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NOTE

Rickettsia slovaca sp. nov., a member of the spotted fever group rickettsiae

Zuzana Sekeyová,[1,2] Véronique Roux,[1] Wenbin Xu,[1] Josef Řeháček[2] and Didier Raoult[1]

Author for correspondence: Didier Raoult. Tel: +33 4 91 32 43 75. Fax: +33 4 91 38 77 72. e-mail: Didier.Raoult@medecine.univ-mrs.fr

[1] Unité des Rickettsies, Faculté de Médecine, CNRS UPRES-A 6020, 13385 Marseille, France

[2] Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 46 Bratislava, Slovak Republic

The name *Rickettsia slovaca* sp. nov. (type strain is strain B) is proposed for a member of the spotted fever group (SFG) rickettsiae which was isolated from *Dermacentor marginatus* ticks in Slovakia in 1968, and was recently implicated in human febrile illness. This rickettsia can be phenotypically distinguished from other SFG rickettsiae by microimmunofluorescence serotyping, SDS-PAGE, Western blotting and mAbs. Genotypic differences between *R. slovaca* and the other SFG representatives can be demonstrated by PCR-RFLP analysis, pulsed-field gel electrophoresis and sequencing of 16S rRNA, *gltA* and *ompA* genes.

Keywords: *Rickettsia slovaca*, spotted fever group rickettsiae, taxonomic and phylogenetic analysis

Rickettsiae belong to the order *Rickettsiales*, whose members were described as obligately intracellular, Gram-negative micro-organisms. Traditionally, the species have been divided into two groups, namely the typhus group (TG) and the spotted fever group (SFG) on the basis of clinical presentation, immunological reactivity, DNA G+C content and intracellular position (Weiss & Moulder, 1984). Recently, serotypes of SFG rickettsiae have been isolated from patient specimens and supposedly non-pathogenic rickettsiae have been isolated from arthropods (Baird, 1992; Beati *et al*., 1992, 1997; Burgdorfer *et al*., 1979; Chen *et al*., 1996; Eremeeva *et al*., 1995; Fan *et al*., 1988; Goldwasser *et al*., 1974; Kelly *et al*., 1996; Tarasevich *et al*., 1991; Uchida *et al*., 1992; Werren *et al*., 1994; Yu *et al*., 1993). Presently, the SFG rickettsiae includes 14 validated species (Raoult & Roux, 1997; Weiss & Moulder, 1984).

Dermacentor marginatus ticks were collected in 1968 near the village of Velký Lom (Slovakia) and subjected to the haemolymph test to detect the presence of *Coxiella burnetii*. Bacteria were characterized in 14 out of 35 adult ticks, but their morphology and localization in tick haemocytes were different from those of the Q fever agent. Isolation in guinea pigs was unsuccessful, but seroconversion with *Rickettsia conorii* as an antigen was noted. Isolation of rickettsiae was performed twice during the same year from a pool of male and female *D. marginatus* ticks collected in the surroundings of the villages of Šula and Madačka, which are in the same district as the village of Velký Lom, and from a pool of males of the same tick species collected near the village of Madačka. The two strains were named 'B' and 'D', respectively (Řeháček, 1984).

Strains B and D were identified as members of the SFG rickettsiae (Brezina *et al*., 1969) and this relationship was confirmed by the discovery of a G+C content similar to that of *Rickettsia sibirica* and *R. conorii* (Schramek, 1974) and by complement fixation test results. This latter approach showed that these two isolates were closely related, but differed from all prototype strains of the SFG examined (Úrvölgyi & Brezina, 1978). Strains isolated from *D. marginatus* in central Slovakia and Armenia and from rodents of the genus *Apodemus* and *Microtus* in eastern Slovakia were demonstrated to be more closely related to *R. sibirica* (Makarova *et al*., 1978). A new SFG member was identified and it was proposed that it be named *Rickettsia slovaca* (Úrvölgyi & Brezina, 1978).

Justification for a new species

R. slovaca has features in common with the other SFG rickettsiae as determined by electron microscopy and

Abbreviations: MIF, microimmunofluorescence; rOmp, rickettsial outer-membrane protein; PFGE, pulsed-field gel electrophoresis; SFG, spotted fever group.

The GenBank accession numbers for the *Rickettsia slovaca* strain B 16S rRNA, *gltA* and *ompA* gene sequences determined in this work are L36224, U59725, and U43808 and U83454, respectively.

