Rickettsia tamurae sp. nov., isolated from *Amblyomma testudinarium* ticks

Pierre-Edouard Fournier,¹ Nobuhiro Takada,² Hiromi Fujita³ and Didier Raoult¹

Correspondence

Pierre-Edouard Fournier
Pierre-Edouard.Fournier@
medecine.univ-mrs.fr

¹Unité des rickettsies, IFR 48, CNRS UMR 6020, Faculté de médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France

²Department of Pathological Sciences, Faculty of Medical Sciences, University of Fukui, Matsuoka, Fukui 910-1193, Japan

³Ohara Research Laboratory, Ohara General Hospital, Fukushima 960-0195, Japan

Rickettsia sp. strain AT-1^T was isolated from *Amblyomma testudinarium* ticks in Japan in 1993. Comparative analysis of sequences obtained from 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* gene fragments demonstrated those from AT-1^T to be markedly different from those of other members of the spotted fever group. Using mouse serotyping, it was also observed that *Rickettsia* sp. strain AT-1^T was different from other *Rickettsia* species with validly published names. Such genotypic and phenotypic characteristics warrant its classification as a representative of a novel species, for which the name *Rickettsia tamurae* sp. nov. is proposed, with the type strain AT-1^T (= CSUR R1^T).

In Japan, *Rickettsia japonica* (Uchida *et al.*, 1992), causing Japanese spotted fever (Mahara *et al.*, 1985), was the first described spotted fever rickettsia. *R. japonica* has been identified in several tick species including *Haemaphysalis longicornis*, *H. flava*, *H. formosensis*, *H. hystricis*, *Dermacentor taiwanensis* and *Ixodes ovatus* (Fournier *et al.*, 2002). In addition to *R. japonica*, another species, *Rickettsia helvetica*, has been isolated from ticks in Japan, being found in *Ixodes persulcatus* and *Ixodes monospinosus* (Fournier *et al.*, 2002). Several other rickettsial isolates have been obtained from *I. ovatus* and *Amblyomma testudinarium* ticks (Fournier *et al.*, 2002). These include a rickettsial isolate, AT-1^T, cultivated in L929 cells from an *A. testudinarium* nymph collected in Anan, in the Tokushima prefecture (Fujita *et al.*, 1999). Forty-five further rickettsial isolates were obtained from *A. testudinarium* ticks collected in Japan (Fujita *et al.*, 1996; Takada *et al.*, 2001) and were demonstrated to be genetically highly similar to AT-1^T (Fujita *et al.*, 1996; Takada *et al.*, 2001; Fournier *et al.*, 2002). So far, strains conspecific with *Rickettsia* sp. strain AT-1^T have not been found in any other tick species. Previously, we demonstrated that *Rickettsia* sp. strain AT-1^T was most closely related to strain IRS4, an uncultivated Slovakian rickettsia from *Ixodes ricinus* (Sekeyova *et al.*, 2000) that was later shown to be closely related to '*Rickettsia monacensis*' (Simser *et al.*, 2002), the name of which has not yet been validly published.

Preliminary mouse serotyping results and sequence comparison of the 16S rRNA gene (*rrs*) and *gltA* genes suggested that *Rickettsia* sp. strain AT-1^T exhibits unique serotypic and genotypic characteristics among spotted fever rickettsiae (Takada *et al.*, 2001; Fournier *et al.*, 2002). Herein, using multigene sequencing and mouse serotyping, we evaluated whether this rickettsia fulfils the minimum requirements to be classified within a novel species.

DNA from *Rickettsia* sp. strain AT-1^T was extracted using the QIAamp tissue kit (Qiagen) according to the manufacturer's instructions. PCR amplification and sequencing of the 5'-end of the *ompA* gene and the complete *ompB* and *sca4* genes were attempted using the primers and conditions described previously (Fournier *et al.*, 1998; Roux & Raoult, 2000; Sekeyova *et al.*, 2001). PCR products were sequenced twice in each direction. *rrs* and *gltA* nucleotide sequences from *Rickettsia* sp. strain AT-1^T, *rrs*, *gltA*, *ompA*, and, when available, *sca4* nucleotide sequences from its closest rickettsia relatives, *Rickettsia* sp. strain IRS4 and '*R. monacensis*', as well as *rrs*, *gltA*, *ompB* and *sca4* nucleotide sequences from the closest *Rickettsia* species with a validly published name, *R. helvetica*, were retrieved from GenBank. Sequences were edited by removal of primer sequences from the 5' and 3' ends. Only pairwise transitions and transversions between sequences, not deletions and insertions, were taken into account to calculate the degree of sequence similarity. Based on *ompA* sequences, *Rickettsia* sp. strain AT-1^T was identical to *Rickettsia* sp. strain ATT (GenBank accession no. AF483202), detected by PCR in *A. testudinarium* in Thailand (Hirunkanokpun *et al.*, 2003). Numbers of nucleotide

