently rickettsial infection was recorded in all organs, the highest infection was in spleen, liver and heart. Our study describes the detection and isolation of tick-borne pathogens *R. raoultii* and *R. slovaca*, show that they are spread in Slovakia and highlight their risk for humans.

Keywords *Rickettsia slovaca* · *Rickettsia raoultii* · *Dermacentor marginatus* · *Dermacentor reticulatus* · Slovakia

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Introduction

Rickettsiae are obligate intracellular Gram-negative bacteria that are associated with arthropod vectors and are responsible for mild to severe diseases in humans (Raoult and Roux 1997).

Rickettsia raoultii, *Rickettsia* sp. genotypes DnS14, DnS28 and RpA4, was first identified as new rickettsiae of the *Rickettsia massiliae* genogroup in 1999, by *rrs* (16S rDNA), *gltA* and *ompA* sequencing from *Dermacentor nutallii* ticks collected in Siberia and *Rhipicephalus pumilio* ticks collected in Astrakhan (Rydkina et al. 1999). *R. raoultii* strains were isolated from PCR-positive *Dermacentor* ticks, *Dermacentor silvarum*, *D. nuttalli*, *Dermacentor reticulatus* and *Dermacentor marginatus*, collected in Russia, Kazakhstan, and France using cell cultures (Rydkina et al. 1999; Mediannikov et al. 2008). Herein, we describe the first cultivation of *R. raoultii* in Slovakia from *D. marginatus*. Since 1999, *R. raoultii* have been detected in *Dermacentor* ticks throughout Europe, in the European part of Russia (Shpynov et al. 2001), Germany (Dautela et al. 2006), Spain (Márquez 2008), Portugal (Vitorino et al. 2007), Netherlands (Nijhof et al. 2007), Slovakia (Boldiš et al. 2008), France and Croatia (Mediannikov et al. 2008), Poland (Chmielewski et al. 2009), UK (Tijsse-Klasen et al. 2011) and in *Haemaphysalis punctata* collected in Spain (Márquez 2008). In 2002, Mediannikov et al. (2008) detected *R. raoultii* DNA in *D. marginatus* tick taken from the scalp of a patient in whom TIBOLA (tick-borne lymphadenopathy)/DEBONEL (*Dermacentor*-borne necrosis erythema and lymphadenopathy) developed in France. It seems to be less pathogenic than *Rickettsia slovaca* (Parola et al. 2009).

Rickettsia slovaca was first isolated in 1968 from the tick *D. marginatus* collected in central Slovakia (Brezina et al. 1969). *R. slovaca* had been considered as a non-pathogenic microorganism for many years. In 1997 it was described as a human pathogen and the etiological agent of TIBOLA/DEBONEL human disease, which is associated with a tick bite, an inoculation eschar on the scalp, and cervical lymphadenopathies (Raoult et al. 1997). *R. slovaca* and human diseases caused by it were recorded across Europe (Parola et al. 2009).

Ixodes ricinus, D. marginatus, D. reticulatus, Haemaphysalis concinna, Haemaphysalis punctata, and *Haemaphysalis inermis* (the family Ixodidae, the order Acari) are exophillic tick species occurring in Slovakia. *Ixodes ricinus* ticks are widely distributed throughout the country whereas *D. reticulatus* and *D. marginatus* ticks are limited to along the rivers and Krupinská planina, respectively (Řeháček et al. 1991).

The aim of this study was to isolate, identify and characterize rickettsial species occurring in *Dermacentor* ticks inhabiting Slovakia.

Materials and methods

Collection of ticks

D. marginatus and *D. reticulatus* ticks were collected by blanket-dragging over the vegetation in Bratislava, Horný Bar, Martinský les, Moravský sv. Ján localities in western Slovakia and Veľký Lom, Kalonda, Píla, Sása, Pinciná, Budiná localities in central Slovakia during 2004–2010 years. They were classified to species, sex and maintained alive at +4°C prior to the examination.

Hemocyte test (HT)

Ticks were microscopically tested by HT, hemocytes from one droplet of hemolymph from ticks were stained by the Gimenez method (Burgdorfer 1970). HT can indicate the presence of microorganisms with rickettsial morphology, such as *Rickettsia* sp., *Coxiella burnetii*, rickettsia-like microorganisms, but can not differentiate microorganism species. Ticks were still alive after the screening by the HT method, which is important for the isolation and cultivation of rickettsiae in cell lines.

