Molecular characterization of *Leptospira* vaccine serovars using the MLST technique

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Abstract

Introduction: Leptospirosis is a widespread zoonotic disease causing substantial morbidity and mortality in humans and animals globally. Understanding genetic diversity, verifying strain authenticity, and ensuring quality control of biological products rely heavily on the molecular characterization of vaccine seed serovars.

Materials and Methods: In this study, five *Leptospira* vaccine serovars maintained at the Razi Vaccine and Serum Research Institute were characterized using MLST scheme 3 based on six housekeeping loci. Following PCR amplification and Sanger sequencing, allelic profiles and sequence types (STs) were determined, concatenated, and compared with 15 reference strains from GenBank.

Results: Among the vaccination strains, three different species were found: *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*. *L. interrogans* serovar Pomona and *L. kirschneri* serovar Grippotyphosa were found to have two new genotypes. Phylogenetic study demonstrated that L. interrogans serovars Icterohaemorrhagiae (ST1), Pomona (new ST), and Canicola (ST31) grouped closely, showing high genetic identity. *L. borgpetersenii* serovar Hardjo type Bovis (ST145), on the other hand, showed clear phylogenetic separation from the other species.

Conclusion: This study demonstrates the first application of MLST to characterize *Leptospira* vaccine serovars in Iran. Comparative analysis confirmed that these vaccine serovars represent the three major pathogenic species prevalent in humans and livestock globally and domestically. By characterizing the genetic identity of these vaccine strains, this work indirectly aids in future vaccine optimization and quality assurance. Furthermore, the identification of novel sequence types emphasizes the necessity of continuous molecular surveillance and routine PubMLST curation.

Keywords: Iran, *leptospira*, MLST, phylogenetic analysis, vaccine strains

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1. Introduction

Pathogenic *Leptospira* species are the cause of leptospirosis, a frequent zoonotic disease. It is transmitted by direct or indirect contact with the urine of infected hosts and continues to be a major veterinary and public health issue, especially in tropical and subtropical regions [1]. Clinical presentations of the illness can range widely, from minor flu-like symptoms to serious, life-threatening consequences, such as vascular, hepatic, renal, pulmonary and skeletal muscle injury, which is known as Weil's syndrome [2]. Additionally, the illness impacts the agriculture sector by resulting in cattle deaths, infertility, and abortions [3].

There are currently 74 species in the genus *Leptospira*. The genus was recently split into two major clades: "Pathogens," which include species that are known to cause disease in humans and animals (Subclade P1) or species whose virulence status is unknown (formerly known as "Intermediates" (Subclade P2)); and "Saprophytes," which are free-living, non pathogenic species [4].

The microscopic agglutination test (MAT) remains the gold standard for the diagnosis of leptospirosis. It, however, requires skilled personnel and maintenance of a broad panel of live *Leptospira* serovars for antigen preparation [5].

To overcome these limitations, molecular typing techniques have been developed for higher discrimination power [6]. Various techniques such as DNA-DNA hybridization investigations, bacterial restriction-endonuclease DNA analysis, ribotyping, and pulsed-field gel electrophoresis are employed to molecularly characterize *Leptospira*. Both characterization and detection can be accomplished with PCR-based techniques. When it comes to determining ancestral relationships and separating isolates linked to outbreaks based on their genome species status, sequence-based genotyping is a very reliable and effective technique [7]. Among these methods, MLST is a PCR-based method that has higher discrimination power [8].

Multilocus sequence typing (MLST) has been widely utilized for molecular typing and other epidemiologic investigations because it offers a versatile method of characterizing diseases by focusing on no more than seven well-conserved loci in the genome [9]. MLST enables the assignment of sequence types (STs) and provides a standardized framework for global comparison of isolates through public databases such as PubMLST [10]. There are three MLST schemes for *Leptospira* bacteria available in the PubMLST database [11]. Six loci make up Multilocus Sequence Typing scheme 3: *adk* (adenylate kinase), *icdA* (isocitrate dehydrogenase), *lipL32* (outer membrane lipoprotein LipL32), *rrs2* (*16S rRNA*), *secY* (pre-protein translocase SecY), and *lipL41* (outer membrane lipoprotein LipL41). As the chosen genes are species specific, genetically stable, and develop slowly, this strategy is regarded as dependable for the molecular characterization of *Leptospira* and offers strong markers for phylogenetic and typing research [12, 13].

