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Original Article

Molecular typing and seroepidemiology of pathogenic *Leptospira* spp. of domestic animals in Gilan province, northern Iran during 2021-2023

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Abstract

Background: *Leptospira*, a pathogenic bacterial organism, causes human leptospirosis when it infects mammals. There are different serovars of *Leptospira*, each of which is adapted to a specific animal host. Adaptation occurs dynamically in different hosts and changes the distribution and clinical manifestations of serovars. Variable-number tandem repeats (VNTRs) have been used to identify *Leptospira interrogans* strains. **Aims:** The purpose of this study was to conduct seroepidemiology and molecular typing of pathogenic *Leptospira* isolated from domestic animals in Gilan province. **Methods:** A total number of 250 urine and blood samples were collected from domestic animals (horse, bovine, and sheep). Urine samples were cultured in EMJH medium. Using the microscopic agglutination test (MAT) and the Multilocus VNTR Analysis (MLVA) methods, unknown samples were analyzed to determine *Leptospira* serovars. **Results:** Based on our data, *Leptospira* Autumnalis was mainly found in horses and dominated at 50%, followed by *Leptospira* Canicola at 29.16%. MAT results showed that among 87 animals with the seropositive, the horses showed the highest level of contamination at 40%, and this difference was not significant (P=0.152). **Conclusion:** According to our data, *Leptospira* Autumnalis was mainly found in horses, and the bacteria were shed in urine, suggesting that horses could transmit leptospirosis. The results of this study confirm that MLVA can replace time-consuming methods such as MAT because it is a simple and fast technique.

Key words: *Leptospira*, Microscopic agglutination test, Molecular typing, Multilocus VNTR analysis

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochaetes of the genus *Leptospira*, considering to be as a reemerging zoonotic infection. Globally, leptospirosis has been reported in many tropical regions (Piredda *et al*., 2021). It is estimated that there are over ~ 1-2 million cases of human infection and 60,000 deaths in the world (Brooks *et al*., 2012). This infection is the most prevalent cause of severe illnesses in companion animals, cattle, and even wildlife. The limited accuracy and accessibility of diagnostic approaches contribute to the negligence about leptospirosis in humans and animals. Considering the complex interactions between pathogenic organisms and animals that can contaminate, mediate, or maintain pathogenic *Leptospira* spp. in the environment, leptospirosis epidemiology, management, monitoring, and control can be examined from a health standpoint (Rees *et al*., 2021). Vincent *et al*. (2019) extended the *Leptospira* phylogeny by designating the two subclades P1 and P2 based on the pathogenicity of their species. Currently, 38 pathogenic species (P1 and P2) and hundreds of serovars have been identified that can cause infection in various animal species (Nadon *et al*., 2013). When *Leptospira* colonizes in renal tubules and sheds in the urine, it can persist for a period of time in the environment. A mucous membrane or an abraded surface is usually the means by which the organisms enter the bloodstream, from where they spread to the kidneys. Due to colonization, shedding, and transmission, infection prevalence can be high in some reservoir host species (Yadeta *et al*., 2016). It appears that brown rats (*Rattus norvegicus*) suffer from renal tubular infections caused by *Leptospira interrogans* serovar Icterohaemorrhagiae in various environments, probably resulting from the

