**Abstract**

Three strains, CLM-U50\(^T\), CLM-R50 and IVIC-Bov1, belonging to the genus *Leptospira*, were isolated in Venezuela from a patient with leptospirosis, a domestic rat (*Rattus norvegicus*) and a cow (*Bos taurus*), respectively. The initial characterisation of these strains based on the *rrs* gene (16S rRNA) suggested their designation as a novel species within the ‘intermediates’ group of the genus *Leptospira*. Further phylogenomic characterisation based on single copy core genes was consistent with their separation into a novel group with the species. The average nucleotide identity between these three strains was >99 %, but below 89 % with respect to any previously described leptospiral species, also supporting their designation as a novel species. Given this evidence, these three isolates were considered to represent a novel species, for which the name *Leptospira venezuelensis* sp. nov. is proposed, with CLM-U50\(^T\) (=CIP 111407\(^T\)=DSM 105752\(^T\)) as the type strain.

**TAXONOMIC DESCRIPTION**

*Leptospira venezuelensis* sp. nov., a new member of the intermediate group isolated from rodents, cattle and humans

Rafael Puche,† Ignacio Ferrés,‡ Lizeth Caraballo,§ Yaritza Rangel,¶ Mathieu Picardeau,** Howard Takiff†* and Gregorio Iraola†*°

Leptospirosis is an emerging and re-emerging worldwide distributed disease caused by spirochetes of the genus *Leptospira* [1]. Based on the clinical presentation of leptospirosis, the species within the genus have been historically divided in three groups: ‘pathogens’ that cause the most severe cases; ‘intermediates’ that cause a milder disease; and non-pathogenic ‘saprophytes’ that do not cause disease in humans or animals [2]. The application of 16S rRNA sequencing, multilocus sequence typing and comparative genomics has revealed a strong correlation between the three groups defined by disease severity and the phylogenetic position of the species within the genus. Accordingly, ‘pathogens’ were renamed as group I, ‘intermediates’ as group II and ‘saprophytes’ as group III [3]. At the time of writing this article, the genus *Leptospira* comprised 22 different species; 10 belonging to group I (*Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira borgpetersenii*, *Leptospira alexanderi*, *Leptospira weili*, *Leptospira santarosai*, *Leptospira kmetyi*, *Leptospira alstoni* and *Leptospira mayottensis*), five to group II (*Leptospira licercaeae*, *Leptospira wolfii*, *Leptospira fainei*, *Leptospira broonii* and *Leptospira inadai*) and seven to group III (*Leptospira idonii*, *Leptospira meyeri*, *Leptospira terpstraec*, *Leptospira biflexa*, *Leptospira vanthielii*, *Leptospira yanagawae* and *Leptospira wolbachii*).

Venezuela is located in the tropical region of South America, a suitable environment for the development of leptospirosis. Leptospires can survive longer in warm and humid conditions [4], making the disease particularly prevalent in wet tropical and subtropical regions [5]. We isolated three strains: (i) CLM-U50\(^T\) from the urine of a patient suffering a moderately severe leptospirosis characterised by fever, icteric and elevated liver enzymes, with vomiting, myalgia and arthralgia but no evidence of renal or pulmonary involvement. The patient was treated with antibiotics and recovered. (ii) CLM-R50 from the kidney of a rat (*Rattus norvegicus*) captured in the same region where the patient resided and, (iii) IVIC-Bov1 from the urine of a cow (*Bos taurus*) at a geographically close (within 40 km) farm from where the patient resided. These strains were grown in Ellinghausen–McCullough–Johnson–Harris medium (EMJH), and when examined under dark-field microscopy, showed the motility and helix shape characteristic of the members of the genus *Leptospira*.

The whole-genome sequences of CLM-U50\(^T\), CLM-R50 and IVIC-Bov1 were obtained with an Illumina MiSeq platform. Libraries were reconstructed with the Nextera XT DNA Library Preparation Kit and sequenced with the MiSeq Reagent Kit v3 (pair-end reads of 150 bp). Reads were de novo assembled with SPAdes version 3.10.1 [6] and scaffolded with a post-assembly improvement pipeline [7]. The

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**Keywords:** Leptospiriosis; intermediates; Venezuela; whole-genome sequencing.

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NCBI accession numbers for sequenced genomes: CLM-U50 \(^T\): NETS00000000, Ivic-Bov1: NFUQ00000000, CLM-R50: NFUP00000000.
quality of the final assemblies was analysed with QUAST version 4.0 [8] and then annotated with Prokka v1.12 [9]. The three genome assemblies were deposited in the GenBank under the accession numbers NETS00000000, NFUQ00000000 and NFUP00000000 for strains CLM-U50\(^T\), IVIC-Bov1 and CLM-R50, respectively.