The *Leptospira* MLST scheme 3 has previously been evaluated for evolutionary neutrality and genetic stability. Low nonsynonymous to synonymous substitution ratios (dN/dS) and acceptable discriminatory indices (D-values) from validation studies indicate that the chosen loci are primarily evolving under neutral selection pressure, supporting their suitability for molecular

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epidemiology and MLST-based phylogenetic inference [12]. In the current study, for the above-mentioned reasons, scheme 3 was chosen to identify vaccine related *leptospira* serovars prevalence in Iran. Relatively few studies have used MLST to differentiate vaccine strains from field isolates, despite the fact that it has been widely used to examine the genetic diversity and phylogeny of *Leptospira*. Determining whether recovered isolates match vaccine serovars or represent new circulating lineages is essential for tracking vaccination coverage and comprehending transmission dynamics in areas where vaccination is widely practiced [14].

Molecular characterization of *Leptospira* strains using multilocus sequence typing (MLST) plays a crucial role in epidemiological surveillance, accurate strain verification, and understanding phylogenetic diversity. Therefore, the present study aimed to molecularly characterize the five Iranian *Leptospira* vaccine serovars using MLST Scheme 3 to establish a national baseline.

2. Materials and Methods

2.1. Bacterial culture

In this study, five different *Leptospira* serovars were used as vaccine strains provided by the *Leptospira* Reference Laboratory, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, Iran. *Leptospira* isolates were cultivated in enriched Ellinghausen–McCullough–Johnson–Harris (EMJH) medium at 28–30 °C under aerobic conditions for 7–14 days until they reached the mid-logarithmic growth phase. To ensure culture integrity, motility and purity were checked weekly by using dark-field microscopy.

2.2. DNA extraction

For the MLST, genomic DNA was extracted using the phenol-chloroform method, and dissolved in TE buffer. DNA concentration and purity were determined using a NanoDrop spectrophotometer, and samples were stored at −20 °C until further use.

2.3. PCR amplification

The amplification of six target genes—*adk*, *icdA*, *lipL32*, *lipL41*, *rrs2*, and *secY*—was performed according to a previously described MLST scheme 3. The PCR reactions were prepared containing 100 ng of genomic DNA, 10 pmol of each primer, and 2× PCR Master Mix so that the final volume was approximately 20 µL. The cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58–60 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min (Table 1). The PCR amplicons were analyzed by electrophoresis on 1% agarose gel, purified and sequenced via the Sanger sequencing method.

2.4. DNA sequencing and quality assessment

PCR products were sequenced by Gene Fanavaran Company, Iran. Sequence quality was initially assessed using Chromas v2.6, and homology of *Leptospira* serovar sequences was analyzed using

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the BLAST webtool available at http://www.ncbi.nih.gov. Sequences were edited and exported in FASTA format using EditSeq (DNASTAR, Madison, WI, USA), and compared with similar sequences from GenBank using MegAlign (DNASTAR). Additional comparative analyses were performed using MEGA version 11.

2.5. MLST data and phylogenetic analysis

To create a single multilocus sequence per isolate for further analysis, sequences were put together and concatenated using Geneious Prime software. The *Leptospira* MLST Scheme 3, which is accessible in the PubMLST database (https://pubmlst.org/leptospira-spp), was used to determine allelic profiles and sequence types (STs). In order to receive ST numbers, new genotypes were submitted in PubMLST. MEGA 11 software's ClustalV method was used to align concatenated sequences.

Table 1. Primer sequences used for amplification of six loci in the *Leptospira* MLST Scheme 3.

