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

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A single *Leptospira* strain (designated Bejo-Iso9T) 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-Iso9T grew in Ellinghausen–McCullough–Johnson–Harris medium at 13, 30 and 37 °C, and also in the presence of 8-azaguanine. Serologically, strain Bejo-Iso9T 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-Iso9T was 36.2 mol%. On the basis of phenotypic, serological and phylogenetic data, strain Bejo-Iso9T represents a novel species of the genus *Leptospira*, for which the name *Leptospira kmetyi* sp. nov. is proposed. The type strain is Bejo-Iso9T (∼WHO LT1101T∼KIT Bejo-Iso9T).

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-Iso9T 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 licerasiae*, 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...
Initially, strain Bejo-Iso9T 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-Iso9T was then forwarded to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia, for further identification. Strain Bejo-Iso9T 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-Iso9T 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-Iso9T 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 T and Leptospira inadai serovar Lyme strain strain T were used as controls in the experiments to represent pathogenic, saprophytic and intermediate species, respectively. Under dark-field microscopy, Bejo-Iso9T 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-Iso9T 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-Iso9T 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, Shermanni and Semaranga. CAAT was used to confirm the results from the MAT serovar-screening reactions (Kmety et al., 1970). When Bejo-Iso9T was tested against hyper-immune antiserum representing the major Leptospira serogroups, it gave a significant titre to Leptospira borgpetersenii serovar Tarassovi (1 : 1600). Strain Bejo-Iso9T 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-Iso9T 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′-CCTCAGG-GTTCCAACATGGA-3′) and Lept 2500r (5′-CGCATCCT-CRAAGTTGTAWCCCTT-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 forward 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-Iso9T 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 Leptomena 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-Iso9T 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
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-Is97 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-Is97 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-Is97 have been ratified by the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Leptospiraceae*.

**Description of *Leptospira kmetyi* sp. nov.**

*Leptospira kmetyi* (kmety’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-Is97 (=WHO LT11017=KIT Bejo-Is97), was isolated from an environmental source (soil) in Johor, Malaysia.

**References**


relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol* 49, 839–858.