Table 1. Reactivities of mAbs directed against *R. slovaca* with all available SFG rickettsiae

mAb/specificity*	Titre of mAb with SFG rickettsial species†:														
	Afr	**Eth**	**Aes**	**Aka**	**AF**	**Aus**	**Bar**	**Bel**	**BJ**	**HA**	**Hel**	**Hon**	**Ind**	**Itt**	**Jpn**
SV2-C21/LPS-like	4096	4096	4096	4096	4096	4096	4096	–	4096	4096	4096	4096	4096	4096	4096
SV2-C22/LPS-like	4096	4096	4096	4096	4096	4096	4096	–	4096	4096	4096	4096	4096	4096	4096
SV6-E9/rOmp	8192	8192	–	–	8192	–	1024	–	8192	8192	–	2048	8192	8192	–
SV10-D2/rOmp	128	2048	–	–	4096	–	–	–	–	–	–	–	4096	2048	–
SV10-E8/rOmp	16384	16384	–	–	–	–	–	–	16384	16384	–	–	16384	–	–
	Titre of mAb with SFG rickettsial species†:														
	Ken	**Man**	**M-1**	**Mor**	**Sev**	**Mtu1**	**GS**	**Mon**	**Par**	**Rhi**	**Ric**	**S**	**Sib**	**Slo**	**Ttt**
SV2-C21/LPS-like	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096
SV2-C22/LPS-like	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096
SV6-E9/rOmp	8192	8192	8192	8192	8192	4096	4096	–	4096	2048	4096	4096	16384	2048	8192
SV10-D2/rOmp	4096	4096	512	2048	4096	4096	4096	–	–	–	–	–	–	16384	–
SV10-E8/rOmp	16384	16384	16384	16384	16384	–	–	–	16384	–	–	8192	16384	32768	–

* rOmp, rickettsial Omp; LPS-like, LPS-like antigen.

† The rickettsial species studied were *R. africae* Z9-Hu (Afr), *R. africae* Ethiopian (Eth), *R. aeschlimannii* MC16 (Aes), *R. akari* Kaplan (Aka), Astrakhan fever rickettsia A-167 (AF), *R. australis* Phillips (Aus), Strain Bar29 (Bar), *R. bellii* 369L42-1 (Bel), Strain BJ90 (BJ), '*R. mongolotimonae*' HA91 (HA), *R. helvetica* C9P9 (Hel), *R. honei* RB (Hon), *R. conorii* Indian tick typhus rickettsia (Ind), Israeli tick typhus rickettsia ISTT CDC 1 (Itt), *R. japonica* YH (Jpn), *R. conorii* Kenya tick typhus rickettsia (Ken), *R. conorii* Manuel (Man), *R. conorii* M-1 (M-1), *R. conorii* Moroccan (Mor), *R. conorii* Seven (Sev), *R. massiliae* Mtu1 (Mtu1), *R. massiliae* GS (GS), *R. montanensis* Tick strain (Mon), *R. parkeri* Maculatum 20 (Par), *R. rhipicephali* 3-7-6 (Rhi), *R. rickettsii* Sheila Smith (Ric), Strain S (S), *R. sibirica* 246 (Sib), '*R. slovaca*' 13-B (Slo) and Thai tick typhus rickettsia TT-118 (Ttt). –, no reactivity of rickettsia with mAb diluted 1:4 in hybridoma culture supernatant.

serological identification. However, microimmunofluorescence (MIF), mAbs, SDS-PAGE, pulsed-field gel electrophoresis (PFGE), PCR-RFLP and sequencing of the genes encoding citrate synthase (*gltA*), 16S rRNA and rOmpA (*ompA*) indicate that this rickettsia is different from all other SFG rickettsiae.

(i) MIF serotyping. Mouse immunization and MIF serotyping were performed (Beati *et al*., 1992, 1994; Eremeeva *et al*., 1995) and specificity differences (SPDs) were calculated using previously described methods (Philip *et al*., 1978). Calculated SPDs between *R. slovaca* and the other considered rickettsiae were variable and illustrated that MIF data are not completely reliable for definitive speciation with SFG rickettsiae.

