Leptospira wolffii sp. nov., isolated from a human with suspected leptospirosis in Thailand

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A single *Leptospira* strain (designated Khorat-H2^T) was isolated from the urine of an adult male patient with suspected leptospirosis from the province of Nakornrachasima, Thailand. The isolate showed typical *Leptospira* motility and morphology under dark-field microscopy. Cells were 10–13 µm long and 0.2 µm in diameter, with a wavelength of 0.5 µm and an amplitude of approximately 0.3 µm. Phenotypically, strain Khorat-H2^T did not grow at 13 °C but grew at 30 and 37 °C and in the presence of 8-azaguanine. Serological identification using the microscopic agglutination test revealed that strain Khorat-H2^T had no cross-reaction with any recognized *Leptospira* serogroups. Phylogenetic analysis of the 16S rRNA gene sequence placed the novel strain within the radiation of the genus *Leptospira*, with sequence similarities of 88.1–97.7% to recognized *Leptospira* species. DNA–DNA hybridization against the type strains of the three most closely related *Leptospira* species was used to confirm the results of the 16S rRNA sequence analysis. The G + C content of strain Khorat-H2^T was 41.8 mol%. On the basis of phenotypic, serological and phylogenetic data, strain Khorat-H2^T represents a novel species of the genus *Leptospira*, for which the name *Leptospira wolffii* sp. nov. is proposed. The type strain is Khorat-H2^T (=WHO LT1686^T =KIT Khorat-H2^T).

Leptospirosis is the zoonotic disease caused by members of the genus *Leptospira*, which are helical spirochaetes that metabolize long-chain fatty acids. Leptospirosis is distributed worldwide (WHO, 1999) and is considered to be an emerging disease (Bharti *et al.*, 2003; Levett, 2001). 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).

There are currently 17 *Leptospira* species as delineated by DNA–DNA hybridization (Brenner *et al.*, 1999; Levett *et al.*, 2006; Perolat *et al.*, 1998; Yasuda *et al.*, 1987). Phylogenetic analysis of the 17 species using the 16S rRNA gene has resulted in the broad classification of the species into pathogenic, saprophytic and intermediates (Perolat *et al.*, 1998). *Leptospira* species are also divided serologically through the cross-reaction of cell antigens using the cross-agglutinin absorption test (CAAT); over 200 serovars have been described for the genus (Levett, 2001).

In this paper, we report on the characterization of a novel species of *Leptospira* using serology, phenotypic studies and molecular studies including 16S rRNA gene sequence analysis, DNA–DNA hybridization and G + C content.

Abbreviations: CAAT, cross-agglutinin absorption test; MAT, microscopic agglutination test.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Khorat-H2^T is EF025496.

Strain Khorat-H2^T was isolated from the urine of an adult male patient with suspected leptospirosis from the Thai province of Nakornrachasima (Khorat) by researchers at the Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand). The isolate was forwarded to the WHO/FAO/OIE Collaborating Centre for Reference & Research on Leptospirosis (Brisbane, Australia) for further identification. Strain Khorat-H2^T and all other *Leptospira* strains used in this study were maintained in Ellinghausen McCullough Johnson Harris (EMJH) medium (Difco) at 30 °C.

Phenotypic characterization of strain Khorat-H2^T was performed by assessing growth at varying temperatures and in the presence of 8-azaguanine (Johnson & Rogers, 1964). Duplicate aliquots of EMJH medium were inoculated with strain Khorat-H2^T and incubated at 13, 30 and 37 °C for 14 days. The cultures were then inspected for growth using dark-field microscopy. Growth was confirmed by subculturing into EMJH medium (without 8-azaguanine) and incubating the subcultures at 30 °C for a further 7 days. *Leptospira interrogans* serovar Pomona strain Pomona, *Leptospira biflexa* serovar Patoc strain Patoc 1^T and *Leptospira inadai* serovar Lyme strain 10^T were used as controls in the experiments to represent pathogenic, saprophytic and intermediate species, respectively. Strain Khorat-H2^T did not grow in EMJH medium at 13 °C but did grow at 30 °C and in the presence of 8-azaguanine.

