Rickettsia asiatica sp. nov., isolated in Japan

Hiromi Fujita,^{1†} Pierre-Edouard Fournier,^{2†} Nobuhiro Takada,³
Takehiro Saito⁴ and Didier Raoult²

Correspondence

Pierre-Edouard Fournier
pierre-edouard.fournier@
medecine.univ-mrs.fr

¹Ohara Research Laboratory, Ohara General Hospital, Fukushima 960-0195, Japan

²Unité des rickettsies, IFR 48, CNRS UMR 6020, Faculté de médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France

³Department of Pathological Sciences, Faculty of Medical Sciences, University of Fukui, Matsuoka, Fukui 910-1193, Japan

⁴Aizawa Hospital, 2-5-1 Honjo, Matsumoto 390-8510, Japan

The characterization of a novel *Rickettsia* species, first isolated from *Ixodes ovatus* ticks in Japan in 1993, is described for which the name *Rickettsia asiatica* sp. nov. is proposed. Using multilocus sequence comparison, five rickettsial isolates from *I. ovatus* collected at various locations in Japan were found to be identical but belonged to a novel species. Serotyping also demonstrated this rickettsia to be distinct from previously described *Rickettsia* species. The type strain of *Rickettsia asiatica* sp. nov. is IO-1^T (= CSUR R2^T). Following discussions held at the meeting of the International Committee on Systematics of Prokaryotes (ICSP) and its Judicial Commission (JC) in San Francisco, 2005, and in anticipation of the published minutes of the JC and ICSP, a committee consisting of the chairman of the ICSP, the chairman of the Judicial Commission of the ICSP and the Editor of the IJSEM has granted an exception in this case to the Rule governing the deposit of type material in two different collections in two different countries.

In Japan, the discovery of *Rickettsia japonica* (Uchida *et al.*, 1992) as the agent of Japanese spotted fever (Mahara *et al.*, 1985) has motivated the study of spotted fever group (SFG) rickettsiae in ticks. To date, 63 SFG rickettsial strains have been isolated from ticks in this country (Fujita *et al.*, 1999). Among these, only three *Rickettsia* species with validly published names have been identified, namely, *R. japonica*, *Rickettsia helvetica* and *Rickettsia tamurae*. *R. japonica* has been identified in several tick species including *Haemaphysalis longicornis*, *Haemaphysalis flava*, *Haemaphysalis formosensis*, *Haemaphysalis hystricis*, *Dermacentor taiwanensis* and *Ixodes ovatus* (Mahara, 1997; Fournier *et al.*, 2002). *R. helvetica* has been found in *Ixodes monospinosus* and *Ixodes persulcatus* (Fournier *et al.*, 2002) and *R. tamurae* has been isolated from *Amblyomma testudinarium* (Fournier *et al.*, 2006). In June 1993, a rickettsial isolate named IO-1^T was obtained from an *I. ovatus* nymph collected in Oozaso, in the Fukushima prefecture (Fujita *et al.*, 1999). Subsequently, 32 additional isolates that exhibited *gltA* gene sequences identical to that of the IO-1^T isolate have been obtained from *I. ovatus* ticks in Japan. To date, this rickettsia has not been found in any other

tick species. A preliminary mouse serotyping assay and sequencing of the entire 16S rRNA (*rrs*) and *gltA* genes demonstrated that *Rickettsia* sp. strain IO-1^T exhibits a unique serotype and unique genotypic characteristics (Takada *et al.*, 2001; Fournier *et al.*, 2002). An electron microscopic study of *Rickettsia* sp. strain IO-1^T demonstrated that it multiplied in the cytoplasm of L929 cells, although a few bacteria were seen in intranuclear location (Yano *et al.*, 2004). However, the ability of this rickettsia to stimulate actin-based motility, as described for other SFG rickettsiae (Heinzen *et al.*, 1993), has not been investigated to date. We herein demonstrate that this rickettsia fulfils the requirements for its proposal as a novel species (Fournier *et al.*, 2003; Raoult *et al.*, 2005).

Five IO-1-like rickettsial isolates obtained from Japanese *I. ovatus* ticks were included in our study. These included strain IO-1^T described above, strain IO-2 from the Kagoshima prefecture (isolated in 1994), strain IO-12 from the Fukushima prefecture (1997), strain IO-25 from the Akita prefecture (1997) and strain IO-38 from Awaji island (2001). For strain IO-1^T, we first retrieved *rrs* and *gltA* nucleotide sequences from GenBank (Table 1). We then extracted DNA from strain IO-1^T and the other four isolates using the QIAmp tissue kit (QIAGEN), according to the manufacturer's instructions. PCR amplification and sequencing of the 5' and 3'-ends of the *ompA* gene and the complete *ompB* and *sca4* genes were attempted from all five isolates using previously described primers and

†These authors contributed equally to this work.