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gltA*, *ompA*, *ompB* and *sca4* gene sequences of strain AT-1^T are respectively AY049981, AF394896, DQ103259, DQ113910 and DQ113911.

substitutions between *Rickettsia* sp. strain AT-1^T and *R. helvetica* were 15 (98.9% nucleotide sequence similarity), 47 (96.1%), 257 (94.7%) and 275 (90.9%), respectively, for the *rrs*, *gltA*, *ompB* and *sca4* genes. Thus, for each of these four genes, *Rickettsia* sp. strain AT-1^T exhibited similarity rates with *R. helvetica* lower than the cut-offs proposed to classify rickettsial isolates within a species (99.8, 99.9, 99.2 and 99.3%, respectively, for the *rrs*, *gltA*, *ompB* and *sca4* genes; Fournier *et al.*, 2003). Therefore, on the basis of genotypic criteria, *Rickettsia* sp. strain AT-1^T did not belong to the species *R. helvetica*. In addition, it was also classified into a species distinct from *Rickettsia* sp. strain IRS4 (Sekeyova *et al.*, 2000), with degrees of sequence similarity between these two rickettsiae being 99.3, 98.7, 94.0 and 97.8%, respectively, for the *rrs*, *gltA*, *ompA* and *sca4* genes. Finally, when compared to '*R. monacensis*' (Simser *et al.*, 2002), *Rickettsia* sp. strain AT-1^T exhibited degrees of sequence similarity of 98.5, 99.1 and 93.6%, respectively, for the *rrs*, *gltA* and *ompA* genes, and thus these two rickettsiae belong to distinct species. A dendrogram inferred from *ompB* sequences by the neighbour-joining method, showing the position of strain AT-1^T within the genus *Rickettsia*, is shown in Fig. 1.

Mouse serotyping was conducted as described previously (Philip *et al.*, 1978). We used as antigens *Rickettsia* sp. strain AT-1^T and *R. helvetica* C9P9^T, cultivated on Vero cells (ATCC CRL-1587) as described previously (Marrero & Raoult, 1989). For each antigen, five BALB/c mice were inoculated intravenously with purified bacterial suspension (0.1 ml containing 10⁴ bacteria) without adjuvant. On day 7, mice were boosted with a similar inoculum. On day 10, mice were anaesthetized and exsanguinated by cardiac puncture. Sera from each group of mice were pooled. Microimmunofluorescence (MIF) was performed and the specificity difference (SPD) was calculated as described

previously (Philip *et al.*, 1978). If the SPD was ≥ 3 , the isolates were assumed to belong to different serotypes. Using serum from mice immunized with *Rickettsia* sp. strain AT-1^T, we found MIF antibody titres of 1:400 and 1:100 to *Rickettsia* sp. strain AT-1^T and *R. helvetica* C9P9^T, respectively. Using serum from mice immunized with *R. helvetica* C9P9^T, we found MIF antibody titres of 1:800 and 1:100 to *R. helvetica* C9P9^T and *Rickettsia* sp. strain AT-1^T, respectively. On the basis of these results, the SPD between the two rickettsiae was 7. Therefore, the genotypic and serotypic specificity of *Rickettsia* sp. strain AT-1^T support its classification within a distinct species.

On the basis of genotypic analyses, we have previously proposed the classification of *Rickettsia* sp. strain AT-1^T within a novel species (Fournier *et al.*, 2002). Our current results support this proposal. Thus, we formally propose the creation of *Rickettsia tamurae* sp. nov., containing strain AT-1^T as the type strain. This rickettsia has been found in Japan and Thailand.

Following discussions held at the meetings of the International Committee on Systematics of Prokaryotes (ICSP) and its Judicial Commission (JC) in San Francisco in 2005, and in anticipation of the published minutes of these meetings, a committee consisting of the Chairman of the ICSP, the Chairman of the JC of the ICSP and the Editor of the *International Journal of Systematic and Evolutionary Microbiology* has granted an exception in this instance to Rule 27(3) of the *Bacteriological Code* governing the deposit of type material in two different collections in two different countries.

Description of *Rickettsia tamurae* sp. nov.

Rickettsia tamurae (ta.mu'rae, N.L. gen. masc. n. *tamurae* of Tamura, named in honour of the Japanese rickettsiologist

Fig. 1. Unrooted dendrogram showing the phylogenetic position of *Rickettsia* sp. strain AT-1^T among *Rickettsia* species with validly published names inferred from comparison of *ompB* sequences by the neighbour-joining method. Bootstrap values $>75\%$ are indicated at nodes. Bar, 2% nucleotide sequence divergence.