formation of biofilms within their renal tubules (Sykes *et al*., 2022). Some adapted host species are susceptible to sexual and vertical transmission. For example, *L. borgpetersenii* Hardjo and *L. interrogans* Bratislava are present in cattle and pigs, respectively (Cilia *et al*., 2020). The severity of the disorder varies from subclinical colonization of reservoir hosts to severe pathogenicity and death of random hosts (Rajeev, 2022). Depending on the strain infecting the host, the infection can result in life-threatening disease or subclinical carrier. Although the leptospiral infection has been known for many years, our understanding of leptospiral diversity has increased significantly in recent years. A comprehensive analysis of *Leptospira* genome sequence has revealed an open pan genome, indicating that it is capable of acquiring new genetic material through horizontal transfer, increasing its ability to infect a wider variety of host organisms (Xu *et al*., 2016). The number of host species that can be infected has also increased significantly, and includes mammals, birds, reptiles, and fish (Gomes-Solecki *et al*., 2017). Managing acute infections, monitoring and controlling transmission from subclinically-infected reservoirs, and understanding the complex epidemiology of leptospirosis require improved diagnosis. Active infection can be detected using several methods, including culture, histopathology, tissue staining, and nucleic acid amplification tests. Indirect serological strategies include microscopic agglutination tests (MAT), ELISA, and lateral flow tests. There are several factors to consider when analyzing leptospirosis, including clinical manifestations, potential vulnerability, diagnostic examinations, and laboratory values (Sykes *et al*., 2022). As a general rule, direct diagnostic procedures are more appropriate for early-onset infections, whereas indirect serological procedures are more appropriate for late stages of infection. In order to gain a better understanding of the prevalence of infection in a specific species, direct and indirect methods should be combined. In parallel, to identify *Leptospira* species and serovars according to whole genome sequencing and serotyping, serological and molecular methods depend on information about the specific epidemiological environments (Nalam *et al*., 2010). Among the challenges faced when recovering leptospiral isolates from clinical specimens are the sophisticated conditions of leptospiral infections, the slow growth of the organisms, and the need for dark field microscopy to assess culture positivity. The cultivation of fastidious organisms has advanced with selective and fastidious media. Rapid diagnostic assays are needed for the identification of acute diseases because antimicrobials and supportive supervision are most effective when started early in the disease cycle (Sivakumar, 2022). Therefore, more sensitive and selective diagnostic tools are needed for determining whether antimicrobial treatments have eradicated renal tubule or genital infections in animals (Viriyakosol *et al*., 2006). The variable-number tandem-repeat (VNTR) method has been demonstrated to identify *L. interrogans* strains by molecular typing procedures. Additionally, Multiloci VNTR analysis (MLVA) is one of the newest and low-cost genotyping methods (Rezasoltani *et al*., 2015). It is hoped that it will replace other costly genotyping methods because of its advantages over other molecular methods. Accordingly, we conducted seroepidemiology and molecular typing of pathogenic *Leptospira* isolated from domestic animals in Gilan province using the MLVA technique. To do so, we investigated the presence of *Leptospira* and its diversity in the urine samples from domestic animals in Gilan province, and used MAT to determine the *Leptospira* serovars in these animals and MLVA to determine the pathogenic *Leptospira* genotypes.

Materials and Methods

Sample collection

In this study, 250 blood and urine samples were collected during the summers from 2021 to 2023 (two years). The sample size was determined based on published articles on leptospirosis in Iran. According to the following formula (a) and PCR sensitivity of 0.8, the confidence interval (CI=95%) (z=1.96) of the sample size was determined:

$$n = \frac{p(1-p)\times z^2}{d^2} \quad (a)$$

Where,
p: Prevalence (PCR sensitivity)
n: Sample size
z: The statistic corresponding to level of confidence
d: Precision

A total of 250 blood samples were taken randomly from the animals's jugular vein including horses (100 samples from Bandar Anzali Taktaz club), bovines (100 samples from Saravan industrial abattoir) and sheep (50 samples from Hendkhale abattoir) at different ages. Saravan abattoir is located around Rasht and Hendkhale is a village around Some-Sara. None of the animals were vaccinated against leptospirosis. After the serum was transferred to the Leptospira Research Laboratory at the Veterinary Research and Teaching Hospital University of Tehran, it was stored in the vicinity of ice at -20°C.

MAT test procedure

Serums were tested using six serotypes of live antigens of *L. interrogans*, including: Pomona, Canicola, Ballum, Gripotiphosa, Hardjo and Autumnalis. MAT was conducted mainly as described by OIE guidelines (16) with some modification at the Veterinary Research and Teaching Hospital, University of Tehran. Pure leptospira cultures of 5-7 days at 30°C were used as antigen. The serum samples were taken out of the freezer a few hours before the start of the MAT test and placed at room temperature to thaw. A mixture of diluted serum (1: 50) and antigen was added to a plate, and kept moist at 30°C for 90 min in an incubator. A dark field

microscopy with a magnification of 100 was then used to examine the samples. To ensure that the above test was accurate, three controls were prepared, including a positive control (positive standard serum), a negative control (negative standard serum), and a third control (antigen only to control spontaneous agglutination). Each sample was graded from +1 to +4 based on the amount of agglutination in the results sheet, such that, in +1, +2, +3, and +4, 75%, 50%, 25% and none of leptospiral bodies were free and mobile. Following the WHO standard, samples with an agglutination of +1 were considered negative, whereas samples with an agglutination of +4 were considered positive and the rest were regarded as suspect. In the negative state, no agglutination was observed and leptospiral objects were alive and active under the dark field microscopy. Finally, all samples that were positive at the 1:50 dilution were tested at the 1:100, 1:200, 1:400, and 1:800 dilutions. The highest dilution indicating an agglutination level of +4 was considered as the final MAT titer.

