Leptospira idonii sp. nov., isolated from environmental water

Mitsumasa Saito,¹ Sharon Y. A. M. Villanueva,¹ Yoshiaki Kawamura,² Ken-ichiro Iida,¹ Junko Tomida,² Takaaki Kanemaru,³ Eri Kohno,¹ Satoshi Miyahara,¹ Akiko Umeda,⁴ Kazunobu Amako,¹ Nina G. Gloriani⁵ and Shin-ichi Yoshida¹

Strain Eri-1T was isolated from a water sample on the campus of Kyushu University, Fukuoka, Japan. The motility and morphology of the isolate were similar to those of members of the genus Leptospira, but the spiral structure of the isolate was sharper under dark-field microscopy. Cells were 10.6±1.3 μm long and 0.2 μm in diameter, with a wavelength of 0.9 μm and an amplitude of 0.4 μm. Strain Eri-1T grew in Korthof’s medium at both 13 and 30 °C, and also in the presence of 8-azaguanine. 16S rRNA gene-based phylogenetic analysis placed strain Eri-1T within the radiation of the genus Leptospira where it formed a unique lineage within the clade of the known saprophytic species of the genus Leptospira. The strain was not pathogenic to hamsters. Strain Eri-1T exhibited low levels (11.2–12.6 %) of similarity by DNA–DNA hybridization to the three most closely related species of the genus Leptospira. The DNA G+C content of the genome of strain Eri-1T was 42.5±0.1 mol%. These results suggest that strain Eri-1T represents a novel species of the genus Leptospira, for which the name Leptospira idonii sp. nov. is proposed. The type strain is Eri-1T (=DSM 26084T=JCM 18486T).

Members of the genus Leptospira are aerobic, Gram-negative bacteria whose cells are long, thin, flexible rods with a regular right-handed helical coiling pattern (Carleton et al., 1979). In 1915, Ryokichi Inada and Yutaka Ido, the first and second professors of The First Medical Clinic of The Imperial University in Kyushu, which is presently known as Faculty of Medicine, Kyushu University, discovered spirochaetes and specific antibodies in the blood of Japanese coal miners with infectious jaundice (Weil’s disease) in Fukuoka, Japan (Inada et al., 1916). This organism is now known as Leptospira interrogans strain Icter No.1. Since this discovery, many serovars of this organism have been reported. Some strains are pathogenic to humans and animals, while others are saprophytic and found in freshwater and marine environments. The taxonomy of the genus Leptospira has undergone substantial revision with the use of 16S rRNA gene sequence comparison and DNA–DNA reassociation studies (Zuerner, 2011). Some researchers have classified 303 strains representing the genus Leptospira to a refined definition of 12 different species, and five new genomospecies have been identified (Brendle & Alexander, 1974; Yasuda et al., 1987; Brenner et al., 1999). One species, Turneriella parva, was placed in a new genus of the family Leptospiraceae (Levett et al., 2005). Recently, four novel species, Leptospira broonii (Levett et al., 2006), Leptospira licerasiae (Matthias et al., 2008), Leptospira wolffii (Slack et al., 2008) and Leptospira

Abbreviations: CAAT, cross-agglutination absorption test; MAT, microscopic agglutination test; STAFF, sulfamethoxazole, trimethoprim, amphotericin B, fosfomycin and 5-fluorouracil.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Leptospira idonii Eri-1T is AB721966.

interrogans strain Icter No.1. Since this discovery, many serovars of this organism have been reported. Some strains are pathogenic to humans and animals, while others are saprophytic and found in freshwater and marine environments. The taxonomy of the genus Leptospira has undergone substantial revision with the use of 16S rRNA gene sequence comparison and DNA–DNA reassociation studies (Zuerner, 2011). Some researchers have classified 303 strains representing the genus Leptospira to a refined definition of 12 different species, and five new genomospecies have been identified (Brendle & Alexander, 1974; Yasuda et al., 1987; Ramadass et al., 1992; Brenner et al., 1999). One species, Turneriella parva, was placed in a new genus of the family Leptospiraceae (Levett et al., 2005). Recently, four novel species, Leptospira broonii (Levett et al., 2006), Leptospira licerasiae (Matthias et al., 2008), Leptospira wolffii (Slack et al., 2008) and Leptospira
kmetyi (Slack et al., 2009) have been described. Thus, at the time of writing, the genus *Leptospira* consists of 20 species (Smythe et al., 2012).