(ii) mAbs. mAbs against *R. slovaca* were a gift from D. H. Walker (University of Texas, USA). These mAbs have cross-reacted in MIF with other SFG rickettsiae (Table 1; extracted from Xu & Raoult, 1997). Two mAbs with specificity to LPS surface structure (SV2-C21 and SV2-C22) cross-reacted with all available SFG rickettsiae, except with the strain *Rickettsia bellii*. Different results were obtained using mAbs SV6-E9, SV10-D2 and SV10-E8, which are directed against rickettsial outer-membrane proteins (rOmps). All three reacted with *Rickettsia africae*, Indian tick typhus rickettsia, Kenya tick typhus rickettsia and *R. conorii*, but gave the highest titres with homologous *R. slovaca*. The anti-rOmp mAbs did not react with *Rickettsia aeschlimannii*, *Rickettsia akari*, *Rickettsia australis*, *R. bellii*, *Rickettsia helvetica*, *Rickettsia japonica* and '*Rickettsia mongolotimonae*'. However, particular positive or negative reactions with each of tested mAbs, suggesting absence of the epitope on the surface of the rickettsiae, were also recorded.

mAbs raised against the other SFG rickettsial species, such as *R. africae*, *R. conorii* and *R. sibirica*, have been shown to cross-react with *R. slovaca* suggesting that this strain shares a number of common epitopes with other SFG rickettsial species. However, when screened with a large number of various mAbs, *R. slovaca* exhibited specific reactivity profiles and could be distinguished from other SFG rickettsial species, thus indicating the strain's antigenic divergence (Xu *et al*., 1997; Xu & Raoult, 1997, 1998).

(iii) SDS-PAGE and Western blot immunoassay. SDS-PAGE protein analysis performed for the identification of rickettsial isolates has revealed that *R. slovaca* profiles differed from those of other members of the SFG (Balayeva *et al*., 1993; Beati *et al*., 1993; Eremeeva *et al*., 1993, 1995) in the high molecular mass range. Western blot immunoassay with mouse serum anti-*R. conorii* or anti-*R. slovaca* revealed two reactive zones: specific high-molecular-mass proteins (two bands) and LPS antigen (Beati *et al*., 1993; Eremeeva *et al*., 1995).

(iv) PCR-RFLP analysis of specific genes. An electrophoresis migration pattern of the PCR-amplified DNA of *R. slovaca* with the Rp CS.877p and Rp CS.1258n

Table 2. Description of the rickettsial strains and the nucleotide accession numbers for the sequences used to construct the dendrogram in Fig. 1

Rickettsia	Strain	Accession no. for *gltA*	Accession no. for *ompA*	References
R. sibirica	246, ATCC VR-151^T		U43807, U83455	Roux *et al.* (1996); Fournier *et al.* (1998)
'*R. mongolotimonae*'	HA-91		U43796, U83439	
R. parkeri	Maculatum 20		U43802, U83449	
R. africae	ESF-5		U43790, U83436	
Strain S	Strain S		U43805, U83452	
R. conorii	Seven, ATCC VR-613^T		U43806, U83448	
R. conorii	Indian tick typhus rickettsia, ATCC VR-597^T		U43794, U83440	
Astrakhan fever rickettsia	A-167		U43791, U83437	
Israeli tick typhus rickettsia	ISTT CDC1		U43797, U83441	
R. slovaca	B		U43808, U83454	
Thai tick typhus rickettsia	TT-118, ATCC VR-599^T		U43809, U83456	
R. rickettsii	R (Bitterroot), ATCC VR-891^T		U43804, U83451	
R. japonica	YM	U59724	U43795, U83442	Roux *et al.* (1996,1997); Fournier *et al.* (1998)
Bar 29	Bar 29	U59720	U43792, U83438	
R. massiliae	Mtu1	U59719	U43799, U83445	
R. rhipicephali	3-6-7	U59721	U43803, U83450	
R. aeschlimannii	MC16	U59522	U43800, U83446	
R. montanensis	M/5-6	U74756	U43801, U83447	
R. helvetica	C9P9	U59723		Roux *et al.* (1997)
R. australis	Phillips	U59718		
R. akari	MK (Kaplan), ATCC VR-148^T	U59717		
R. typhi	Wilmington, ATCC VR-144^T	U59714		
R. prowazekii	Breinl, ATCC VR-142^T	M17149		
AB bacterium		U59712		
R. canadensis	2678, ATCC VR-610^T	U59713		
R. bellii	369L42-1	U59716		
Coxiella burnetii	Hamilton	M36338		R. A. Heinzen and others, GenBank