Genes	Primer sequences(5′–3′)	PCR product size(bp)	References
adk	F-GGGCTGGAAAAGGTACACAA R-ACGCAAGCTCCTTTTGAATC	531	[12, 13]
icdA	F-GGGACGAGATGACCAGGAT R-TTTTTTGAGATCCGCAGCTTT	674	[12, 13]
lipL32	F-ATCTCCGTTGCACTCTTTGC R-ACCATCATCATCATCGTCCA	474	[12, 13]
lipL41	F-TAGGAAATTGCGCAGCTACA R-GCATCGAGAGGAATTAACATCA	520	[12, 13]
rrs2	F-CATGCAAGTCAAGCGGAGTA R-AGTTGAGCCCGCAGTTTTC	541	[12, 13]
secY	F-ATGCCGATCATTTTTGCTTC R-CCGTCCCTTAATTTTAGACTTCTTC	549	[12, 13]

2.6. Comparative sequence and phylogenetic analysis with NCBI reference strains

The NCBI GenBank database's publicly accessible *Leptospira* sequences were compared with the concatenated sequences (2,980 bp) acquired in this investigation. The corresponding loci (*adk*, *icdA*, *lipL32*, *lipL41*, *rrs2*, and *secY*) were concatenated using the same gene order after being extracted from either individually deposited gene sequences or whole genome sequences (WGS). MEGA 11 software was used to align several sequences. The genetic link between vaccine serovars and reference *Leptospira* strains was evaluated using phylogenetic and pairwise distance analysis.

3. Results

3.1. Amplification by PCR

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PCR amplification was successfully achieved for all six loci included in the *Leptospira* MLST scheme 3 (*adk*, *icdA*, *lipL32*, *lipL41*, *rrs2* and *secY*). Clear single bands of the expected sizes were observed for each gene without non-specific products, confirming the specificity of the primers and the reliability of the amplification conditions (Supplementary Figure S1).

3.2. Multilocus Sequence typing and Phylogenetic Analysis

The allelic profiles of RTCC 2808 and RTCC 2815 represented novel sequence types. In RTCC 2808 (*L. kirschneri* serovar Grippotyphosa), allelic divergence was driven by variations at the *secY* locus compared to published Grippotyphosa profiles. In RTCC 2815 (*L. interrogans* serovar Pomona), novel allelic variations were identified at the *icdA* and *lipL41* loci relative to previously reported reference Pomona genotypes. For both isolates, these unique allelic combinations resulted in the designation of new STs. Table 2 summarizes the STs used to assign species, serovars, and serogroups.

Geneious Prime was used to concatenate the polymorphic regions of all six loci, yielding a total concatenated length of 2,980 base pairs per sample. MegAlign was utilized to align these concatenated sequences to determine pairwise genetic similarity and divergence. Sequence identity between isolates ranged from 89% to 99.9% (Table 3). Phylogenetic analysis based on concatenated sequences of the six MLST loci revealed the genetic relationships among the five *Leptospira* vaccine serovars, delineating two major clusters (Figure 1). The first cluster consisted of two subclusters: the first subcluster encompassed *L. interrogans* serovars Canicola (RTCC 2805), Pomona (RTCC 2815), and Icterohaemorrhagiae (RTCC 2812), while the second subcluster contained *L. kirschneri* serovar Grippotyphosa (RTCC 2808). The second major cluster comprised *L. borgpetersenii* serovar Hardjo type Bovis (RTCC 2810).

Across distinct species (*L. interrogans*, *L. kirschneri* and *L. borgpetersenii*), interspecies divergence was driven by allelic variation spanning all six MLST loci, as reflected by the distinct allele numbers in Table 2. Within *L. interrogans*, intraspecific diversity primarily exhibited double-locus variation (DLV). Specifically, serovar Canicola differed from serovar Icterohaemorrhagiae at two loci (*adk* and *secY*), representing a DLV relationship. Similarly, serovars Pomona and Canicola were related as DLVs, differing at the *rrs2* (*16S rRNA*) and *lipL32* loci. In contrast, when compared to the domestic *L. interrogans* serovar Icterohaemorrhagiae (RTCC 2812 / ST1), serovar Pomona differed across four distinct loci (*adk*, *lipL32*, *rrs2*, and *secY*), sharing identical allele designations only at *icdA* and *lipL41*.