Pathogenic *Leptospira* are carried in the kidney tubules of a variety of animals (Levett, 2001; Adler & de la Peña Moctezuma, 2010) and contaminate the environment (water, soil, marsh and other elements) by being excreted in large numbers in urine (Adler & de la Peña Moctezuma, 2010). Humans and other animals become infected, mainly through the skin and mucous membranes, when they encounter a leptospire-contaminated environment (Adler & de la Peña Moctezuma, 2010). Due to this correlation between organisms in the environment and disease transmission, isolation of leptospires from the environment is important for epidemiological studies as well as for prevention and control of the disease. However, isolation from environmental samples is challenging due to the slow growth of leptospires and overgrowth of coexisting microorganisms.

We previously reported a novel combination of antimicrobial agents (STAFF; 40 μg sulfamethoxazole ml⁻¹, 20 μg trimethoprim ml⁻¹, 5 μg amphotericin B ml⁻¹, 400 μg fosfomycin ml⁻¹ and 100 μg 5-fluorouracil ml⁻¹, final concentration) for selective isolation of leptospires from contaminated samples (Chakraborty et al., 2011). This cocktail, after being incorporated into *Leptospira* growth medium, inhibited the growth of contaminants and allowed successful detection of leptospires in environmental samples. During our subsequent epidemiological investigation into the distribution of leptospires in environmental soil and water utilizing STAFF, we discovered a novel species of the genus *Leptospira*. In this paper, we report the characterization of strain Eri-¹ by using morphological, serological, phenotypic and molecular studies, including 16S rRNA gene sequence analysis, DNA–DNA hybridization and DNA G+C content analysis, to define a novel species within the genus *Leptospira*.

*Leptospira* strain Eri-¹ was isolated from environmental water beside a swimming pool at Kyushu University, Fukuoka, Japan. We collected 10 ml of environmental water in a sterile 15 ml screw-capped tube. The tube was kept in a vertical position for 1 h to allow the sediment to settle. After that, 2.0 ml supernatant from the sample was added to 2.5 ml 2 × concentrated Korthof’s medium (WHO & ILS, 2003) supplemented with 500 μl 10 × concentrated STAFF (Chakraborty et al., 2011). This tube was incubated at 30 °C and checked daily by dark-field microscopy (BX41, Olympus, Tokyo, Japan) for the presence of *Leptospira*, which was confirmed by observing their characteristic thin helical structures with prominent hooked ends and motility. For single colony isolation, solid medium was prepared by the incorporation of 1 % (w/v) agar in liquid Korthof’s medium. The bacteria in the liquid culture medium were counted and diluted to approximately 10⁴ cells ml⁻¹. The solid medium was inoculated by spreading 0.1 ml diluted bacterial culture evenly over the surface of the medium with a glass spreader. The inoculated plate was sealed with tape and incubated at 30 °C. After seven days, subsurface colonies appeared. A single colony was picked up with a Pasteur pipette, transferred into fresh liquid Korthof’s medium, and incubated at 30 °C.

The isolated strain, Eri-¹, showed motility and morphology that were similar to those of members of the genus *Leptospira* under dark-field microscopy. However, the spiral structure of the isolate was sharper (Fig. 1). Fig. 2 shows an electron micrograph of strain Eri-¹. The cells were helical, 10.6 ± 1.3 μm long and 0.2 μm in diameter, with a wavelength of 0.9 μm and an amplitude of 0.4 μm. The diameter and wavelength of the strain were slightly larger and longer, respectively, than those of previously recognized species of the genus *Leptospira*. The larger diameter and longer wavelength of strain Eri-¹ may be the reason why it is seen more sharply under dark-field microscopy.

![Image](https://example.com/leptospira_eiri1.jpg)

**Fig. 1.** Dark-field micrograph of strain Eri-¹ (a) and *Leptospira biflexa* strain Patoc I (b). Bars, 10 μm.
Phenotypic characterization of strain Eri-1T was performed by assessing its growth at varying temperatures and in the presence of 8-azaguanine (Johnson & Rogers, 1964). Duplicate Korthof's medium or Ellinghausen-McCullough-Johnson-Harris (EMJH) medium were inoculated with strain Eri-1T (2.0 \times 10^6 \text{ cells ml}^{-1}) and incubated at 13 or 30 °C for 30 days (WHO & ILS, 2003). L. interrogans serovar Icterohaemorrhagiae strain Ictero No.1 and Leptospira biflexa serovar Patoc strain Patoc I were used as controls to represent pathogenic and saprophytic species, respectively. Strain Eri-1T as well as L. biflexa grew in Korthof's medium, and also in the presence of 8-azaguanine at 30 °C, and entered stationary phase after 5 days (1.5 \times 10^8 \text{ cells ml}^{-1}). At 13 °C in Korthof's medium, the novel strain grew slowly and entered stationary phase after 13 days (6.3 \times 10^7 \text{ cells ml}^{-1}). Based on these results, strain Eri-1T was considered to be a non-pathogenic Leptospira. In EMJH medium the isolate required 30 days to reach stationary phase, and the bacterial count in the stationary phase was 5.6 \times 10^7. This result suggested that EMJH medium was inadequate for the growth of strain Eri-1T compared with Korthof’s medium.