primer pair (chosen from the sequence of the *gltA* gene of *Rickettsia prowazekii*) digested with *Alu*I restriction endonuclease revealed the profile found in most of the SFG rickettsiae (Eremeeva *et al.*, 1994; Regnery *et al.*, 1991). In an extension of the previous work of Regnery *et al.* (1991) and Eremeeva *et al.* (1994), Roux *et al.* (1996) amplified by PCR and sequenced a fragment of the gene encoding the protein rOmpA from 21 serotypes of the SFG. The authors considered that *R. slovaca* presented a specific profile after enzymic digestion with *Pst*I and showed the specificity of the sequence. By PCR-RFLP of a fragment of the gene encoding the membrane protein rOmpB (PCR was performed with the primers BG1-21 and BG2-20 and enzymic digestion was performed with *Rsa*I), Eremeeva *et al.* (1994) found a specific profile for *R. slovaca* compared with the 12 other SFG rickettsiae studied.

The authors determined the sequence of the 590 bp used for SFG rickettsiae identification as described by Roux *et al.* (1996) and confirmed the identification of strain D as *R. slovaca*.

(v) PFGE. Roux & Raoult (1993) studied the chromosomes of SFG rickettsiae using PFGE. The mean size of the *R. slovaca* genome was estimated to be 1248 kb. This value is consistent with the genome size of other SFG rickettsiae. Specific profiles were obtained after total DNA digestion with low-frequency restriction endonucleases but the same patterns were obtained for different isolates of *R. slovaca* (Eremeeva *et al.*, 1993). A genetic relationship dendrogram established from PFGE patterns shows that the estimated levels of genetic divergence between *R. slovaca* and other rickettsiae studied are as follows: 32% with the closest relative, Thai tick typhus rickettsia; 36% with *R. sibirica* and *R. conorii*; and 36–52% with the other SFG rickettsiae tested.

(vi) Phylogenetic analyses inferred from sequence comparisons. Several studies have investigated the phylo-

