Rickettsia raoultii sp. nov., a spotted fever group rickettsia associated with *Dermacentor* ticks in Europe and Russia

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We describe the characterization of a novel *Rickettsia* species cultivated from *Dermacentor* ticks collected in Russia and France, for which we propose the name *Rickettsia raoultii* sp. nov. Using multigene sequencing, we demonstrated that five rickettsial isolates from *Dermacentor silvarum*, *Dermacentor reticulatus*, *Dermacentor marginatus* and *Dermacentor nuttalli* ticks were classified within this novel spotted fever rickettsia species. This rickettsia also exhibited a serotype distinct from previously described *Rickettsia* species. The type strain of *Rickettsia raoultii* sp. nov. is strain Khabarovsk^T (=CSUR R3^T =ATCC VR-1596^T).

In 1999, three novel rickettsial genotypes, RpA4, DnS14 and DnS28, were identified in ticks collected in Russia using PCR amplification and sequencing of the *rrs* (16S rRNA), *gltA* and *ompA* genes (Rydkina *et al.*, 1999). *Rickettsia* sp. genotypes DnS14 and DnS28 were detected in *Dermacentor nuttalli* ticks collected in Siberia, whereas genotype RpA4 was detected in *Rhipicephalus pumilio* ticks collected in Astrakhan (Rydkina *et al.*, 1999). A later study demonstrated that *Dermacentor* ticks naturally infected with genotypes DnS14, DnS28 and RpA4 harbour these rickettsiae throughout the life cycle and that transovarial and transstadial transmission occurs (Samoilenko *et al.*, 2003). These rickettsial agents form a reliable cluster within the *Rickettsia massiliae* group (Rydkina *et al.*, 1999). This rickettsial group has been defined phylogenetically and phenotypically (Roux *et al.*, 1996b; Rolain *et al.*, 1998; Drancourt & Raoult, 1999) and it consists of *R. massiliae*, *Rickettsia rhipicephali*, *Rickettsia aeschlimannii* and *Rickettsia montanensis*, which exhibit a unique feature within the genus *Rickettsia*, their resistance to rifampicin caused by a Phe-to-Leu mutation within the *rpoB* gene (Drancourt & Raoult, 1999; Rolain *et al.*, 1998). Due to their phylogenetic homogeneity, it was suggested that *Rickettsia* sp. genotypes DnS14, DnS28 and RpA4 belonged to a novel species (Rydkina *et al.*, 1999). Genotypes identical to DnS14, DnS28 and RpA4 have also been detected in *Dermacentor* ticks from the European part of Russia (Shpynov *et al.*, 2001, 2004), Spain (Ibarra *et al.*, 2006) and Croatia (D. Raoult, personal communication). In 2002, we detected *Rickettsia* sp. genotype DnS14 by PCR in a *Dermacentor marginatus* tick taken from the scalp of a patient who had developed a typical clinical picture of tick-borne lymphadenitis (TIBOLA) in France.

Herein, we describe the first cultivation of two rickettsial isolates genetically identical to *Rickettsia* sp. genotype DnS14 (Rydkina *et al.*, 1999), two rickettsial isolates genetically identical to *Rickettsia* sp. genotype RpA4 (Rydkina *et al.*, 1999) and one rickettsial isolate genetically identical to *Rickettsia* sp. genotype DnS28 (Rydkina *et al.*, 1999). Using a polyphasic strategy combining genotypic and phenotypic tests, we demonstrate that these new rickettsial isolates fulfil the requirements for their classification within a novel species (Fournier *et al.*, 2003; Raoult *et al.*, 2005).

Abbreviations: MIF, microimmunofluorescence; SPD, specificity difference.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *gltA*, *ompA*, *ompB* and *sca4* sequences of strain Khabarovsk^T are DQ365810, DQ365804, DQ365801, DQ365798 and DQ365808, respectively, and those of the *ftsY* and *rpoB* gene sequences of strains Khabarovsk^T and Marne are respectively DQ387058 and DQ387059 (*ftsY*) and DQ365812 and DQ365811 (*rpoB*). Accession numbers for sequences from other strains are detailed in Table 1.