Table 2. Alleles and sequence types (STs) of vaccine *Leptospira* serovars

RTCC No.	Species	Serogroup	Serovar	Allelic profile						ST
				adk	*icdA*	*lipL32*	*lipL41*	*rrs2*	*secY*	
2805	*L. interrogans*	Canicola	Canicola	2	1	1	1	1	6	31
2808	*L. kirschneri*	Grippotyphosa	Grippotyphosa	13	24	11	16	12	5	New ST I
2810	*L. borgpetersenii*	Sejroe	Hardjo type Bovis	57	54	35	39	20	47	145
2812	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	1	1	1	1	1	1	1
2815	*L. interrogans*	Pomona	Pomona	2	1	3	1	2	6	New ST II

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L. interrogans serovars Canicola (RTCC 2805) and Pomona (RTCC 2815) showed the highest genetic similarity (99.9%), whereas serovar Icterohaemorrhagiae (RTCC 2812) showed 99.8% and 99.7% sequence similarity to *L. interrogans* serovars Canicola and Pomona, respectively. Despite belonging to a separate species, *L. kirschneri* serovar Grippotyphosa (RTCC 2808) clustered within the first lineage, sharing over 96% sequence similarity with the *L. interrogans* serovars. On the other hand, *L. kirschneri* serovar Grippotyphosa (RTCC 2808) and *L. borgpetersenii* serovar Hardjo type Bovis (RTCC 2810) showed the least similarity (89%), indicating significant divergence between these taxa (Table 3). These findings demonstrate the genetic variation among the vaccination strains and validate MLST scheme 3's ability to distinguish between *Leptospira* species and serovars.

Table 3. Percent sequence similarity and divergence among the five *Leptospira* vaccine serovars based on concatenated MLST loci (Clustal V algorithm, MegAlign, DNASTAR).

Percent Identity

	1	2	3	4	5	
1		96.6	89.2	99.8	99.9	1
2	3.3		89.0	96.7	96.6	2
3	11.1	11.2		89.2	89.1	3
4	0.2	3.2	11.0		99.7	4
5	0.1	3.3	11.1	0.3		5
	1	2	3	4	5	

Divergence

1 2805-L.Canicola
2 2808-L.Grippotyphosa
3 2810-L.Hardjo Type Bovis
4 2812-L.icterohaemorrhagiae
5 2815-L.Pomona

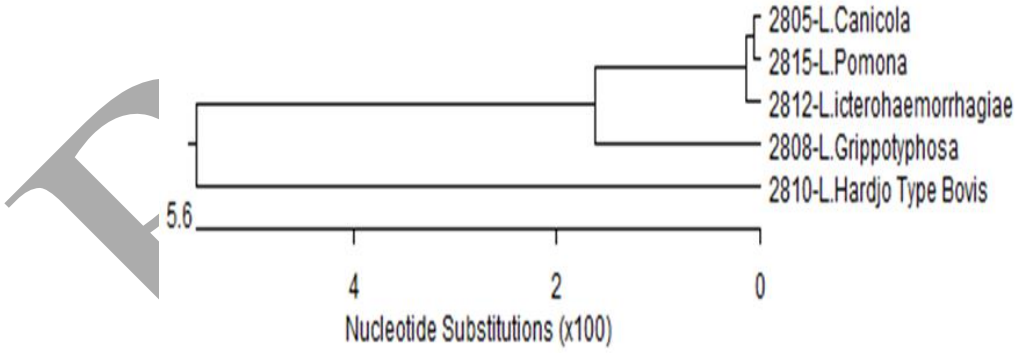


Figure 1. Distance-based dendrogram of the five *Leptospira* vaccine serovars based on concatenated MLST loci generated using the Clustal V method in MegAlign (DNASTAR). The horizontal scale indicates nucleotide substitutions per 100 residues.