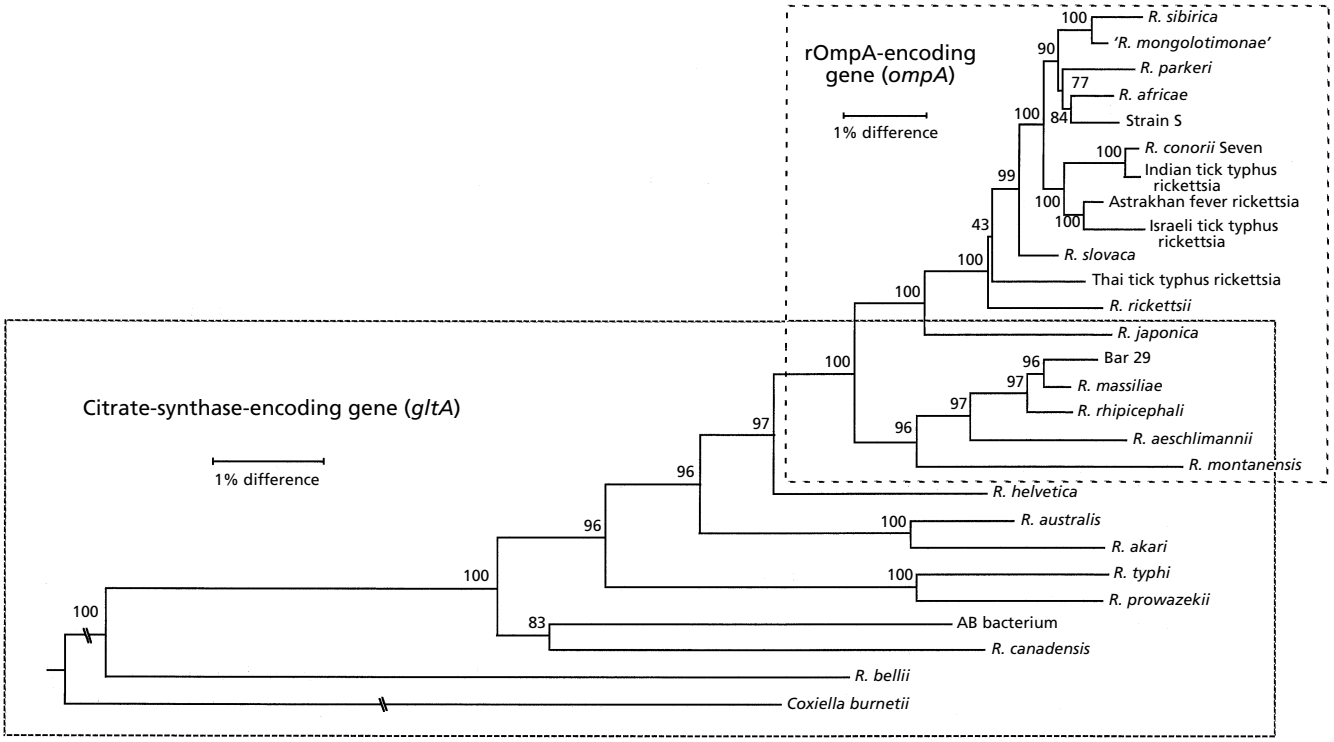

Fig. 1. Dendrogram representing phylogenetic relationships between *Rickettsia* species. The tree includes data determined from analysis of the *gltA* and *ompA* genes. Sequences extracted from GenBank were aligned with the multisequence alignment program CLUSTAL, which is part of the BISANCE software package. Phylogenetic relationships were inferred with version 3.4 of the PHYLIP software package. The evolutionary distance values were determined by the Jukes & Cantor method. These values were used to construct a dendrogram by the neighbour-joining method. Nucleotide accession numbers for sequences used to construct this dendrogram are listed in Table 2.

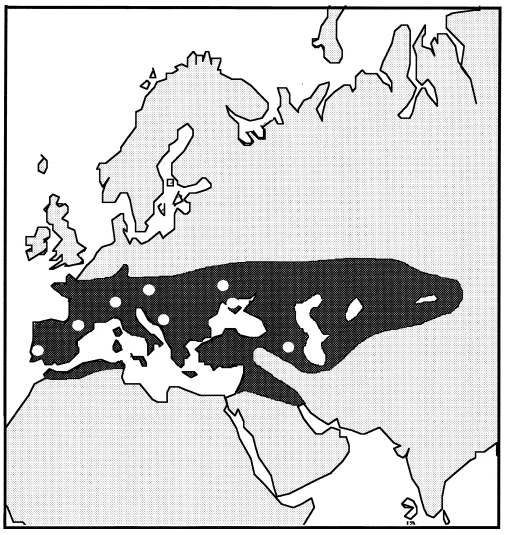

Fig. 2. Geographical distribution of the *D. marginatus* tick (dark shading). White circles indicate the locations where *R. slovaca* was isolated.

genetic organization of the bacteria of the genus *Rickettsia*. Sequence analysis has shown that *R. slovaca* has 16S rRNA, *gltA* and *ompA* gene sequences that differ from those of all the other SFG rickettsiae (Fig. 1). For the 16S rRNA gene, the level of similarity ranges from 98·1 % with AB bacterium to 99·8 % with *R. conorii* (Roux & Raoult, 1995). For *gltA* and *ompA*, the levels of similarity range from 86·6 % with *R. bellii* to 99·8 % with *R. sibirica* and from 95·3 % with *Rickettsia montanensis* to 98·4 % with *Rickettsia rickettsii*, respectively (Fournier *et al.*, 1998; Roux *et al.*, 1997). The position of *R. slovaca* was best established when phylogenetic analysis was inferred from *ompA* sequence comparisons. The species did not specifically cluster with any other rickettsia species but branched alone between the *Rickettsia massiliae* group and the large cluster including the *R. conorii* complex and the *R. sibirica* group (Fournier *et al.*, 1998).

On the basis of the distinctive clinical, epidemiological, phenotypic and genotypic features described above, we propose that *R. slovaca* should be considered a separate taxonomic species.

Description of *Rickettsia slovaca* sp. nov.

Rickettsia slovaca (slo.va.ca′. L. gen. n. *slovaca* from Slovakia, the country where the organism was first isolated).