Serological identification of the isolates was performed using the microscopic agglutination test (MAT) method using serovars representative of the major *Leptospira* serogroups (Faine *et al.*, 1999). The *Leptospira* serogroups tested included Icterohaemorrhagiae, Javanica, Celledoni, Canicola, Ballum, Pyrogenes, Cynopteri, Autumnalis, Djasiman, Australis, Pomona, Grippotyphosa, Hebdomadis, Mini, Sejroe, Bataviae, Tarrasovi, Panama, Shermani and Semeranga. Strain Khorat-H2^T produced no reactions against any of the serogroups tested and therefore represents a novel serovar.

Cultures were prepared for DNA isolation by centrifugation as described previously (Slack *et al.*, 2006) followed by genomic DNA extraction using the ChargeSwitch gDNA mini bacteria kit (Invitrogen). 16S rRNA gene amplification was performed in a final volume of 25 µl containing 1 × PCR buffer (NEB), 2.0 mM MgCl₂, 200 µM dNTPs, 10.0 pmol primers FD1MOD (Kotilainen *et al.*, 1998) and 13R (Relman *et al.*, 1992), 1 U *Taq* polymerase (NEB), 2 µl DNA extract and double-distilled water (ddH₂O) to make up the final volume. Thermal cycling was as follows: initial denaturation at 94 °C for 10 min followed by 35 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 30s, with a final extension at 72 °C for 10 min. PCR products were confirmed by agarose gel electrophoresis (1.5 % w/v) of 5 µl PCR product for 60 min at 80 V. Excess primers and dNTPs were removed from the remaining PCR product (20 µl) using an enzymic method. Briefly, 2.5 µl 10 ×

Antarctic phosphatase buffer (NEB), 10 U *E. coli* exonuclease I (Fermentas), 2.5 U Antarctic phosphatase (NEB) and 1.5 µl ddH₂O were added to each sample. The PCR product plus enzyme mixture was incubated at 37 °C for 45 min followed by 85 °C for 15 min to inactivate the enzyme. DNA sequencing was performed using the Big Dye Terminator (BDT) sequencing kit version 3.1 (Applied Biosystems) using the original primers and internal primers 515F, 91e, 11e, 16s1RRB, 907R and 342R (Lane, 1991; Relman *et al.*, 1990, 1992; Wilbrink *et al.*, 1998) with the following modifications. Each 20 µl reaction contained a 1:16 dilution of BDT mix (0.5 µl), 3.75 µl 5 × dilution buffer, 3.2 pmol primer, 5–10 ng DNA and ddH₂O to make up the final volume. Cycle sequencing was performed using 30 cycles of 95 °C for 10 s, 50 °C for 10 s and 60 °C for 4 min. The cycle-sequencing products were purified using sodium acetate/alcohol precipitation according to the manufacturer's instructions (Applied Biosystems). The purified products were forwarded to the Griffith University DNA sequencing facility (GUDSF; Brisbane, Australia) for capillary electrophoresis using the ABI 3130xl instrument. Sequences were assembled and trimmed to a minimum of two contiguous sequences using Vector NTI software (Invitrogen). Sequences from strain Khorat-H2^T and representative sequences from the genus *Leptospira* were aligned with CLUSTAL W (Thompson *et al.*, 1994). Using MEGA4 (Tamura *et al.*, 2007), distances of aligned sequences were estimated by the method of Jukes & Cantor (1969) and bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method (Fig. 1). 16S rRNA gene sequence similarity between strain Khorat-H2^T and strains of the 17 previously described *Leptospira* species was 88.1–97.7 %. The type strains of *Leptospira broomii* (97.7 %), *Leptospira fainei* (97.7 %) and *L. inadai* (97.4 %) showed the highest 16S rRNA gene sequence similarity to strain Khorat-H2^T. Phylogenetic analysis showed that strain Khorat-H2^T was placed within the radiation of the genus *Leptospira* and formed a unique lineage within the clade formed by the intermediates/potentially pathogenic *Leptospira* species.