Serological identification of strain Eri-1T was performed by using the microscopic agglutination test (MAT) (WHO & ILS, 2003). Positive agglutination of strain Eri-1T was observed with three antisera against reference Leptospira serovars, anti-Hebdomadis, anti-Sejroe and anti-Mini. No agglutination was observed with the antisera against other serovars tested, namely Icterohaemorrhagiae, Australis, Djasiman, Louisiana, Grippotyphosa, Celledoni, Javanica, Pomona, Shermanti, Canicola, Sarmin, Manhao, Patoc, Tarassovi, Autumnalis, Panama, Cynopteris, Manilae and losanos. Hyperimmune antiserum against strain Eri-1T (with titres of 12 800) was produced in rabbits by using standard methods (Graves & Faine, 1970). The hyperimmune sera against Eri-1T was reactive to serovar Hebdomadis (strain Hebdomadis, 1: 25 600), serovar Andamana (strain CH11, 1: 50) and serovar Sao Paulo (strain Sao Paulo, 1: 50).

Because of the serological similarity of strain Eri-1T and strain Hebdomadis, a cross-agglutinin absorption test (CAAT) was performed between strain Eri-1T and serovar Hebdomadis (strain Hebdomadis) according to Dikken and Kmety (1978). The results of the CAAT showed that strain Eri-1T does not belong to serovar Hebdomadis (Table 1). Whether strain Eri-1T belongs to serovar Jules or serovar Kremastos (other serovars in serogroup Hebdomadis) remains to be determined.

PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out (Kawamura et al., 1999). After confirming amplicons of the 16S rRNA gene on 1% agarose gels, the sequence was determined using a 3130 Genetic Analyzer (Applied Biosystems). The sequences of the other species of the genus Leptospira used for alignment and for calculating levels of similarity were obtained from GenBank. Multiple sequence alignments of

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<th>Antiserum against serovar/strain</th>
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<th>Absorbing strain</th>
<th>Homologous titre after absorption</th>
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Fig. 2. Electron micrograph of strain Eri-1T (a) and L. biflexa strain Patoc I (b). Bars, 1 μm.
DNA sequences were performed using CLUSTAL W software (Thompson et al., 1997). Phylogenetic distances were calculated using the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree was reconstructed using TreeView software (Page, 1996). 16S rRNA gene sequence similarity between strain Eri-1T and the 20 previously described species of the genus *Leptospira* was found to be less than 97%. The type strains of *L. biflexa* (96.9%), *Leptospira meyeri* (96.8%), *Leptospira yanagawae* (96.6%), *Leptospira terpstrae* (96.9%), *Leptospira wolbachii* (96.8%) and *Leptospira vanthielii* (96.9%) showed the highest 16S rRNA gene sequence similarity to strain Eri-1T. Phylogenetic analysis showed that strain Eri-1T was placed within the radiation of the genus *Leptospira* and formed a unique lineage within the clade of the saprophytic species of the genus *Leptospira* (Fig. 3).

To confirm that strain Eri-1T was not pathogenic to humans or animals, PCR assays and animal experiments were performed. Sets of PCR primers specific for both pathogenic (*flaB*) and saprophytic (*ssl*) *Leptospira* have been described (Murgia et al., 1997; Kawabata et al., 2001). It was possible to identify pathogenic strains and saprophytic strains by using these primer sets. Bacterial cultures of strain Eri-1T, *L. interrogans* strain K37 (Villanueva et al., 2010) and *L. biflexa* strain Patoc I were prepared for DNA isolation by centrifugation, followed by genomic DNA extraction using Illustra Bacteria genomicPrep Mini Spin kit (GE Healthcare). PCR was performed as described by Murgia et al. (1997) and Kawabata et al. (2001). The ssl-PCR assay yielded products of the expected sizes from DNA extracted from strain Eri-1T and *L. biflexa* strain Patoc I. The *flaB*-PCR assay produced a positive PCR product only for the pathogenic *L. interrogans* strain K37. The results from these two PCR assays strongly suggested that strain Eri-1T should be classified as a saprophytic *Leptospira*. For the pathogenicity experiment, four-week-old male golden Syrian hamsters were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Five hamsters were inoculated subcutaneously with 10⁸ cells of strain Eri-1T in 1 ml PBS (in-house) NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.13 g/L, KH₂PO₄ 0.2 g/L, and observed for 28 days. The experimental protocol was reviewed by the Committee of Ethics on Animal Experiments of the Faculty of Medical Sciences, Kyushu University, and carried out in accordance

![Phylogenetic tree based on 16S rRNA gene sequence analysis showing the phylogenetic position of strain Eri-1T.](image-url)
with the Animal Experiment Guidelines of the Faculty, Law No. 105 and Notification No. 6 of the Japanese Government. No hamster died from infection even at such a high bacterial dose. Taken together, these results confirmed that strain Eri-1T represents a non-pathogenic or saprophytic species of the genus Leptospira.