Obligately intracellular Gram-negative bacteria. Most of the strains were isolated from *Dermacentor margina-*

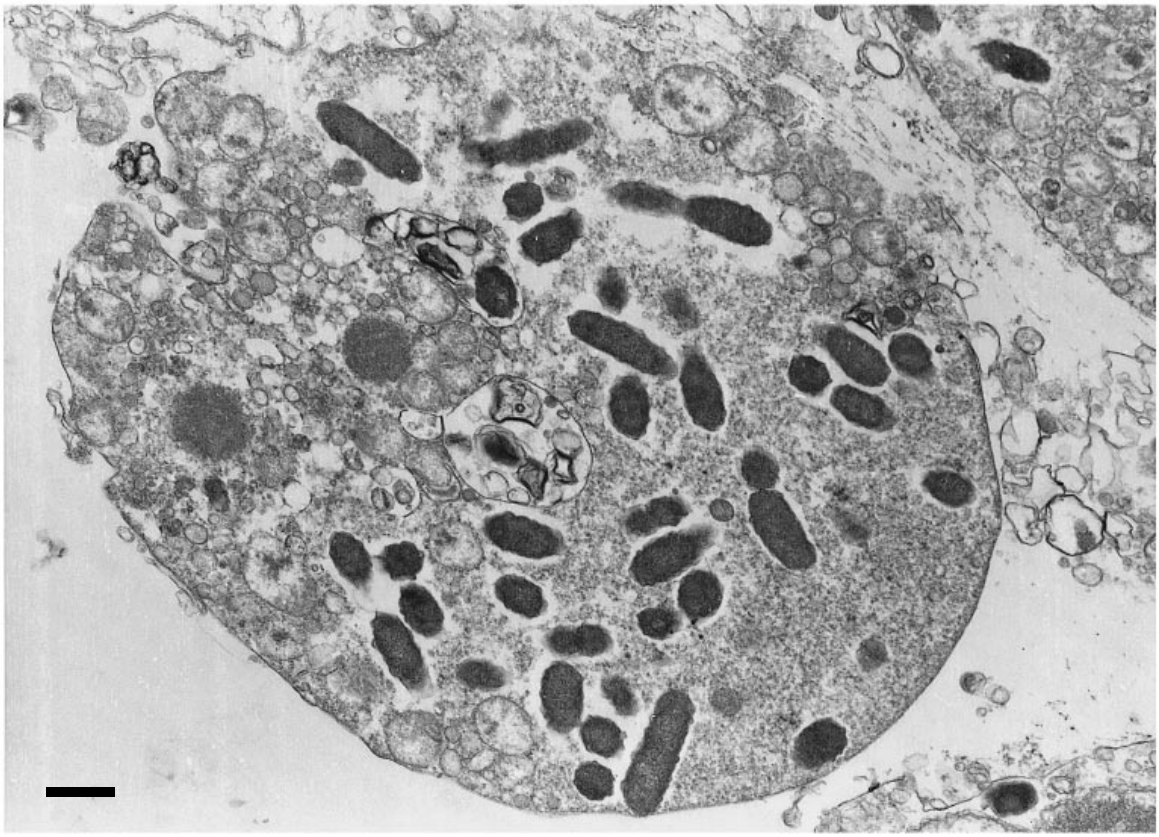

Fig. 3. Electron micrograph of *R. slovaca* cultivated on Vero cells. Bar, 1 μm.

tus ticks (Řeháček *et al*., 1972). However, rickettsial strains closely related to *R. slovaca* were detected and isolated from *Ixodes ricinus* (Úrvölgyi & Brezina, 1978; Řeháček, 1984), *Haemaphisalis punctata* (Řeháček *et al*., 1972), *Haemaphisalis inermis* (Řeháček, 1984), *Ixodes hexagonus* (Sixl *et al*., 1973) and *Argas persicus* (Řeháček *et al*., 1977b). Distribution of *R. slovaca* widely exceeds the borders of the Slovak and Czech Republics. It has since been isolated in Armenia (Balayeva *et al*., 1994; Makarova *et al*., 1978; Řeháček *et al*., 1977b; Tarasevich *et al*., 1976), Crimea (Balayeva *et al*., 1993), The Ukraine (Balayeva *et al*., 1993; Úrvölgyi & Brezina, 1978), France (Beati *et al*., 1993; Raoult *et al*., 1997), Switzerland (Beati *et al*., 1994), Yugoslavia (Manor *et al*., 1992; Sixl *et al*., 1976) and Portugal (Bacellar *et al*., 1995). Positive serology was reported from the Lithuanian Republic (Tarasevich *et al*., 1981), Germany (Řeháček *et al*., 1977a) and Austria (Sixl *et al*., 1973). It therefore seems to closely follow the distribution of its main host tick, *D. marginatus*, which can be found throughout Europe and as far as the western border of the People's Republic of China (Eremeeva *et al*., 1993) (Fig. 2). *R. slovaca* exhibits features characteristic of those of the SFG rickettsiae. It is a typical rod-shaped rickettsia, with dimensions of approximately 0·37–0·45 × 0·8–1·2 μm (Řeháček, 1984). *R. slovaca* can be propagated on chicken embryo, HEL, Vero, L929 and tick cells, although no cytopathologic changes in any of the cell cultures inoculated (despite the high multiplicity of infection) have been noted (Beati *et al*., 1993; Řeháček, 1984; Řeháček *et al*., 1977a). Infected cells stained by the Gimenez method revealed rickettsiae either scattered in host cytoplasm, in clusters or occasionally within the nuclei of cells. Electron microscopical studies showed that the bacteria were free in the cytoplasm and were surrounded by a slime layer (Řeháček, 1984) (Fig. 3).