Culture of urine samples

Urine samples from domestic animals were cultured in EMJH medium, and then kept in an incubator at 28-30°C for at least a month. Ten microliters of culture medium was taken and placed on a clean slide every week to see whether bacteria grew or not.

DNA extraction and PCR

From 7-day cultures of *Leptospira* (2×10^8 cells/ml), genomic DNA was extracted using QIAamp DNA extraction kit (QIAGEN, USA). The primary PCR primers FD1 and RD1 were used for the amplification of the eubacterial rDNA. With the Qiaquick PCR purification kit (QIAGEN, USA), we purified PCR products and sequenced them using an ABI 3100 automated sequencer (PerkinElmer, USA). Using internal sequencing primers, we sequenced base pairs between 32 and 1355 of the leptospiral *16S rRNA* gene since the most distinct 16S sequence occurs in the middle.

Building a phylogenetic tree

The *16S rRNA* sequences of the eight studied isolates were compared using MEGA 11 software and the UPGMA method, and the corresponding phylogenetic tree was drawn.

Design of VNTR primers

To identify genetic typing of *Leptospira* spp. isolates was used MLVA technique for seven VNTR loci. In order to analyze the sequences of VNTR, Tandem Repeats Finder software and Tandem Repeats database (http://minisatellites.i2bc.paris-saclay.fr/) were used.

VNTR amplification was carried out with PCR Master Mix (super mix, sigma Aldrich, Germany) using SensoQuest instrument (Germany). The PCRs program were performed as follows: One cycle of denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. The primers used are listed in Table 1. The following formula (b) was used to calculate the number of repetitions for each locus:

$$\text{Number of repetitions} = \frac{(\text{size of left flanking} + \text{size of right flanking})}{\text{size of repetitionunit}}$$

– size of PCR product (b)

Results

MAT results showed that out of 250 sera, 87 (34.8%) livestock were positive and 163 (65.2%) samples were negative. 40% of horses, 35% of cattle and 24% of sheep showed positive reaction with one serovar (Fig. 1). The most prevalent of serovars were Autumnalis, Canicola and Grippotyphosa, respectively. Also, the difference was significant (P=0.00). The frequency of each serovars in the studied animals is shown in Table 2. Each of the positive MAT sera showed a serological reaction with only one serovar. The highest serological titer was 1/200.

Table 1: Target primers

Name	Primer sequence (5´-3´)	References
A(R)	TCGCTCTRCAGGTCGGTGTT	Caimi *et al*. (2012)
B(F)	TCGCTCTRCAGGTCGGTGTT	Varni *et al*. (2014)
B(R)	GTGGCCGACACCCTCTCAGGCCGGCTA	Strain *et al*. (2022)
C(F)	CCAGACTCGGAGGAAGATTACCCACG	Tomazou *et al*. (2008)
C(R)	TTGAACACGCGCCGCAACCCGTCCGC	Calì *et al*. (1997)
VNTR7	GATGATCCCAGAGAGTACCG	Fawzy *et al*. (2016)
VNTR7	TCCCTCCACAGGTTGTCTTG	Fang *et al*. (2008)
VNTR10	GAGTTCAGAAGAGACAAAAGC	Muz *et al*. (2021)
VNTR10	ACGTATCTTCATATTCTTTGCG	Borumand Azad *et al*. (2017)
VNTRLb5	ATTGCGAAACCAGATTTCCAC	Salaün *et al*. (2006)
VNTRLb5	AGCGAGTTCGCCTACTTGC	Loffler *et al*. (2017)
VNTR4	AAGTAAAAGCGCTCCCAAGA	Ramírez-Patiño (2013)
VNTR4	ATAAAGGAAGCTCGGCGTTT	Khaki (2013), Postollec *et al*. (2011)
VNTRLb4	TCGAGCGCCCATAGAAGCGAGACGCTGAG	Postollec *et al*. (2011)
VNTR Loci	TTACT	