(Note: This dendrogram illustrates descriptive genetic divergence among the vaccine strains; inferential phylogenetic relationships and statistical branch robustness are evaluated via bootstrap testing in the MEGA analysis).

3.3. Comparative sequence and phylogenetic analysis with NCBI reference strains

Comparative sequence analysis was performed using concatenated MLST loci from the five vaccine serovars analyzed in this study, together with 15 reference *Leptospira* serovars retrieved from the NCBI GenBank database (Table 4), resulting in a total of 20 *Leptospira* serovars included in the analysis. Multiple sequence alignment was carried out using MEGA and MegAlign software to assess nucleotide sequence similarity and genetic divergence among the isolates. The resulting similarity and divergence percentages, as summarized in Table 4, ranged from 88.6% to 100%. A phylogenetic tree was subsequently constructed using the Neighbor-Joining method with the Kimura 2-parameter evolutionary model and 1000 bootstrap replications in MEGA software to infer the evolutionary relationships among the *Leptospira* serovars (Figure 2).

Table 4. Percent sequence similarity and divergence among five *Leptospira* vaccine serovars and fifteen NCBI reference serovars based on MLST analysis (Clustal V, MegAlign, DNASTAR).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1		96.6	89.2	99.8	99.9	89.2	89.2	89.0	99.9	100.0	99.6	99.6	99.6	99.6	99.8	99.8	99.8	94.9	95.0	95.0	1
2	3.3		89.0	96.7	96.6	89.0	89.0	88.9	96.6	96.6	96.7	96.7	96.7	96.7	96.6	96.6	96.6	98.2	98.2	98.2	2
3	11.1	11.2		89.2	89.1	100.0	100.0	99.8	89.1	89.2	89.2	89.2	89.2	89.2	89.1	89.1	89.1	89.4	89.4	89.4	3
4	0.2	3.2	11.0		99.7	89.2	89.2	89.0	99.8	99.8	99.8	99.8	99.8	99.8	99.7	99.7	99.7	95.0	95.0	95.0	4
5	0.1	3.3	11.1	0.3		89.1	89.1	88.9	100.0	99.9	99.6	99.6	99.6	99.6	99.9	99.9	99.9	94.9	94.9	94.9	5
6	11.1	11.2	0.0	11.0	11.1		100.0	99.8	89.1	89.2	89.2	89.2	89.2	89.2	89.1	89.1	89.1	89.4	89.4	89.4	6
7	11.1	11.2	0.0	11.0	11.1	0.0		99.8	89.1	89.2	89.2	89.2	89.2	89.2	89.1	89.1	89.1	89.4	89.4	89.4	7
8	11.2	11.4	0.2	11.2	11.3	0.2	0.2		89.0	89.0	89.0	89.0	89.0	89.1	89.0	89.0	89.0	89.2	89.2	89.2	8
9	0.1	3.3	11.1	0.2	0.0	11.1	11.1	11.2		99.9	99.6	99.6	99.6	99.6	99.9	99.9	99.9	95.0	95.0	95.0	9
10	0.0	3.3	11.1	0.2	0.1	11.1	11.1	11.2	0.1		99.6	99.6	99.6	99.6	99.8	99.8	99.8	94.9	95.0	95.0	10
11	0.4	3.2	11.1	0.2	0.4	11.1	11.1	11.2	0.4	0.4		99.9	99.9	99.9	99.6	99.6	99.6	95.0	95.0	95.0	11
12	0.4	3.2	11.1	0.2	0.4	11.1	11.1	11.2	0.4	0.4	0.1		100.0	99.9	99.6	99.6	99.6	95.0	95.0	95.0	12
13	0.4	3.2	11.1	0.2	0.4	11.1	11.1	11.2	0.4	0.4	0.1	0.0		99.9	99.6	99.6	99.6	95.0	95.0	95.0	13
14	0.4	3.2	11.0	0.2	0.4	11.0	11.0	11.2	0.4	0.4	0.1	0.1	0.1		99.6	99.6	99.6	95.0	95.0	95.0	14
15	0.2	3.3	11.1	0.3	0.1	11.1	11.1	11.2	0.1	0.2	0.4	0.4	0.4	0.4		100.0	100.0	94.9	94.9	94.9	15
16	0.2	3.3	11.1	0.3	0.1	11.1	11.1	11.2	0.1	0.2	0.4	0.4	0.4	0.4	0.0		100.0	94.9	94.9	94.9	16
17	0.2	3.3	11.1	0.3	0.1	11.1	11.1	11.2	0.1	0.2	0.4	0.4	0.4	0.4	0.0	0.0		94.9	94.9	94.9	17
18	5.1	1.8	10.8	5.0	5.1	10.8	10.8	11.0	5.0	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1		99.9	99.9	18
19	5.0	1.8	10.8	5.0	5.1	10.8	10.8	11.0	5.0	5.0	5.0	5.0	5.0	5.0	5.1	5.1	5.1	0.1		100.0	19
20	5.0	1.8	10.8	5.0	5.1	10.8	10.8	11.0	5.0	5.0	5.0	5.0	5.0	5.0	5.1	5.1	5.1	0.1	0.0		20
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	