Isolation and cultivation of rickettsiae in Vero and L929 cell lines

Isolation of rickettsiae was attempted on the hemolymph-positive *Dermacentor* tick. One droplet of hemolymph obtained from previously unbroken leg of tick, was inoculated into one cultivation well (shell vial) containing monolayer of confluent Vero or L929 cells. After inoculation, the shell vials were centrifuged for 45 min at 1,000*g*, 25°C. Then the monolayer was incubated in a CO_2 incubator for 120 min at 33°C. Finally, the cells were incubated in CO_2 incubator at 33°C for 7–10 days. For study of intracellular growth, *R. raoultii* was cultivated in Vero and L929 cells (inoculum $\sim 10^5$ *R. raoultii* per flask). Negative controls of Vero and L929 cells without rickettsiae were also done. In three parallel series of static cultivation the growth medium was not replaced over the 14 days. Each 24 h intervals of cultivation infected cells in growth medium were scrapped, frozen at −70°C and after defrost centrifuged (5,000*g*, 10 min). Pellet was processed to extraction of DNA for quantification of rickettsial DNA copies by qPCR.

Infection of guinea pigs with *Rickettsia raoultii*

Experimental infection of guinea pigs through infected ticks was done analogous to study of Niebylski et al. (1999). *Rickettsia raoultii*-positive adult ticks fed 7 days on 3 guinea pigs. Blood from guinea pigs was collected every seventh day during 4 weeks and serum, heart, marrow, liver, lungs, bones, spleen and brain were collected from guinea pigs after their death. Guinea pigs died spontaneously. All organs and blood have been subjected to DNA extraction and subsequently to qPCR.

DNA extraction

All infected cell line, tick, blood and organ samples were individually processed by PCR. The DNA from infected mammalian cells, blood and organs from guinea pigs was isolated according to Wilson (1995) by phenol–chloroform extraction. The DNA from ticks collected from the vegetation was extracted using alkaline hydrolysis. Each ticks were washed with 70% ethanol and sterile water, crushed with sterile forceps and treated with 0.7 M ammonium hydroxide (NH_4OH) for 15 min at 100°C in sealed PCR tubes. Subsequently, NH_4OH was evaporated for 25 min at 100°C. DNA from ticks acquired from animals was extracted using Dneasy Blood and Tissue Kit (Qiagen) according to manufacturing protocol. DNA extractions were stored at −20°C and later used as templates for the PCR amplification.

PCR amplification, PCR–RFLP and DNA sequencing

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). Detection of *Rickettsia* sp. was done with specific primers RpCS.877p–RpCS.1258n amplified 381 bp part of the *gltA* gene, RR190.70F–RR190.701R amplified 632 bp part of *ompA* gene and D767f–D1390r primers amplified a part of 623 bp of the *sca*4 gene (Regnery et al. 1991; Roux et al. 1996). Enzymatic digestion for the identification of *R. slovaca* was performed as described by Špitalská et al. (2008). PCR and PCR–RFLP products were analyzed by eletrophoresis in a 1% agarose gel, stained with GelRed (Biotium), and visualized with UV transilluminator. Amplicons were purified using a QIAquick Spin PCR Purification Kit (Qiagen) as described by the manufacturer. The sequencing was performed by Macrogen (South Korea; 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/. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

qPCR assay

PCR amplification was conducted using DyNAmo HS SYBR Green qPCR kit reagents according to the manufacturer's instructions (Finnzymes, Finland). The reaction mixture and conditions for amplification were described by Boldiš and Špitalská (2010). Negative controls contained all PCR reaction components, as template DNA were DNAs of uninfected mammalian cells and nuclease-free water. The 632-bp fragment of the *ompA* gene of *R. slovaca* strain B13 was amplified and cloned into pGEM-T easy plasmid (Promega), vector was transformed into *Escherichia coli* competent cells and used as a standard for absolute quantification. To quantify the copy numbers of rickettsial gene in samples, tenfold serial dilutions of a plasmid were used to generate a standard curve.

Results

A total of 540 adult ticks, 272 *D. marginatus*, and 268 *D. reticulatus* were collected from the vegetation. Totally 249 adult *Dermacentor* ticks, 96 *D. marginatus*, 153 *D. reticulatus* were collected from horses, sheep, goats and dogs, and only 5 *I. ricinus* from horses (Table 1).