Dr Akira Tamura, who contributed to our knowledge of rickettsiae and rickettsioses in Japan).

Gram-negative, obligately intracellular bacterium. Grows on Vero cells at 32 °C in minimal essential medium supplemented with 2 % fetal calf serum and 2 mg L-glutamine ml⁻¹. Non-motile.

The type strain is strain AT-1^T (=CSUR R1^T), which was isolated from an *Amblyomma testudinarium* tick in 1993 in Tokushima prefecture, Japan (Fujita *et al.*, 1999). The type strain has been deposited in the Collection de souches de l'Unité des Rickettsies (CSUR), World Health Organization Collaborative Center for Rickettsioses, Borrelioses and Tick-borne Infections, Marseille, France. Attempts are also being made to deposit the type strain in the American Type Culture Collection.

Acknowledgements

The part of this work carried out in Japan was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant nos 13576005 and 16406008).

References

- Fournier, P. E., Roux, V. & Raoult, D. (1998). Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. *Int J Syst Bacteriol* **48**, 839–849.
- Fournier, P. E., Fujita, H., Takada, N. & Raoult, D. (2002). Genetic identification of rickettsiae isolated from ticks in Japan. *J Clin Microbiol* **40**, 2176–2181.
- Fournier, P. E., Dumler, J. S., Greub, G., Zhang, J., Wu, Y. & Raoult, D. (2003). Gene sequence-based criteria for identification of new rickettsia isolates and description of *Rickettsia heilongjiangensis* sp. nov. *J Clin Microbiol* **41**, 5456–5465.
- Fujita, H., Takada, N. & Tsuboi, Y. (1996). Survey of ixodid ticks (Acarina: Ixodidae) and tick-borne spotted fever group rickettsiae in Tokunoshima Island, Japan. *Med Entomol Zool* **47**, 15–21.
- Fujita, H., Watanabe, Y., Ishikura, M. & Takada, N. (1999). List of all isolates of spotted fever group rickettsiae from ticks in Japan 1993–1998. *Ann Rep Ohara Hosp* **42**, 45–50.
- Hirunkanokpun, S., Kittayapong, P., Cornet, J. P. & Gonzalez, J. P. (2003). Molecular evidence for novel tick-associated spotted fever group rickettsiae from Thailand. *J Med Entomol* **40**, 230–237.
- Mahara, F., Koga, K., Sawada, S. & 8 other authors (1985). The first report of the rickettsial infections of spotted fever group in Japan; three clinical cases. *Jpn J Assoc Infect Dis* **59**, 1165–1172.
- Marrero, M. & Raoult, D. (1989). Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever rickettsia in blood culture. *Am J Trop Med Hyg* **40**, 197–199.
- Philip, R. N., Casper, E. A., Burgdorfer, W., Gerloff, R. K., Hugues, L. E. & Bell, E. J. (1978). Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. *J Immunol* **121**, 1961–1968.
- Roux, V. & Raoult, D. (2000). Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (*ompB*). *Int J Syst Evol Microbiol* **50**, 1449–1455.
- Sekeyova, Z., Fournier, P. E., Rehacek, J. & Raoult, D. (2000). Characterization of a new spotted fever group rickettsia detected in *Ixodes ricinus* (Acari: Ixodidae) collected in Slovakia. *J Med Entomol* **37**, 707–713.
- Sekeyova, Z., Roux, V. & Raoult, D. (2001). Phylogeny of *Rickettsia* spp. inferred by comparing sequences of 'gene D', which encodes an intracytoplasmic protein. *Int J Syst Evol Microbiol* **51**, 1353–1360.
- Simser, J. A., Palmer, A. T., Fingerle, V., Wilske, B., Kurtti, T. J. & Munderloh, U. G. (2002). *Rickettsia monacensis* sp. nov., a spotted fever group rickettsia, from ticks (*Ixodes ricinus*) collected in a European city park. *Appl Environ Microbiol* **68**, 4559–4566.
- Takada, N., Fujita, H., Yano, Y., Ishiguro, F., Iwasaki, H. & Masuzawa, T. (2001). First records of tick-borne pathogens, *Borrelia*, and spotted fever group rickettsiae in Okinawajima Island, Japan. *Microbiol Immunol* **45**, 163–165.
- Uchida, T., Uchiyama, T., Kumano, K. & Walker, D. H. (1992). *Rickettsia japonica* sp. nov., the etiological agent of spotted fever group rickettsiosis in Japan. *Int J Syst Bacteriol* **42**, 303–305.