The quantitative distribution of *R. slovaca* in organs and haemolymph of infected adult *D. marginatus* collected in a natural focus of this rickettsia in central Slovakia has been demonstrated (Župančičová & Kováčová, 1972). The majority of rickettsiae were seen in the haemolymph, gut, Malpigian tubes and nerve ganglia; rickettsiae were less frequent in the connective tissues, salivary glands and testes (Župančičová & Kováčová, 1972).

Intraperitoneal injection of rickettsiae does not kill white mice. Pathological changes in mice are limited to

a slightly enlarged spleen. Guinea pigs react only occasionally to intraperitoneal injection of rickettsiae, by demonstrating a brief and mild fever. *Lepus europaeus* infected subcutaneously with *R. slovaca* suffer a asymptomatic infection accompanied by irregular rickettsiaemia and distribution of rickettsiae to different organs (Brezina *et al.*, 1969; Řeháček, 1984). The course of infection with rickettsiae in hamsters after intraperitoneal inoculation is asymptomatic (Jablonskaja, 1976).

Human illness caused by *R. slovaca* was not described until recently. This agent has been suspected to be responsible for meningoencephalitis, accompanied by a prolonged persistance of neurasthenic disorders (Mittemayer *et al.*, 1980; Řeháček, 1984; Řeháček & Tarasevich, 1988). The most recent reported case in France confirmed its pathologic role (Raoult *et al.*, 1997). The symptoms of the patient's illness were severe headache, a necrotic eschar on the occiput surrounded by a reddish halo and four enlarged occipital lymph nodes, and a general weakness and tiredness that persisted for 2 months. A patient with meningoradiculitis following a tick bite was observed in south-west France (D. Raoult, unpublished data). Cases of patients bitten by *D. marginatus* ticks who developed the same clinical symptoms as those presented by this French patient were described in France as early as 1955. A rickettsial origin of these tick-transmitted diseases was suspected, but the aetiological agent was not identified (Giroud *et al.*, 1965). In 1981, again in France, the pathogenicity of *R. slovaca* was hypothesized by Edlinger in the aetiology of erythema chronicum migrans (Edlinger, 1981). Raoult *et al.* (1997) suggested that *Borrelia*-seronegative patients with manifestations resembling those of Lyme disease (a local erythema accompanied by meningoencephalitis) should perhaps be tested for *R. slovaca*. This might also apply in the case of patients from Hungary whose clinical symptoms – a benign infection with positive serology to Lyme borreliosis – detected in only some of the patients raised questions about the aetiology of the ongoing disease (Lakos, 1997).

In vitro susceptibility of *R. slovaca* to various antibiotics has been previously tested; the MIC for doxycycline was found to be 0·125 μg ml^{-1} and the MIC for rifampicin was 0·5 μg ml^{-1}. Doxycycline is the recommended treatment, and fluoroquinolones can be used as alternatives (MICs of 1 μg ml^{-1}). MICs for thiamphenicol, amoxicillin, cotrimoxazole are relatively high (1–128 μg ml^{-1}), whereas macrolide compounds are also effective, and can therefore be considered as alternative treatments during childhood and pregnancy (Rolain *et al.*, 1998).

Type strain. The type strain of *R. slovaca* is strain B, an isolate obtained from *D. marginatus* ticks; this strain has been deposited in the Collection of the World Health Organization Collaborative Center for Rickettsial Reference, Marseille, France and in the American Type Culture Collection.

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