Leptospira kmetyi sp. nov., isolated from an environmental source in Malaysia

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A single *Leptospira* strain (designated Bejo-Iso9^T) was isolated from a soil sample taken in Johor, Malaysia. The isolate showed motility and morphology typical of the genus *Leptospira* under dark-field microscopy. Cells were found to be 10–13 µm in length and 0.2 µm in diameter, with a wavelength of 0.5 µm and an amplitude of approximately 0.2 µm. Phenotypically, strain Bejo-Iso9^T grew in Ellinghausen–McCullough–Johnson–Harris medium at 13, 30 and 37 °C, and also in the presence of 8-azaguanine. Serologically, strain Bejo-Iso9^T produced titres towards several members of the Tarassovi serogroup, but was found to be serologically unique by cross-agglutinin absorption test and thus represented a novel serovar. The proposed name for this serovar is Malaysia. Phylogenetic analysis of 16S rRNA gene sequences placed this novel strain within the radiation of the genus *Leptospira*, with sequence similarities within the range 90.4–99.5% with respect to recognized *Leptospira* species. DNA–DNA hybridization against the three most closely related *Leptospira* species was used to confirm the results of the 16S rRNA gene sequence analysis. The G + C content of the genome of strain Bejo-Iso9^T was 36.2 mol%. On the basis of phenotypic, serological and phylogenetic data, strain Bejo-Iso9^T represents a novel species of the genus *Leptospira*, for which the name *Leptospira kmetyi* sp. nov. is proposed. The type strain is Bejo-Iso9^T (=WHO LT1101^T=KIT Bejo-Iso9^T).

Leptospirosis is the zoonotic disease caused by members of the genus *Leptospira*, helical spirochaetes that metabolize long-chain fatty acids. Leptospirosis is an emerging disease of worldwide distribution, affecting both developing and developed countries (Bharti *et al.*, 2003; LaRocque *et al.*, 2005; Levett, 2001; Slack *et al.*, 2006a). The disease is transmitted by direct contact with infected animal urine or body fluids or by indirect contact with contaminated water or soil (Levett, 2001).

Abbreviations: CAAT, cross-agglutinin absorption test; EMJH, Ellinghausen–McCullough–Johnson–Harris; MAT, microscopic agglutination test.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, DNA gyrase subunit B and RNA polymerase subunit B gene sequences of *Leptospira kmetyi* serovar Malaysia strain Bejo-Iso9^T are AB279549, AB279550 and AB291211, respectively.

Supplementary tables are available with the online version of this paper.

At the time of writing, there are 18 species in the genus *Leptospira*, as determined by DNA–DNA hybridization (Brenner *et al.*, 1999; Levett *et al.*, 2006; Perolat *et al.*, 1998; Slack *et al.*, 2008; Yasuda *et al.*, 1987). More recently, a further novel species, *Leptospira licherasiae*, has been validly published (Matthias *et al.*, 2009). Phylogenetic analysis of the *Leptospira* species has resulted in their broad classification into four distinct clades: pathogenic, saprophytic, intermediate and novel (Perolat *et al.*, 1998; Slack *et al.*, 2008). Members of the genus *Leptospira* are also divided serologically through the homology of cell antigens by using the cross-agglutinin absorption test (CAAT). There have been over 200 serovars described for this genus (Levett, 2001).

In this paper, we report the characterization of a novel species of the genus *Leptospira* by using serology, phenotypic studies and molecular studies, including 16S

rRNA, DNA gyrase subunit B (*gyrB*) and RNA polymerase subunit B (*rpoB*) gene sequence analysis, DNA–DNA hybridization and DNA G+C content analysis, to define a novel species within the genus *Leptospira*.

Initially, strain Bejo-Iso9^T was isolated from soil in Johor, Malaysia, by researchers at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, using the following isolation procedure. Soil samples (20 g in sterile water at approximately three times the volume of the sample) were filtered initially through filter paper (Whatman no. 1) and then through a 0.45 µm membrane filter. The filtered water was inoculated into semi-solid JS medium. The inoculated medium was incubated at 30 °C and examined weekly by using dark-field microscopy for the presence of leptospira. Strain Bejo-Iso9^T was then forwarded to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia, for further identification. Strain Bejo-Iso9^T and all other strains used in this study were maintained in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco) at 30 °C.