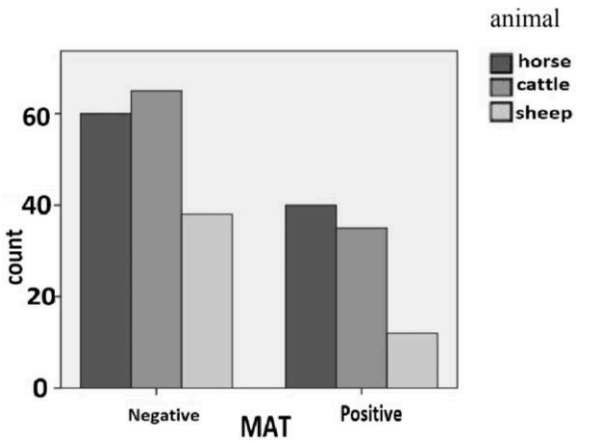


Fig. 1: Distribution of the MAT positive and negative samples in three types of animals in Gilan province

Table 2: Frequency of different *Leptospira interrogans* serovars in the studied animals using MAT method

Serovar	Animal			Total
	Horse	Bovine	Sheep	
Autumnalis	20 (20.00%)	5 (5.00%)	0 (0.00%)	25 (28.80%)
Ballum	0 (0.00%)	5 (5.00%)	3 (6.00%)	8 (9.20%)
Grippotyphosa	9 (9.00%)	5 (5.00%)	0 (0.00%)	14 (16.10%)
Pomona	2 (2.00%)	10 (10.00%)	1 (2.00%)	13 (14.90%)
Hardjo	4 (4.00%)	2 (2.00%)	2 (4.00%)	8 (9.20%)
Canicola	5 (5.00%)	8 (8.00%)	6 (12.00%)	19 (21.80%)
Total	40 (40.00%)	35 (35.00%)	12 (24.00%)	87 (100%)

Table 3: Distribution of *Leptospira* serovars isolated from domestic animal in Gilan province

Type of animal	Serovar				Total
	Ballum	Autumnalis	Hardjo	Canicola	
Horse	-	9	2	1	12 (50%)
Bovine	1	3	-	2	6 (25%)
Sheep	1	-	1	4	6 (25%)
Total	2 (8.33%)	12 (50%)	3 (12.50%)	7 (29/17%)	24 (100%)

PCR results for VNTR loci amplification

Twenty-four *Leptospira* were isolated from urine of animals (Table 3) and they were genotyped using the MLVA method. The repeat numbers in VNTR products were analyzed with gel electrophoresis (Fig. 2) and sequencing (Fig. 3). The size of the bands was converted into the number of repetitions after electrophoresis. The comparison of the *16S rRNA* sequences with data from the NCBI gene bank validated that all eight studied isolates correspond to distinct species of *Leptospira* (Fig. 3). This phylogenetic analysis, conducted using the UPGMA method with a bootstrap of 500, revealed that isolates G10-D, G11-D, G12-D, and G13-D were clustered together in the same phylogenetic group. Furthermore, isolates G10-C, G12-C, and G13-C demonstrated greater genomic similarity to a cluster containing four *Leptospira* species, specifically *L. interrogans*, *L. kirschneri*, *L. noguchii*, and *L. kmetyi*.

Simpson's diversity index (D) has been used in comparative studies diversity and calculated (Table 4) based on following formula (c):