Abbreviations: MIF, microimmunofluorescence; SFG, spotted fever group.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gltA*, *ompB* and *sca4* gene sequences of strain IO-1^T are AF394906, AF394901, DQ110870 and DQ110869, respectively.

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

NA, Not available.

Strain	16S rRNA	<i>gltA</i>	<i>ompB</i>	<i>sca4</i>
<i>R. asiatica</i> sp. nov. IO-1 ^T	AF394906	AF394901	DQ110870	DQ110869
<i>R. aeschlimannii</i> MC16 ^T	U74757	U59722	AF123705	AF163005
<i>R. africana</i> ESF-5	L36098	U59733	AF123706	AF151724
<i>R. akari</i> MK (Kaplan) ^T (=ATCC VR-148 ^T)	L36099	U59717	AF123707	AF213016
<i>R. australis</i> NIAID Phillips 32 ^T	L36101	U59718	AF123709	AF187982
<i>R. bellii</i> 369L42-1	L36103	U59716	NA	NA
<i>R. canadensis</i> 2678 ^T	L36104	U59713	NA	NA
<i>R. conorii</i> NIAID Malish 7 ^T (=ATCC VR-613 ^T)	AF541999	U59730	AF123721	AF163008
<i>R. felis</i> URRWXC2 (=ATCC VR-1525)	L28944	AF210692	AF210695	AF196973
<i>R. helvetica</i> C9P9	L36212	U59723	AF123725	AF163009
<i>R. honei</i> TT-118 (=ATCC VR-599)	L36220	U59726	AF123724	AF163004
<i>R. japonica</i> YM	L36213	U59724	AF123713	AF155055
<i>R. massiliae</i> Mtu1 ^T	L36214	U59719	AF123714	AF163003
<i>R. montanensis</i> M/5-6	L36215	U74756	AF123716	AF163002
<i>R. parkeri</i> NIAID maculatum 20 ^T	L36673	U59732	AF123717	AF155059
<i>R. peacockii</i> Skalkaho ^T	U55820	AY590152	NA	NA
<i>R. prowazekii</i> Brein 1 ^T (=ATCC VR-142 ^T)	M21789	M17149	AF123718	AF200340
<i>R. rhipicephali</i> Burgdorfer 3-7-female 6 ^T	L36216	U59721	AF123719	AF155053
<i>R. rickettsii</i> R (Bitterroot) (=ATCC VR-891)	L36217	U59729	X16353	AF163000
<i>R. sibirica</i> 246 ^T (=ATCC VR-151 ^T)	L36218	U59734	AF123722	AF155057
<i>R. slovacica</i> 13-B	L36224	U59725	AF123723	AF155054
<i>R. tamurae</i> AT-1 ^T	AY049981	AF394896	DQ113910	DQ113911
<i>R. typhi</i> Wilmington ^T (=ATCC VR-144 ^T)	L36221	U59714	L04661	AF188482

conditions (Fournier *et al.*, 1998; Roux & Raoult, 2000; Sekeyova *et al.*, 2001). In addition, *rrs* and *gltA* genes were amplified from strains IO-2, IO-12, IO-25 and IO-38. No *ompA* PCR product could be obtained from any of the studied isolates. However, whether this negative result is the result of the absence of the *ompA* gene in IO-1-like isolates or the incapacity of the primers used to amplify the gene is not yet known. In contrast, PCR products of the expected sizes were obtained for the other genes. Sequences were edited by removal of regions of ambiguity at the 5' and 3' ends so that their lengths were 1417, 1047, 4845 and 2946 bp for *rrs*, *gltA*, *ompB* and *sca4*, respectively. The five isolates exhibited identical sequences for each of the four genes. For further tests, we selected the IO-1^T strain as representative of these five isolates. The phylogenetic relationships of *Rickettsia* sp. strain IO-1^T with all *Rickettsia* species with validly published names for which *rrs*, *gltA*, *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. strain IO-1^T was grouped with *R. helvetica* with elevated bootstrap values (Fig. 1). When calculating similarity values between nucleotide sequences of strain IO-1^T and *Rickettsia* species, transitions or transversions, not insertions or deletions, were included. For all four loci