1	L.Canicola RTCC 2805
2	L.Grippotyphosa RTCC 2808
3	L.Hardjo Type Bovis RTCC 2810
4	L.icterohaemorrhagiae RTCC 2812
5	L.Pomona RTCC 2815
6	L.borgpetersenii Hardjo bovis CP000348
7	L.borgpetersenii Hardjo bovis-†CP03344
8	L.borgpetersenii Hardjo bovis- CP000350
9	L.interrogans Canicola CP186605.1
10	L.interrogans Canicola CP197415.1
11	L.interrogans Canicola CP043884.1
12	L.interrogans icterohaemorrhagiae CP043
13	L.interrogans icterohaemorrhagiae CP044
14	L.interrogans icterohaemorrhagiae CP197
15	L.interrogans Pomona CP130642.1
16	L.interrogans Pomona CP186594.1
17	L.interrogans Pomona LUHH01000031.1
18	L.kirschneri CP112976.1
19	L.kirschneri Grippotyphosa MVIS01000017
20	L.kirschneri Grippotyphosa NZ_AHQA01000

L. interrogans serovar Canicola (RTCC 2805) showed 99.6–100% sequence similarity with registered strains from Malaysia, Sweden, and Kazakhstan, according to the alignment, suggesting that this serovar is highly conserved worldwide. While *L. interrogans* serovar Pomona (RTCC 2815) showed 99.9% similarity to Pomona strains reported worldwide, *L. interrogans* serovar Icterohaemorrhagiae (RTCC 2812) showed 99.8% similarity with matching sequences available in the NCBI database. A slightly higher level of genetic variation was shown by the 98.2%

sequence similarity between registered *L. kirschneri* Grippotyphosa isolates and *L. kirschneri* serovar Grippotyphosa (RTCC 2808). *L. borgpetersenii* serovar Hardjo type Bovis (RTCC 2810), on the other hand, showed 99.8–100% match to equivalent *Leptospira* sequences found in GenBank.

Notably, the lowest sequence identity (88.6%) across the 20 strains was identified between *L. kirschneri* serovar Grippotyphosa (RTCC 2808) and *L. borgpetersenii* serovar Hardjo type Bovis strain CP000350 from the USA (Table 4). This low sequence identity directly reflects the substantial interspecific phylogenetic divergence and profound genomic variation existing between *L. kirschneri* and *L. borgpetersenii*.