Using cell culture (Marrero & Raoult, 1989), we isolated five rickettsial strains from PCR-positive *Dermacentor* ticks collected on vegetation in Russia and France. Strain Khabarovsk^T was cultivated from a *Dermacentor silvarum* tick collected in Russian Far East in 2005, strain Shayman was obtained from a *D. silvarum* tick collected in eastern Siberia in 2002, strain Elanda-23/95 was cultivated from a *D. nuttalli* tick collected in Altay in 1995, strain Marne was cultivated from a *Dermacentor reticulatus* tick collected in the Marne region of eastern France in 2004 and strain 8/9 Karaganda was obtained from a *Dermacentor marginatus* tick collected in Kazakhstan in 2002. DNA was extracted from the five isolates using the QIAmp tissue kit (Qiagen) according to the manufacturer's instructions. Sequencing of the 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* genes was attempted from all five isolates using previously described primers and PCR conditions (Roux & Raoult, 1995, 2000; Roux *et al.*, 1996a, 1997; Sekeyova *et al.*, 2001). PCR products of the expected sizes were obtained from all five genes. Sequences were edited by removal of regions of ambiguity at the 5′ and 3′ ends so that their lengths were 1424, 1134, 590, 4890 and 3028 bp, respectively, for the 16S rRNA gene, *gltA*, *ompA*, *ompB* and *sca4*. Accession numbers for these sequences are reported in Table 1. Isolates Khabarovsk^T and Shayman exhibited identical sequences and their 16S rRNA gene and *gltA* sequences were identical to those of *Rickettsia* sp. genotype DnS14 (Rydkina *et al.*, 1999). Isolates Marne and 8/9 Karaganda also exhibited identical sequences and their 16S rRNA gene and *gltA* sequences were identical to those of *Rickettsia* sp. genotype RpA4 (Rydkina *et al.*, 1999). Isolate Elanda-23/95 exhibited 16S rRNA gene and *gltA* sequences identical to those of *Rickettsia* sp. genotype DnS28 (Rydkina *et al.*, 1999). The phylogenetic relationships of *Rickettsia* sp. strains Khabarovsk^T, Marne and Elanda-23/95 with all *Rickettsia* species with validly published names for which 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* gene sequences are available (Table 1) were evaluated for each gene using the neighbour-joining and maximum-parsimony methods within the MEGA 3.1 software (Kumar *et al.*, 2004) and the maximum-likelihood method within the PHYLIP software package (Felsenstein, 1989). For all studied genes and using the three analysis methods, *Rickettsia* sp. strains Khabarovsk^T, Marne and Elanda-23/95 clustered with the *R. massiliae* group with elevated bootstrap values (Fig. 1). When calculating similarity values between nucleotide sequences of strains Khabarovsk^T, Marne and Elanda-23/95 and *Rickettsia* species, transitions and transversions, not insertions or deletions, were included. For all five loci examined, strains Khabarovsk^T, Marne and Elanda-23/95

Table 1. GenBank accession numbers of the gene sequences used in the present study