examined, strain IO-1^T shared highest sequence similarity with *R. helvetica* (99.0, 99.0, 98.2 and 98.3 %, respectively, for the *rrs*, *gltA*, *ompB* and *sca4* genes). However, these values were lower than the cut-offs proposed for *Rickettsia* species definition (Fournier *et al.*, 2003). Therefore, on the basis of genotypic criteria, *Rickettsia* sp. strain IO-1^T, although phylogenetically closely related to the *R. helvetica* species (Fig. 1), belonged to a distinct species. In addition, we compared *Rickettsia* sp. strain IO-1^T to another Japanese rickettsia, *R. tamurae*, also phylogenetically related to *R. helvetica* (Fournier *et al.*, 2006). We found degrees of nucleotide sequence similarity between the two rickettsiae of 98.5, 96.7, 95.7 and 91.2 %, respectively, for the *rrs*, *gltA*, *ompB* and *sca4* genes. Therefore, the two rickettsiae were sufficiently genetically different to be classified within different species. Mouse serotyping was conducted by microimmunofluorescence (MIF) as described by Philip *et al.* (1978). We used as antigens *Rickettsia* sp. strain IO-1^T, *R. helvetica* type strain C9P9^T and *R. tamurae* type strain AT-1^T, cultivated on Vero cells (ATCC CRL-1587) as described previously (Marrero & Raoult, 1989). Strain IO-1^T caused cytopathic effects after 5 days of incubation. If the specificity difference (SPD) was ≥ 3 , the isolates were assumed to belong to different serotypes. Using serum from mice immunized with strain IO-1^T, we found MIF antibody titres of 1:400, 1:50 and 1:100 to the homologous antigen and to *R. tamurae* and *R. helvetica*, respectively. Using serum from mice immunized with *R. tamurae*, we found MIF antibody

Fig. 1. Unrooted dendrogram showing the phylogenetic position of *Rickettsia* sp. strain IO-1^T among *Rickettsia* species with validly published names inferred from the comparison of *ompB* gene sequences by the neighbour-joining method. GenBank accession numbers are indicated in parentheses. Bootstrap values are indicated at nodes. Bar, 5% nucleotide sequence divergence.

titres of 1 : 400, 1 : 50 and 1 : 100 to the homologous antigen and to strain IO-1^T and *R. helvetica*, respectively. Finally, using serum from mice immunized with *R. helvetica*, we found MIF antibody titres of 1 : 800, 1 : 200 and 1 : 100 to the homologous antigen and to strain IO-1^T and *R. tamurae* antigens, respectively. On the basis of these results, the SPD between these rickettsiae was 4 between strain IO-1^T and *R. helvetica* and 6 between strain IO-1^T and *R. tamurae*. Therefore, the genotypic and serotypic specificity of *Rickettsia* sp. strain IO-1^T justify its classification in a distinct species.

Our data support the proposal to classify *Rickettsia* sp. strain IO-1^T within a new species. Thus, we formally propose the creation of *Rickettsia asiatica* sp. nov., which contains the strains IO-1^T, IO-2, IO-12, IO-25 and IO-38. This rickettsia has only been found in Japan to date. Following discussions held at the meeting of the International Committee on Systematics of Prokaryotes (ICSP) and its Judicial Commission (JC) in San Francisco, 2005, and in anticipation of the published minutes of the JC and ICSP, a committee consisting of the chairman of the ICSP, the chairman of the Judicial Commission of the ICSP and the Editor of the *International Journal of Systematic and Evolutionary Microbiology* has granted an exception in this case to the Rule governing the deposit of type material in two different collections in two different countries.

Description of *Rickettsia asiatica* sp. nov.

Rickettsia asiatica (a.si.a'ti.ca. L. fem. adj. *asiatica* Asian).

Gram-negative, obligately intracellular bacterium. Grows in Vero and L929 cells at 32 °C in minimal essential medium supplemented with 2% fetal calf serum and 2 mg L-glutamine ml⁻¹. Non motile. 16S rRNA, *gltA*, *ompB* and *sca4* gene sequencing indicate that this rickettsia is clearly different from all other recognized rickettsial species, the most closely related organisms being *R. helvetica* and *R. tamurae*. No information is available about the possible pathogenicity of this organism for vertebrate hosts. The known geographical distribution of this bacterium is restricted to Japan.

The type strain is strain IO-1^T (=CSUR R2^T), which was isolated from *Ixodes ovatus* ticks in 1993 in Fukushima prefecture, Japan (Fujita *et al.*, 1999). The type strain has been deposited in the Collection de souches de l'Unite des rickettsies (CSUR), WHO Collaborative Center for Rickettsioses, Borrelioses and Tick-borne Infections, Marseilles, France, and is being deposited in the American Type Culture Collection (ATCC). Strains IO-2, IO-12, IO-25 and IO-38, have been deposited in the CSUR under references CSUR R4, CSUR R5, CSUR R6 and CSUR R7, respectively, and are also being deposited in the ATCC.

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