Phenotypic characterization of strain Bejo-Iso9^T was performed by assessing growth at varying temperatures and in the presence of 8-azaguanine (Johnson & Rogers, 1964). Duplicate EMJH media were inoculated with strain Bejo-Iso9^T and incubated at 13, 30 and 37 °C for 14 days. The cultures were then inspected for growth by using dark-field microscopy. Growth was confirmed by subculturing into EMJH medium (without 8-azaguanine) and incubating at 30 °C for a further 7 days. *Leptospira interrogans* serovar Pomona strain Pomona, *Leptospira biflexa* serovar Patoc strain Patoc I^T and *Leptospira inadai* serovar Lyme strain 10^T were used as controls in the experiments to represent pathogenic, saprophytic and intermediate species, respectively. Under dark-field microscopy, Bejo-Iso9^T showed typically leptospiral motility and morphology, and cells were found to be 10–13 µm in length and 0.2 µm in diameter, with a wavelength of 0.5 µm and an amplitude of approximately 0.2 µm. Phenotypically, strain Bejo-Iso9^T grew in EMJH medium at 13, 30 and 37 °C and also in the presence of 8-azaguanine.

Serological identification of the isolate was performed by using the microscopic agglutination test (MAT) method, using serovars representative of the major *Leptospira* serogroups (Faine *et al.*, 1999). Hyperimmune antiserum against strain Bejo-Iso9^T was produced in rabbits by using standard methods (Graves & Faine, 1970). The *Leptospira* serogroups tested included Icterohaemorrhagiae, Javanica, Celledoni, Canicola, Ballum, Pyrogenes, Cynopteri, Autumnalis, Djasiman, Australis, Pomona, Grippotyphosa, Hebdomadis, Mini, Sejroe, Bataviae, Tarassovi, Panama, Shermani and Semarang. CAAT was used to confirm the results from the MAT serovar-screening reactions (Kmety *et al.*, 1970). When Bejo-Iso9^T was tested against hyperimmune antiserum representing the major *Leptospira* serogroups, it gave a significant titre to *Leptospira borgpetersenii* serovar Tarassovi (1:1600). Strain Bejo-Iso9^T

culture and antiserum were then screened by using MAT for homology against the previously described members of the Tarassovi serogroup (the serovar MAT results are shown in Supplementary Table S1, available in IJSEM Online). It produced a significant titre against the culture and antiserum of *Leptospira* (undesignated species) serovar Banna strain A31. CAAT between the two isolates revealed that they were not serologically identical and, therefore, Bejo-Iso9^T represents a novel serovar, designated Malaysia. Additionally, given the initial serological reactions, it is proposed that this serovar be placed within the Tarassovi serogroup.

Cultures were prepared for DNA isolation by centrifugation as described previously (Slack *et al.*, 2006b), followed by genomic DNA extraction using a ChargeSwitch gDNA mini bacteria kit (Invitrogen). Amplification of the 16S rRNA and *gyrB* genes was performed as described previously (Slack *et al.*, 2006b, 2008). *rpoB* gene amplification was performed by using a previously described method (La Scola *et al.*, 2006) with the following modifications: PCR amplification was performed in 25 µl volumes containing 1 × PCR buffer (New England Biolabs), 1.5 mM MgCl₂, 200 µM dNTPs, 10 pmol oligonucleotides [Lept 1900f (5'-CCTCATGG-GTTCCAACATGCA-3') and Lept 2500r (5'-CGCATCCT-CRAAGTTGTAWCCTT-3')] (La Scola *et al.*, 2006), 1 U *Taq* Polymerase (New England Biolabs) and 2 µl template DNA. The DNA was amplified by using the following thermal-cycling profile: 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s, and a final extension for 7 min at 72 °C. DNA sequencing was performed by using BigDye Terminator sequencing, version 3.1 (Applied Biosystems), as described previously (Slack *et al.*, 2006b, 2008). The cycle-sequencing products were purified by using sodium acetate/alcohol precipitation as per the manufacturer's instructions (Applied Biosystems) and the purified products were forwarded to the Griffith University DNA sequencing facility, Brisbane, Australia, for capillary electrophoresis using an ABI 3130xl instrument. The sequences were assembled and trimmed to a minimum of two contiguous sequences by using Vector NTI software (Invitrogen). Sequences from strain Bejo-Iso9^T and representative 16S rRNA (1331 bp), *gyrB* (421 bp) and *rpoB* (495 bp) gene sequences from members of the genus *Leptospira* were aligned with CLUSTAL W (Thompson *et al.*, 1994). By using MEGA4 (Tamura *et al.*, 2007), distances of aligned 16S rRNA gene sequences were estimated by the Jukes–Cantor method (Jukes & Cantor, 1969), bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method. The final phylogenetic tree was rooted by using *Leptonema illini* serovar Habaki strain Habaki as an outgroup and bootstrap values were displayed as percentages (Fig. 1). 16S rRNA gene sequence similarity between strain Bejo-Iso9^T and the 18 previously described species in the genus *Leptospira* was found to be in the range 90.4–99.5%. *Leptospira* genomospecies 1 (99.5%), *Leptospira noguchii* (99.2%) and