The DNA of *Rickettsia* sp. was found in 229 ticks, 69 *D. reticulatus* and 88 *D. marginatus* ticks from the vegetation and 44 *D. reticulatus* and 28 *D. marginatus* ticks collected from animals using PCR. The infection rate was from 25.49 to 36.17% depending on the collection and from 16.67 to 44% depending on sex of ticks (Table 1). PCR–RFLP and/or sequencing for identification of *Rickettsia* species showed that *D. reticulatus* ticks were more infected with *R. raoultii,* 86.67 and 94.12% infection of tick from vegetation and animals, respectively. Seventy-five percent of *D. marginatus* ticks from vegetation were infected with *R. slovaca* and *R. slovaca*-infection was found in 70.59% of *D. marginatus* ticks from animals. Table 2 shows the prevalence of each rickettsia in each species of *Dermacentor* ticks.

Isolation of rickettsiae was attempted on *D. marginatus* ticks, the hemolymphs of which were positive in the HT. Rickettsiae were detected in eukaryotic cells by genus-specific PCR followed by sequencing. The obtained isolate showed infection with *R. slovaca*

Table 1 Infection rate with *Rickettsia* sp. in *Dermacentor* ticks

Ticks from	*D. marginatus*	*D. reticulatus*
Vegetation	88/272 (32.35%)	69/268 (25.75%)
	21/102 (20.59%) M	31/108 (28.70%) M
	67/170 (39.41%) F	38/160 (23.75%) F
Horses	1/4	26/102 (25.49%)
	0/1 M	8/48 (16.67%) M
	1/3 F	18/54 (33.33%) F
Sheep	25/89 (31.46%)	
	7/41 (17.07%) M	
	18/48 (43.9%) F	
Goats	2/3 M	1/4
		0/1 M
		1/3 F
Dogs		17/47 (36.17%)
		11/25 (44.0%) M
		6/22 (27.27%) F
Total from animals	26/93 (27.96%)	44/153 (28.76%)
	9/45 (20.0%) M	19/74 (25.68%) M
	19/51 (37.25%) F	25/79 (31.65%) F

No. of *Rickettsia* sp. positive ticks/no. of tested ticks

Table 2 The prevalence of *Rickettsia raoultii* and *R. slovaca* in *Dermacentor marginatus* and *D. reticulatus* ticks in Slovakia

	In ticks from vegetation (%)		In ticks from animals (%)	
	R. raoultii	*R. slovaca*	*R. raoultii*	*R. slovaca*
D. marginatus	8.09	24.26	8.58	20.59
D. reticulatus	22.31	3.43	27.02	1.69

(Boldiš and Špitalská 2010) and *R. raoultii*. Partial sequencing of *ompA* gene of *R. raoultii* isolate (*R. raoultii* DMS, Acc. No. JN398480) confirmed 99.6% identity with *R. raoultii* strain Marne (Acc. No. DQ365799), (Fig. 1). Intracellular growth of *R. raoultii* (Fig. 2) was evaluated by quantitative PCR assay using L929 cells (triangle) and Vero cells (circles). Curves of bacterial growth were modeled with lag, exponential, stationary and death phases. *R. raoultii* achieved the highest point of multiplication on the 7th and 8th day postinfection in Vero and L929 cells, respectively. The rickettsial copy number in Vero and L929 cells per flask at this point was 1.6 and 1.4 times greater than rickettsial DNA copy number of inoculum, respectively.

Figure 3 shows the rickettsial copy number in blood and organs of guinea pigs, on which *R. raoultii*-positive adult ticks fed. The rickettsial infection was recorded in all organs, the highest infection was in spleen, liver and heart. The infection increased in time in blood samples.