DNA was isolated for DNA–DNA hybridization and G + C content analysis from strain Khorat-H2^T, *L. broomii* 5399^T, *L. fainei* serovar Hurstbridge strain BUT 6^T and *L. inadai* serovar Lyme strain 10^T as described by Brenner *et al.* (1982). The isolated DNA was labelled with [³²P]dCTP and DNA relatedness and divergence studies were performed using the hydroxyapatite method at the optimal reassociation temperature of 55 °C (Brenner *et al.*, 1982). Strain Khorat-H2^T showed no significant relatedness to *L. fainei* BUT 6^T (relative binding ratio 19 %, divergence 23.5 %), *L. inadai* 10^T (relative binding ratio 22 %, divergence 23.0 %) or *L. broomii* 5399^T (relative binding ratio 18 %, divergence 22.5 %). The G + C content was determined by the thermal denaturation method as described by Mandel *et al.* (1970). The G + C content of strain Khorat-H2^T was 41.8 mol%, which is within the range of 33–43 mol% reported for the genus *Leptospira* (Yasuda *et al.*, 1987).

Fig. 1. 16S rRNA gene sequence-based phylogeny of strain Khorat-H2^T and representative *Leptospira* species. The dendrogram was reconstructed from distance matrices using the neighbour-joining method, using *Leptonema illini* strain Habaki as an outgroup. Bootstrap values are indicated as percentages of 1000 resamplings. Bar, 0.05 inferred substitutions per nucleotide position.

Although strain Khorat-H2^T shares a high level of 16S rRNA gene sequence similarity with three previously recognized *Leptospira* species (*L. inadaï*, *L. fainei* and *L. broomii*), it forms its own distinct phylogenetic lineage. This novel lineage is supported by the DNA–DNA hybridization investigations, which showed significant divergence from *L. inadaï*, *L. fainei* and *L. broomii*. The limited phenotypic characterization of *Leptospira* species can be offset by the fact that strain Khorat-H2^T was found to be serologically unique and represented a novel serovar. This research provides sufficient evidence in the form of molecular and serological taxonomic characterization to justify the inclusion of strain Khorat H2^T within a novel species of the genus *Leptospira*, for which the name *Leptospira wolffii* sp. nov. is proposed. However, this research does not show whether this strain represents a true human pathogen, as the isolation was not supported at the time by other investigations such as MAT or PCR. The novel species and serovar designation of strain Khorat-H2^T as *Leptospira wolffii* serovar Khorat has been ratified by the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Leptospiraceae*.

Description of *Leptospira wolffii* sp. nov.

Leptospira wolffii (wolf'fi.i. N.L. gen. masc. n. *wolffii* of/ from Wolff, named after Dr Jan Willem Wolff, a Dutch bacteriologist who made significant contributions to the study of *Leptospira*).

Shows typical *Leptospira* motility and morphology under dark-field microscopy. Cells are 10–13 µm long and 0.2 µm in diameter, with a wavelength of 0.5 µm and an amplitude of approximately 0.3 µm. Does not grow at 13 °C but grows at 30 and 37 °C and in the presence of 8-azaguanine. Serological identification using MAT reveals that the type strain has no cross-reaction with any recognized *Leptospira* serogroups. Phylogenetic analysis of the 16S rRNA gene sequence places the type strain within the radiation of the genus *Leptospira*, as confirmed by DNA–DNA hybridization. The DNA G+C content of the type strain is 41.8 mol%.

The type strain, Khorat-H2^T (=WHO LT1686^T =KIT Khorat-H2^T), was isolated from the urine of a human patient with suspected leptospirosis in Nakornrachasima, Thailand. This strain is also the reference strain of *Leptospira wolffii* serovar Khorat.

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