Strain	16S rRNA gene	*gltA*	*ompA* (5′ end)	*ompB*	*sca4*
Rickettsia raoultii sp. nov.					
Khabarovsk^T (=CSUR R3^T)	DQ365810	DQ365804	DQ365801	DQ365798	DQ365808
Marne (=CSUR R9)	DQ365809	DQ365803	DQ365799	DQ365797	DQ365807
Elanda-23/95 (=CSUR R171)	EU036982	EU036985	EU036986	EU036984	EU036983
R. aeschlimannii MC16^T	U74757	U59722	U43800	AF123705	AF163005
R. africae ESF-5	L36098	U59733	U43790	AF123706	AF151724
R. akari MK (Kaplan)^T (=ATCC VR-148^T)	L36099	U59717	NA	AF123707	AF213016
R. asiatica IO-1^T (=CSUR R2^T)	AF394906	AF394901	NA	DQ110870	DQ110869
R. australis NIAID Phillips 32^T	L36101	U59718	AF149108	AF123709	AF187982
R. bellii 369L42-1	L36103	U59716	NA	NA	NA
R. canadensis 2678^T (=ATCC VR-610^T)	L36104	U59713	NA	NA	NA
R. conorii NIAID Malish 7^T (=ATCC VR-613^T)	AF541999	U59730	U43806	AF123721	AF163008
R. felis URRWXCal2 (=ATCC VR-1525)	L28944	AF210692	AF210694	AF210695	AF196973
R. heilongjiangensis 054^T (=ATCC VR-1524^T)	AF178037	AF178034	AF179362	AY260451	AY331396
R. helvetica C9P9	L36212	U59723	NA	AF123725	AF163009
R. honei RB^T (=ATCC VR-1472^T)	U17645	AF018074	AF018075	AF123711	AF163004
R. japonica YM	L36213	U59724	U43795	AF123713	AF155055
R. massiliae Mtu1^T	L36214	U59719	U43799	AF123714	AF163003
R. montanensis M/5-6	L36215	U74756	U43801	AF123716	AF163002
R. parkeri NIAID maculatum 20^T	L36673	U59732	U43802	AF123717	AF155059
R. prowazekii Breinl^T (=ATCC VR-142^T)	M21789	M17149	NA	AF123718	AF200340
R. rhipicephali Burgdorfer 3-7-female 6^T	L36216	U59721	U43803	AF123719	AF155053
R. rickettsii R (Bitteroot) (=ATCC VR-891)	L36217	U59729	U43804	X16353	AF163000
R. sibirica 246^T (=ATCC VR-151^T)	L36218	U59734	U43807	AF123722	AF155057
R. slovaca 13-B	L36224	U59725	U43808	AF123723	AF155054
R. tamurae AT-1^T (=CSUR R1^T)	AY049981	AF394896	DQ103259	DQ113910	DQ113911
R. typhi Wilmington^T (=ATCC VR-144^T)	L36221	U59714	NA	L04661	AF188482

NA, Not amplifiable.

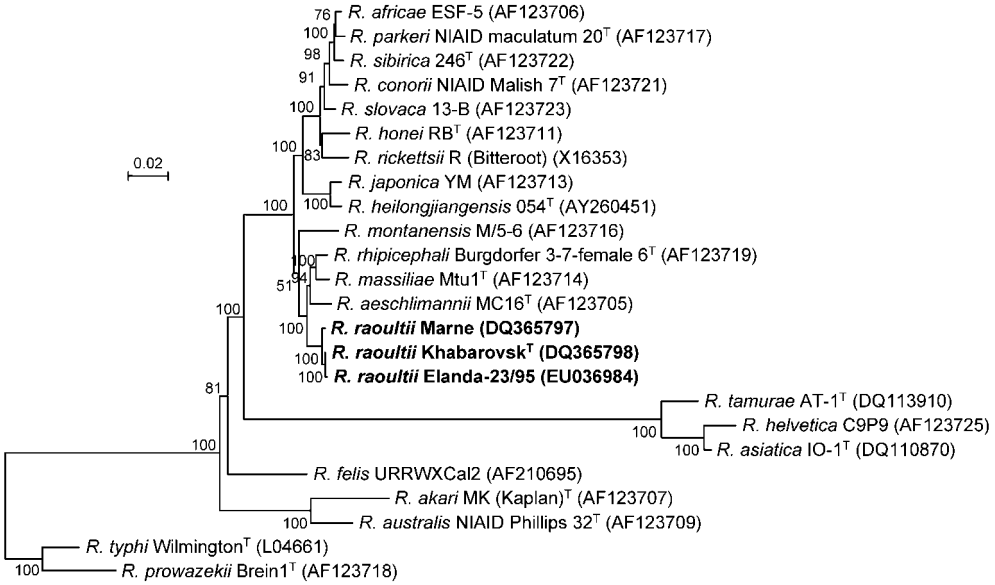

Fig. 1. Unrooted dendrogram showing the phylogenetic position of *Rickettsia* sp. isolates Khabarovsk^T, Marne and Elanda-23/95 among *Rickettsia* species inferred from the comparison of *ompB* sequences by the neighbour-joining method. Bootstrap values are indicated at nodes. Bar, 2 % nucleotide sequence divergence.