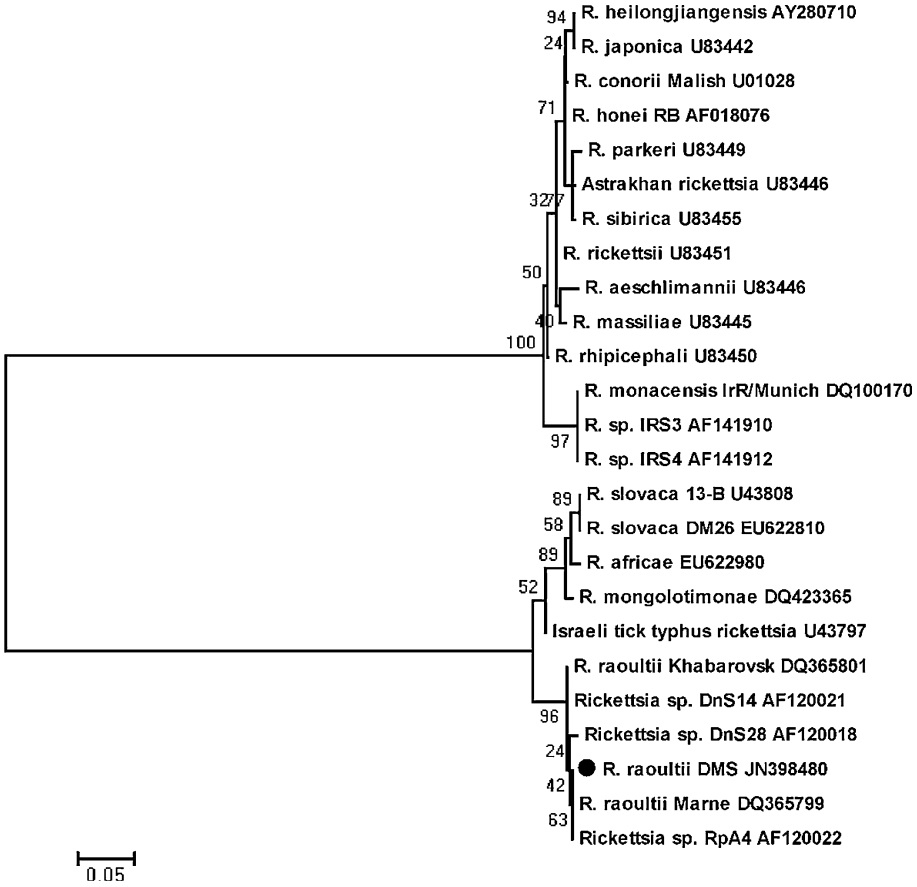

Fig. 1 Evolutionary relationship of *Rickettsia* spp. inferred from the comparison of a portion of the *ompA* gene using the Neighbor-Joining method. Bootstrap values are reported at the nodes. Sequences of *R. raoultii* DMS (highlighted by the *black circle*) were compared with sequences downloaded from the GenBank

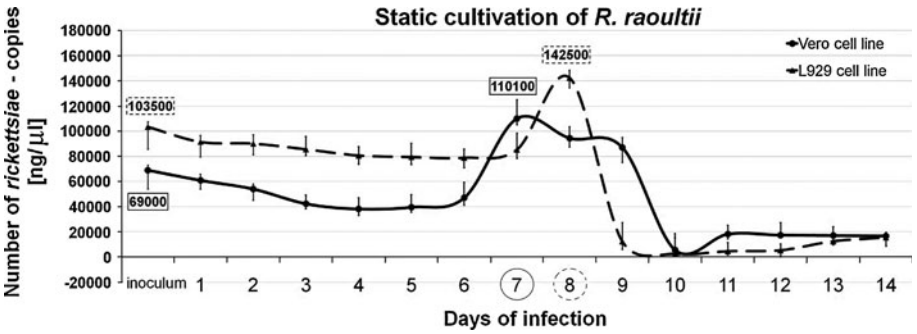

Fig. 2 The growth kinetics of *Rickettsia raoultii* in Vero and L929 determined by the quantitative PCR assay. Eukaryotic cells approaching confluence were infected with $\sim 10^5$ copy number of bacteria per flask. Values are the average ± SE of three replicates

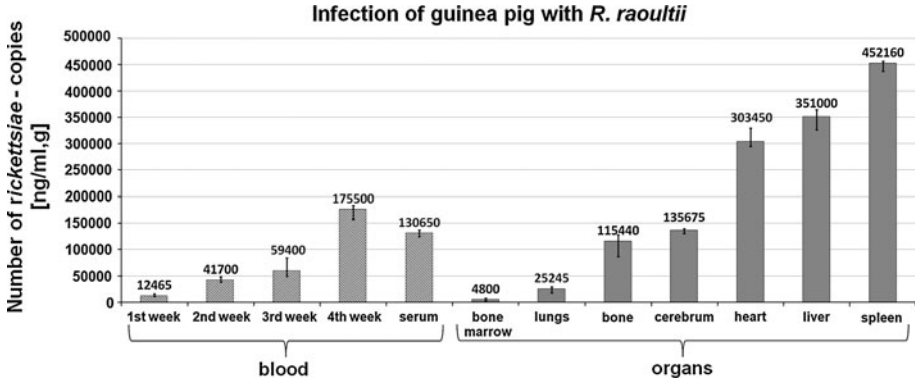

Fig. 3 Infection with *Rickettsia raoultii* in blood and organs of guinea pigs. Each point corresponds to the mean of three distinct experiments