For DNA–DNA hybridization and DNA G+C content analysis, DNA was isolated from strain Eri-1T, L. biflexa strain Patoc I, L. yanagawae (genomospecies 5) strain Sao Paulo and L. meyeri strain Verdrat Semarang 173 by using standard methods (Wilson, 1987). DNA–DNA hybridization was performed, as described by Tomida et al. (2011). Briefly, purified DNA (100 µg ml⁻¹) of each strain was heat denatured and then diluted to 10 µg ml⁻¹ with PBS containing 0.1M MgCl₂. The diluted DNA solution was distributed onto a microplate (Nunc-Immunoplate) at 100 µl per well, and the plate was incubated at 30 °C for 12 h. The solution was discarded and the plate was dried. DNA from the four strains was labelled with photobiotin (Vector Laboratories). The plate was pre-hybridized for 30 min and then hybridized for 2 h at 29 °C (optimal conditions) or 39 °C (stringent conditions) using 2 × SSC containing 50 % formamide. The plate was washed three times with 1 × SSC, and 100 µl of streptavidin–ß-D-galactosidase (diluted 1:1000 with 0.5 % BSA in PBS; Calbiochem) was added to each well. The plate was incubated at 37 °C for 30 min and washed three times with 1 × SSC. Following this, 100 µl of the substrate (4-methylumbelliferyl-ß-D-galactopyranoside, 100 µg ml⁻¹) was added to each well and the fluorescence intensity was measured using a DTX 800 Multimode Detector (Beckman Coulter). DNA relatedness was expressed as a mean percentage of the homologous DNA binding value. Strain Eri-1T showed no significant relatedness to L. biflexa strain Patoc I (12.4 % under optimal conditions), L. yanagawae strain Sao Paulo (11.2 %) or L. meyeri strain Veldrat Semarang 173 (12.6 %).

The DNA G+C content (mol%) was determined by HPLC (Mesbah et al., 1989; Tomida et al., 2011). A total of 5 µg denatured DNA was hydrolysed with P1 nuclease (Yamasu Syoyu, Chiba, Japan) for 1 h at 50 °C. Alkaline phosphatase (Sigma) was then added, and the mixture was incubated at 37 °C for 30 min for nucleotide dephosphorylation. The nucleosides were quantified with a GC analysis standard (Yamasu Syoyu) using a model L-2400 HPLC system (Hitachi) and an Inertsil ODS-3 HPLC Column (GL Sciences). The nucleosides were eluted with a solvent containing 0.2 M NH₄H₂PO₄ and acetonitrile (20:1, v/v). DNA G+C content (mol%) was determined using the mean values of three experiments. The DNA G+C content of strain Eri-1T was 42.5 ± 0.1 mol%, which is within the range 33–43 mol% reported for members of the genus Leptospira (Zuerner, 2011).

These results provide sufficient evidence through molecular taxonomic characterization to justify the inclusion of strain Eri-1T within a novel species of the genus Leptospira, for which the name Leptospira idonii sp. nov. is proposed.

Description of Leptospira idonii sp. nov.

Leptospira idonii (i.do’ni.i. N.L. gen. masc. n. idonii of Ido, named after Yutaka Ido, a Japanese physician and microbiologist who is one of the discoverers of leptospires in the blood of patients with Weil’s disease).

Motility and morphology of the isolate are similar to those of other members of the genus Leptospira but spiral structure of the isolates is sharper under dark-field microscopy. Grows well in Korthof’s media at 13 °C and 30 °C, and in EMJH media at 13 °C, but not in EMJH media at 30 °C. Grows in the presence of 8-azaguanine. Cells are 10.6 ± 1.3 µm long and 0.2 µm in diameter, with a wavelength of 0.9 µm and an amplitude of 0.4 µm. Shares less than 97 % 16S rRNA gene sequence similarity with all other recognized species of the genus Leptospira and exhibits low levels of DNA–DNA relatedness to the three most closely related species.

The type strain, Eri-1T (=DSM 26084 =JCM 18486T), was isolated from an environmental water sample on the campus of Kyushu University, Fukuoka, Japan. The DNA G+C content of the genome of strain Eri-1T is 42.5 ± 0.1 mol%.

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References