The phylogenetic tree constructed from the concatenated MLST sequences of all 20 serovars revealed three distinct clades corresponding to the species *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* (Figure 2). These clades were clearly resolved, with major species-defining nodes strongly supported by bootstrap values up to 100%, indicating that the MLST scheme efficiently discriminates *Leptospira* species while preserving evolutionary relationships.

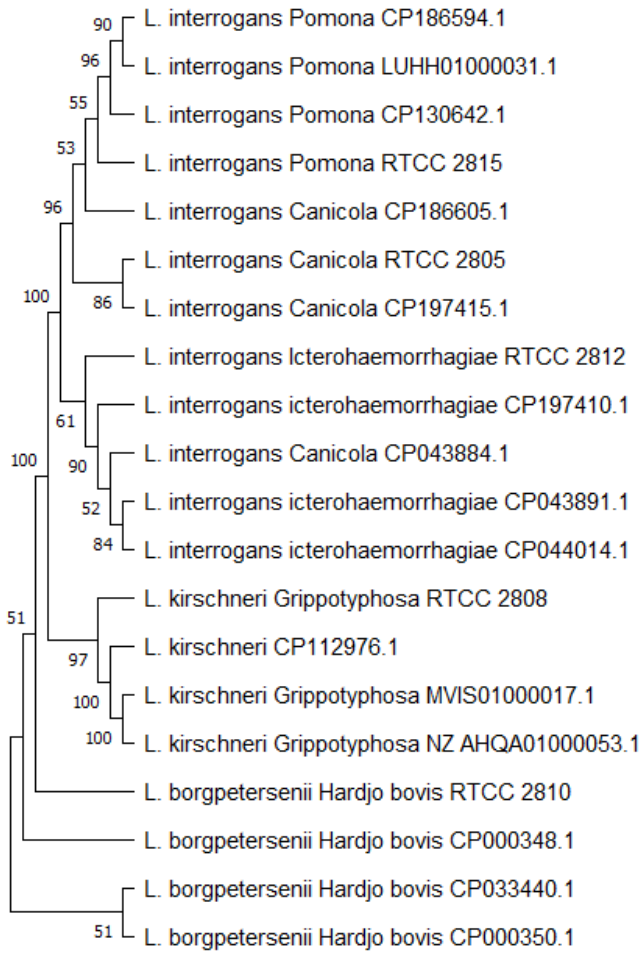


Figure 2. Phylogenetic tree of five *Leptospira* vaccine serovars and fifteen NCBI reference serovars based on concatenated MLST sequences constructed using the Neighbor-Joining method with the Kimura 2-parameter model in MEGA software. Bootstrap values (1000 replications) are shown next to the branches.

Specifically, the *L. interrogans* clade formed the largest cluster, where Iranian vaccine strains clustered with global counterparts: serovar Canicola (RTCC 2805) grouped closely with Canicola CP197415.1, serovar Pomona (RTCC 2815) clustered within the Pomona lineage alongside strains CP130642.1 and CP186594.1, and serovar Icterohaemorrhagiae (RTCC 2812) clustered within the Icterohaemorrhagiae branch. In the second clade, *L. kirschneri* serovar Grippotyphosa (RTCC 2808) formed a distinct lineage basal to the reference Grippotyphosa isolates. The third clade comprised *L. borgpetersenii*, where the vaccine strain RTCC 2810 clustered reliably with reference Hardjo type Bovis genomes. Bootstrap support values for each node are detailed in Figure 2.

4. Discussion

Accurate molecular profiling of biological products is fundamental for verifying seed stock integrity and tracking evolutionary lineage stability. Applying MLST Scheme 3 to the Iranian pentavalent *Leptospira* vaccine strains resolved their taxonomic positions across three primary pathogenic species—*L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*—which consistently represent the etiologic agents behind severe clinical leptospirosis and reproductive failure in livestock globally [15, 16]. The close phylogenetic clustering among *L. interrogans* serovars Canicola, Pomona, and Icterohaemorrhagiae mirrors findings from global MLST surveys, which demonstrate a highly conserved core genome within this species despite marked lipopolysaccharide-driven serological diversity [12]. In sharp contrast, the deep phylogenetic branch separating *L. borgpetersenii* serovar Hardjo type Bovis from the *L. interrogans* and *L. kirschneri* lineages corresponds to the substantial genomic reduction (approximately 700 kb smaller) and high pseudogene density documented by Bulach et al., reflecting its host-restricted transmission ecology in cattle [17].