Discussion

For long time is known, that *Dermacentor* ticks are the main vectors of some species of *Rickettsia*. To this time only prevalence of *Rickettsia* spp. in *Dermacentor* ticks in Slovakia was determined, but no information exists regarding the prevalence of *R. slovaca* and *R. raoultii* in *D. reticulatus* and *D. marginatus* ticks. Herein we provide detailed data related to the infection with these rickettsiae in *Dermacentor* ticks. Infection rates of *R. raoultii* in *D. reticulatus* were 86.7 and 94.1% from vegetation and animals, respectively. Contrary, infection rates of *R. slovaca* in *D. marginatus* were 75 and 70.6% from vegetation and animals, respectively. Similar study was conducted by Milhano et al. (2010) in Portugal, where 58.5% of *D. marginatus* ticks were infected with *R. raoultii* and 41.5% were *R. slovaca* positive. Socolovschi et al. (2009) defined infection rate with *R. slovaca* in *D. marginatus* ticks, which is 7.2–40.6% and with *R. raoultii* in *D. reticulatus* is 5.6–23%, in *D. marginatus* 22.5–83.3%. Part of our findings is in accordance with Socolovschi's et al. (2009) data. In our study, more *D. marginatus* ticks were infected by *R. slovaca* with prevalence 20.6 and 24.3% in ticks from animals and from vegetation, respectively. More *D. reticulatus* were infected by *R. raoultii* with 22.3% prevalence in ticks from vegetation and 27.0% in ticks from animals. Infection of *D. marginatus* with *R. raoultii* (8.0–8.6%) is lower in comparisson with Socolovschi's et al. (2009) data (22.5–83.3%). In previously published studies, *R. raoultii* has been more frequently detected in *D. marginatus* in Spain (73%) and Portugal (65%), in 57% of *D. reticulatus* ticks in Poland (Márquez et al. 2006; Vitorino et al. 2007; Chmielewski et al. 2009). The most (13 from 17) *D. reticulatus* ticks from Wales and England showed 100% homology with *R. raoultii* (Tijsse-Klasen et al. 2011). Infection of ticks removed from animals was studied by Dautela et al. (2006), Nijhof et al. (2007) and Selmi et al. (2009). They found that 14–23% *D. reticulatus* ticks were infected with *R. raoultii* and recorded 1.8 and 32.1% infection prevalence of *D. marginatus* with *R. raoultii* and *R. slovaca*, respectively. Their results are similar to ours. Differences in infection rates of both rickettsia in both *Dermacentor* species could be explained by the sampling methods, size of the samples, the potential PCR inhibitors, PCR reaction mixture and different detection surveys. Herein, we also described successful the first cultivation of *R. raoultii* in Slovakia from *D. marginatus* ticks. According to our knowledge, the bacterial growth kinetics of *R. raoultii* in mammalian cells was not done up

to date. In general, bacterial growth can be modeled with known four different phases. Similar phases of *R. raoultii* growth curves were seen in our study. Our findings added growth data that bacteria might be equally accustomed to growth in L929 and Vero cell lines at 33°C supplemented with RPMI 1,640 cell culture medium (PAA Laboratories, Austria) containing 5% fetal bovine serum (Gibco, BRL, USA). *R. raoultii* was transmitted during feeding of *R. raoultii*-positive ticks to guinea pigs. It is human pathogen causing a mild rickettsiosis in humans, but pathogenicity for guinea pigs is not known and requires more studies. Pathological assessment of *R. raoultii* was not the objective of the study, therefore it is not possible to identify whether *R. raoultii* caused any disease in guinea pigs.

Anyway, the high percentage of *D. reticulatus* and *D. marginatus* ticks infected with *R. slovaca* and *R. raoultii* strongly indicates increasing needs of medical attention for people in localities where these ticks occur. Data of this study indicate that clinicians should be aware that patient with tick-borne lymphadenopathy may be in Slovakia.

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