shared highest sequence similarity with the type strain of *R. rhipicephali* (99.5, 99.2, 96.8, 98.1 and 97.7 %; 99.6, 99.1, 97.0, 98.1 and 97.9 %; and 99.5, 99.0, 96.6, 98.0 and 97.3 %, respectively, for the 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* genes). However, these values were lower than the cut-offs proposed for *Rickettsia* species definition (Fournier *et al.*, 2003). In contrast, similarity values higher than these cut-offs were found between strains Khabarovsk^T and Marne (99.9, 99.9, 99.5, 99.6 and 99.5 %), Khabarovsk^T and Elanda-23/95 (99.9, 99.7, 99.5, 99.9 and 99.5 %) and Marne and Elanda-23/95 (99.9, 99.9, 99.3, 99.5 and 99.2 %). Therefore, on the basis of genotypic criteria, *Rickettsia* sp. strains Khabarovsk^T, Marne and Elanda-23/95, although closely related to members of the *R. massiliae* group (Fig. 1), belonged to a single distinct species. In addition, we compared *Rickettsia* sp. strains Khabarovsk^T, Marne and Elanda-23/95 to '*Rickettsia amblyommii*' (Burgdorfer *et al.*, 1981), also classified phylogenetically within the *R. massiliae* group. We found degrees of nucleotide sequence similarity of *Rickettsia* sp. strains Khabarovsk^T, Marne and Elanda-23/95 with '*R. amblyommii*' of 99.1, 98.8, 97.3 and 96.3 %, 99.3, 98.8, 97.1 and 96.2 % and 99.2, 98.6, 97.3 and 96.0 %, respectively, for the 16S rRNA, *gltA*, *ompA* and *ompB* genes, thus classifying it in a different species.

For further tests, we selected strains Khabarovsk^T and Marne as representatives of this novel species. The G+C contents of strains Khabarovsk^T and Marne estimated by sequencing the *ftsY* gene as previously described (Fournier *et al.*, 2006) were 33.9 and 33.8 mol%, respectively. By amplification and sequencing of a 364 bp *rpoB* fragment of *Rickettsia* sp. strains Khabarovsk^T and Marne as described previously, we identified the mutation that confers rifampicin resistance in both strains previously identified in members of the *R. massiliae* group (Drancourt & Raoult, 1999). Mouse serotyping was conducted by microimmunofluorescence (MIF) as described by Philip *et al.* (1978). We used as antigens *Rickettsia* sp. strains Khabarovsk^T and Marne, *R. rhipicephali* Burgdorfer 3-7-female 6^T, *R. aeschlimannii* MC-16^T, *R. massiliae* Mtu1^T and *R. montanensis* M/5-6, cultivated on L929 cells as described previously (Marrero & Raoult, 1989). Strains Khabarovsk^T and Marne caused cytopathic effects after 5 days of incubation. If the specificity difference (SPD) was $\geqslant$3, the isolates were assumed to belong to different serotypes. Using serum from mice immunized with strain Khabarovsk^T, we found MIF antibody titres of 1 : 512 to the homologous antigen and to strain Marne and of 1 : 256 to the other four tested antigens. Using serum from mice immunized with strain Marne, we found MIF antibody titres of 1 : 512 to the homologous antigen and strain Khabarovsk^T and of 1 : 128 to the other four antigens. Serum from mice immunized with *R. massiliae* Mtu1^T produced antibody titres of 1 : 1024 to the homologous antigen and of 1 : 256 to strains Khabarovsk^T and Marne. Serum from mice immunized with *R. aeschlimannii* MC-16^T produced antibody titres of 1 : 1024 to the homologous antigen and of 1 : 256 to strains Khabarovsk^T and Marne. Serum from mice immunized with *R. rhipicephali* Burgdorfer 3-7-female 6^T produced antibody titres of 1 : 1024 to the homologous antigen and of 1 : 256 to strains Khabarovsk^T and Marne. Finally, using serum from mice immunized with *R. montanensis* M/5-6, we found antibody titres of 1 : 256 to the homologous antigen and of 1 : 32 to