The designation of novel sequence types for *L. kirschneri* serovar Grippotyphosa (RTCC 2808) and *L. interrogans* serovar Pomona (RTCC 2815) illustrates the discriminatory power of MLST Scheme 3 over single-locus typing. In the case of serovar Grippotyphosa, divergence was concentrated at the *secY* locus [18]. This pattern aligns with broader phylogeographic studies reporting that *secY* displays higher nucleotide diversity and substitution rates compared to other housekeeping loci, providing sufficient discriminatory capacity to capture lineage-specific polymorphisms.

Seroepidemiological reviews across the Middle East indicate that although diagnostic methodologies and reported seroprevalence vary, predominant serogroups—notably Sejroe, Grippotyphosa, Icterohaemorrhagiae, and Pomona—are consistently identified in humans and livestock across regional countries such as Iran, Turkey, and Egypt [19, 20]. This demonstrated prevalence closely matches the serogroups represented by the Iranian vaccine strains evaluated here. Although detailed molecular and sequence data for domestic vaccine strains in neighboring countries are not currently available, the predominant circulating serovars across the region remain broadly identical.

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Crucially, because protective immunity is primarily mediated by surface-exposed lipopolysaccharides and outer membrane proteins (such as LipL32 and LipL41) rather than conserved internal metabolic enzymes, detecting novel STs in housekeeping genes does not imply diminished protective efficacy. Instead, depositing these distinct allelic profiles in PubMLST provides an essential benchmark to differentiate seed stocks from circulating field isolates during diagnostic investigations [21, 22]. Establishing this baseline indirectly supports quality control and ongoing formulation reviews, ensuring that domestic vaccine manufacturing retains fidelity against evolving regional lineages.

A recognized limitation of this study is the constrained sample size, which was inherently defined by the five specific vaccine serovars maintained at the national reference center rather than an expansive epidemiological cohort. Consequently, deriving population-wide genetic diversity metrics or statistical mean genetic distances offers limited analytical power. While selection pressure dynamics (dN/dS) have been established in prior scheme validation studies, and descriptive pairwise genetic distances and identity matrices were reliably computed using MEGA and MegAlign, robust statistical population inferences will require future investigations that incorporate an extensive collection of geographic field isolates alongside these vaccine reference baselines.

5. Conclusion

This study successfully applied MLST Scheme 3 to establish the first comprehensive molecular baseline for the pathogenic *Leptospira* vaccine strains utilized in Iran, leading to the designation of two novel sequence types. While MLST indexes conserved metabolic loci and does not directly measure protective clinical efficacy, these molecular profiles confirm the taxonomic fidelity and genomic stability of domestic seed stocks relative to regional lineages, indirectly supporting ongoing quality assurance protocols. Although MLST remains a cost-effective and dependable framework for routine epidemiological surveillance and reference typing, integrating whole-genome sequencing (WGS) in future investigations will offer higher resolution into surface antigen dynamics, mobile elements, and microevolutionary variations, ultimately facilitating more targeted regional leptospirosis control and vaccine management.

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Conflict of Interests:

The authors declare no conflicts of interest.

Ethics Approval:

This study did not involve human or live animal subjects. All genetic characterization and experimental procedures were conducted in accordance with the approved biosafety and institutional protocols of the Razi Vaccine and Serum Research Institute.

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Data Availability:

The multilocus sequence typing (MLST) data and novel sequence types (STs) generated during this study have been submitted to the PubMLST database and are currently awaiting final accession assignment. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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