An initial phylogenetic characterisation of full-length 16S rRNA sequences was performed to determine the phylogenetic position of the three strains. The 16S rRNA gene sequences of CLM-U50\(^T\), CLM-R50 and IVIC-Bov1 were extracted from their genomes and aligned with those of other leptospiral species. A neighbour-joining tree was built with MEGA6 [10] using 1000 repetitions to determine bootstrap values. The 16S rRNA gene sequence similarity between the three strains was 100 %, while the similarity with respect to the closest species, \(L.\) licerasiae, was 93 %. The three strains formed a distinct monophyletic clade within the ‘intermediates’ (group II) with \(L.\) licerasiae as a sister species (Fig. 1). To further confirm the phylogenetic position of the three strains within the genus Leptospira, we screened the genomes of CLM-U50\(^T\), CLM-R50 and IVIC-Bov1, and available genomes of other leptospiral species against the eggNOG version 3.0 database [11].

\[\text{Fig. 1. 16S rRNA phylogeny. Phylogenetic tree built with 16S rRNA gene full-length sequences using the neighbour-joining method. Bootstrap values (1000 replicates) are displayed for most important internal nodes. The tree was rooted with \(Leptonema illini\) 3055\(^T\). Phylogenetic groups I ('pathogens'), II ('intermediates') and III ('saprophytes') are shaded in light grey.}\]
specifically customised for spirochetes (spiNOG), using HMMER version 3.1b2 [12]. A total of 530 single copy genes were recovered as the core genome, a concatenated alignment was generated with MUSCLE [13], and phylogenetic reconstruction was performed with phangorn [14]. Fig. 2 shows the resulting phylogeny evidencing the membership of the three strains in the ‘intermediates’ group, and demonstrating their phylogenetic position with L. licerasiae as a sister species.

The average genomic relatedness between CLM-U50T, CLM-R50 and IVIC-Bov1, and with respect to the other available sequenced Leptospira genomes was calculated using the average nucleotide identity (ANI) value [15] as previously implemented [16]. ANI can be used to replace DNA–DNA hybridisation (DDH), whereby the DDH species threshold of 70 % corresponds to an ANI of 95 % [17]. Strains CLM-U50T, CLM-R50 and IVIC-Bov1 were extremely similar, showing an ANI >99 % to each other, confirming that they belong to the same bacterial species and could represent a single clone circulating in Venezuela. The ANI value was <89 % with respect to the genome of any other leptospiral species, thereby justifying their designation as representatives of a distinct new species (Table 1). The mean G+C content of the genomic DNA was 39.5 %, which falls within the range reported for the members of the genus Leptospira [18].

Fig. 2. Single copy core genes phylogeny. Phylogenetic tree built with a concatenated alignment of 530 single copy genes present in all the analysed genomes, using the maximum-likelihood method. Bootstrap values (100 replicates) are displayed for most important internal nodes. Phylogenetic groups I (‘pathogens’), II (‘intermediates’) and III (‘saprophytes’) are shaded in light grey.
ANI values are sufficient to define strains CLM-U50 relatedness measured by 16S, single copy core genes and within this genus. The phylogenetic position and the genetic support the designation of the three in Venezuela as representatives of a novel species requiring a deeper analysis of its epidemiological characteristics in Venezuela and a broader sampling effort to determine its presence and incidence in the rest of the world.

Because CLM-U50 was isolated from a human patient with leptospirosis, which is infrequent for an ‘intermediate’ species, we screened the genomes for previously characterised leptospiral virulence factors [18] using BLAST+ blastn [19] and Pfam [20]. We found putative distant homologs of L. interroga-
ans proteins LipL32/LIC11352 (amino acid identity=67 %) and the collagenase ColA/LIC12760 (amino acid identity=75 %). Also, L. venezuelensis has a gene encoding a leptospiral immunoglobulin-like (Lig) protein, which contains bacterial immunoglobulin-like (Big) domains. This family of surface-exposed lipoproteins, including LigA, LigB and LigC, is present in pathogenic species, but not in saprophytes.

In conclusion, the genotypic and genomic analyses strongly support the designation of the three Leptospira strains isolated in Venezuela as representatives of a novel species within this genus. The phylogenetic position and the genetic relatedness measured by 16S, single copy core genes and ANI values are sufficient to define strains CLM-U50T, CLM-R50 and IVIC-Bov1 as members of a new species distinct from all others currently described for the genus Leptospira. The finding that a novel species belonging to the ‘intermediates’ group can cause leptospirosis in humans, and the identification of key leptospiral virulence genes in its genome, highlight the need to further study this group of leptospires whose actual incidence, epidemiology and patho-
genetic potential in humans and animals remain largely unknown. Furthermore, the wide host range of this novel species provides a suitable scenario for its dissemination, requiring a deeper analysis of its epidemiological characteristics in Venezuela and a broader sampling effort to determine its presence and incidence in the rest of the world. The proposed name for this species is Leptospira venezue-
lenensis sp. nov., with CLM-U50T (=CIP 111407T=DSM 105752T) as the type strain.

**DESCRIPTION OF LEPTOSPIRA VENEZUELENSESIS SP. NOV.**

Leptospira venezuelensis (ve.ne.zu.e.len’sis. N.L. fem. adj. venezuelensis, belonging to the country of Venezuela in South America).

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