strains Khabarovsk^T and Marne. On the basis of these results, the SPD between these rickettsiae was 1, 4, 4, 3 and 4, respectively, between strain Khabarovsk^T and strain Marne, *R. massiliae*, *R. aeschlimannii*, *R. rhipicephali* and *R. montanensis*. The SPD was 4, 5, 4 and 3, respectively, between strain Marne and *R. massiliae*, *R. aeschlimannii*, *R. rhipicephali* and *R. montanensis*. Therefore, the genotypic and serotypic specificity of *Rickettsia* sp. strains Khabarovsk^T and Marne justify their classification within a distinct species. Using scanning microscopy, the cell length of isolate Khabarovsk^T varied from 0.6 to 2.0 μm and the mean diameter was 0.4 μm (Fig. 2a) and, using transmission electron microscopy, it was found free within the cytoplasm of L929 cells, but not in the nucleus (Fig. 2b).

Our data support the proposal to classify *Rickettsia* sp. strain Khabarovsk^T within a novel species. Thus, we formally propose the creation of *Rickettsia raoultii* sp. nov., which contains strains Khabarovsk^T, Marne, Shayman, 8/9 Karaganda and Elanda-23/95. This rickettsia has been found in France, Spain, Croatia, Russia and Kazakhstan.

Description of *Rickettsia raoultii* sp. nov.

Rickettsia raoultii (ra.oul′ti.i. N.L. masc. gen. n. *raoultii* of Raoult, named after Professor Didier Raoult, founder of the WHO-Collaborative Centre for Rickettsioses, Borrelioses and Tick-borne Infections in Marseilles, France, and a major contributor to the study of rickettsiae).

Gram-negative, obligately intracellular bacterium. Grows in L929 and Vero cells at 32 °C in minimal essential medium supplemented with 2 % heat-inactivated fetal calf serum and 2 mM glutamine. Non-motile. 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* gene sequencing indicate that this rickettsia is clearly different from all other recognized rickettsial species, the most closely related organism being *R. massiliae*. Estimated G+C content is 33.8 mol%. Resistant to rifampicin. No information is available about the possible pathogenicity of this organism for vertebrate hosts. The known geographical distribution of this bacterium is France, Spain, Croatia, Russia and Kazakhstan.

The type strain is isolate Khabarovsk^T (=CSUR R3^T =ATCC VR-1596^T), which was isolated from *Dermacentor silvarum* ticks from the Russian Far East in 2005. The type strain has also been deposited in the rickettsial collection of the World Health Organization-Collaborative Gamaleya Institute under reference 147. Strains Shayman, Marne, 8/9 Karaganda and Elanda-23/95 have been deposited in the Collection de souches de l'unité des Rickettsies (CSUR), WHO-Collaborative Centre for Rickettsioses, Borrelioses and Tick-borne Infections, Marseille, France, under references CSUR R8, CSUR R9, CSUR R10 and CSUR R171, respectively, and are also being deposited in the ATCC.

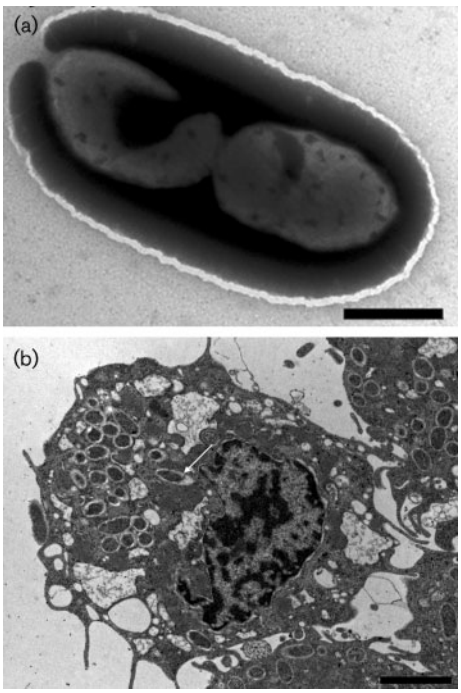

Fig. 2. (a) *Rickettsia* sp. isolate Khabarovsk^T cultivated in L929 cells. Rickettsial bacillus in the process of division, with lophotrichous flagella. Scanning electron microscopy; bar, 1.0 μm. (b) *Rickettsia* sp. isolate Khabarovsk^T appears as bacilli in the cytoplasm (arrow). Transmission electron microscopy; bar, 2.0 μm.

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