Fig. 1. 16S rRNA gene sequence-based phylogeny of strain Bejo-Iso9^T and 18 representative species of the genus *Leptospira*. The dendrogram was constructed from distance matrices by using the neighbour-joining method, with *Leptonema illini* strain Habaki as outgroup. Bootstrap values are displayed as percentages. Bar, 0.02 inferred nucleotide substitutions per 100 nt.

Leptospira kirschneri (99.1 %) showed the highest levels of 16S rRNA gene sequence similarity to strain Bejo-Iso9^T. The 16S rRNA gene sequence analysis results were confirmed by *gyrB* (87.6–88.2 %) and *rpoB* (72.7–89.5 %) gene sequence analysis (shown in Supplementary Table S2, available in IJSEM Online). Phylogenetic analysis using 16S rRNA gene sequences showed that strain Bejo-Iso9^T was placed within the radiation of the genus *Leptospira* and was found to cluster with the pathogenic *Leptospira* species (Fig. 1).

DNA was isolated for DNA–DNA hybridization and G + C content analysis from strain Bejo-Iso9^T and eight known pathogenic *Leptospira* species as described previously (Brenner *et al.*, 1982). The DNA was labelled with [³²P]dCTP, and DNA relatedness and divergence studies were performed by using the hydroxyapatite method at the optimal reassociation temperature of 55 °C (Brenner *et al.*, 1982). G + C content was determined by the thermal-denaturation method (Mandel *et al.*, 1970). Strain Bejo-Iso9^T showed no significant similarity to the previously described pathogenic *Leptospira* species (DNA–DNA hybridization results are shown in Supplementary Table S3, available in IJSEM Online.). The genomic G + C content of strain Bejo-Iso9^T was 36.2 mol%, which is within the range 33–43 mol% described for the genus *Leptospira* (Yasuda *et al.*, 1987).

Despite having high 16S rRNA gene sequence similarity to recognized *Leptospira* species, there is sufficient evidence, in the form of DNA–DNA hybridization and *rpoB* and *gyrB* gene sequence analysis, to justify the inclusion of strain Bejo-Iso9^T as a member of a novel species within the genus *Leptospira*. Interestingly, this strain has the phenotypic characteristics typical of a non-pathogenic or potentially pathogenic *Leptospira* species, i.e. it shows growth at 13 °C (Johnson & Harris, 1967) and in the presence of 8-azaguanine (Johnson & Rogers, 1964), yet based upon 16S rRNA gene sequence analysis, it is phylogenetically positioned amongst the pathogenic *Leptospira* species. Further research is required to determine whether this strain is pathogenic to humans.

This research provides sufficient evidence in the form of molecular and serological taxonomic characterization to justify the inclusion of strain Bejo-Iso9^T as the type strain of a novel species with the genus *Leptospira*, for which the name *Leptospira kmetyi* sp. nov. is proposed (serovar Malaysia). The novel species and serovar designation of strain Bejo-Iso9^T have been ratified by the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Leptospiraceae*.

Description of *Leptospira kmetyi* sp. nov.

Leptospira kmetyi (kmet'yi. N.L. gen. masc. n. *kmetyi* after Professor Emil Kmety, a Slovak bacteriologist who made significant contributions to the study of the genus *Leptospira*).

Morphology and motility under dark-field microscopy are consistent with those for the genus. Cells are 10–13 µm in length and 0.2 µm in diameter, with a wavelength of 0.5 µm and an amplitude of approximately 0.2 µm. Grows at 13, 30 and 37 °C and in the presence of 8-azaguanine. Serologically, titres are produced towards several members of the Tarassovi serogroup, but serologically unique by CAAT (designated novel serovar Malaysia). Phylogenetically placed within the radiation of the genus *Leptospira* (based on 16S rRNA gene sequence analysis and confirmed by using DNA–DNA hybridization against the three most closely related *Leptospira* species). The DNA G + C content of the type strain is 36.2 mol%.

The type strain, Bejo-Iso9^T (=WHO LT1101^T=KIT Bejo-Iso9^T), was isolated from an environmental source (soil) in Johor, Malaysia.

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