$$D = 1 - \sum \left[nj(nj - 1) \right] / \left[N(N - 1) \right] \quad (c)$$

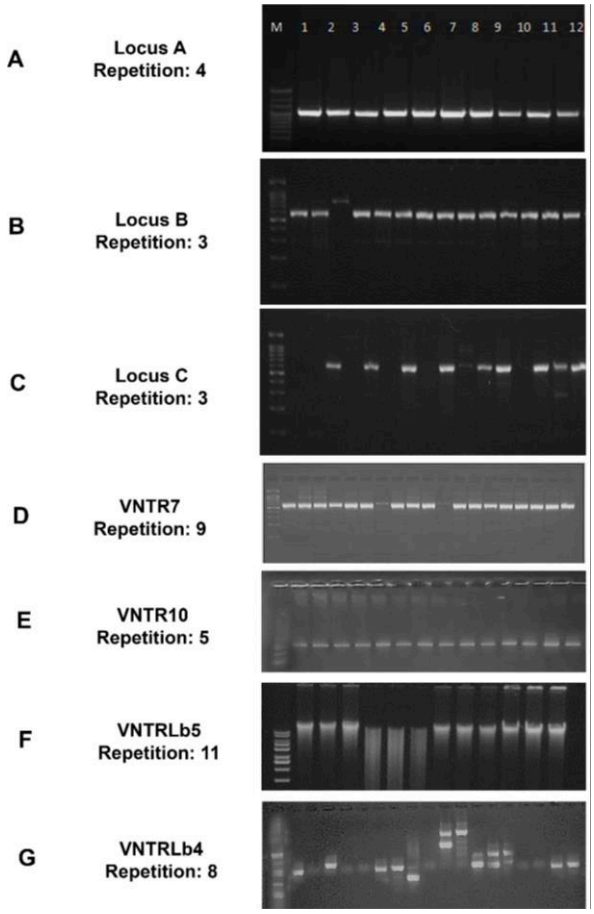


Fig. 2: VNTR products in *Leptospira* spp. isolates. A 100 bp ladder (Sigma Aldrich, Germany) was used different length of VNTR products was detected with (**A**) 4 repeats (Locus A), (**B**) 3 repeats (Locus B), (**C**) 3 repeats (Locus C), (**D**) 9 repeats (VNTR7), (**E**) 5 repeats (VNTR10), (**F**) 11 repeats (VNTRLb5), and (**G**) 8 repeats (VNTRLb4)

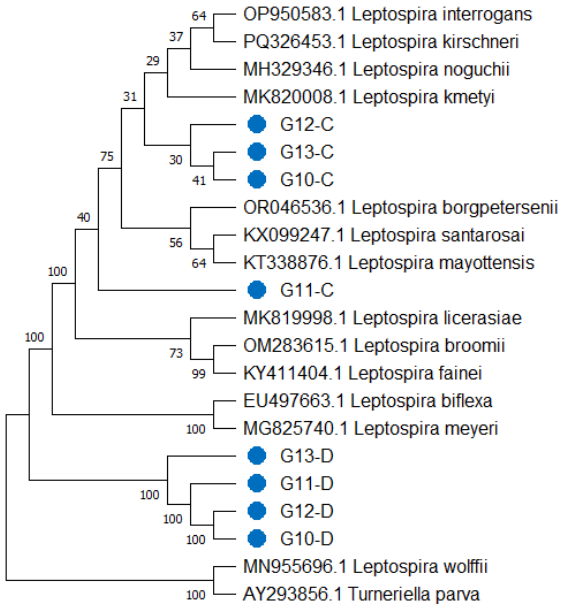


Fig. 3: A dendrogram of 8 isolates based on *16S rRNA* were analyzed using unweighted pair group method with arithmetic averages (UPGMA) and 500 bootstrap replications as implemented MEGA software version 11

Where,
D: Simpson's diversity index
nj: The total number of isolates of a particular genotype
N: The total number of isolates of all genotypes

Table 4: Repeats of each VNTR locus in each sample

VNTRLb4	VNTRLb5	VNTR10	VNTR7	C	B	A	No.
9	9	5	9	3	4	4	1
9	9	5	9	3	4	4	2
8	11	5	9	3	4	3	3
8	11	5	9	3	5	3	4
8	11	5	9	3	4	3	5
8	11	5	9	3	4	4	6
8	11	Null	9	Null	2	3	7
8	11	5	9	3	3	3	8
8	11	5	9	3	3	4	9
8	11	5	9	3	3	3	10
8	11	Null	9	3	3	4	11
8	11	5	9	3	3	3	12
8	8	5	9	3	3	3	13
8	11	5	9	3	3	3	14
8	11	5	9	3	3	3	15
9	Null	5	9	3	3	3	16
9	Null	5	9	3	3	4	17
8	11	5	9	3	3	4	18
9	11	5	9	3	3	3	19
7	11	5	9	3	3	3	20
8	9	5	9	3	4	3	21
8	9	5	9	3	4	3	22
8	9	5	9	3	4	3	23
8	9	5	9	3	4	3	24

A: Locus A, B: Locus B, and C: Locus C. VNTRLb4: Locus VNTRLb4, VNTRLb5: Locus VNTRLb5, VNTR10: Locus VNTR10, and VNTR7: Locus VNTR7

VNTR allele diversity

There were 7 VNTR loci analyzed for allele variation, with the VNTRLb5 locus having the highest number (9 repeats, 8 repeats, 11 repeats, and null) while the C locus had only one type (three repeats). According to Simpson's diversity coefficient, the VNTRLb5 locus had the highest diversity (0.222) whereas the VNTRLb4 locus had the lowest diversity (0.009). A description of each VNTR locus in the studied population can be found in Table 5.

Analyzing VNTR data with NJ

VNTR data was analyzed using the NJ method and Dice similarity coefficient to draw dendrograms. As a result, *Leptospira* isolates can be classified into two clusters (A and B), according to their similarity. Each of these clusters is subdivided into two sub-clusters (A1, A2, B1 and B2). In the NJ method (Fig. 4), the results are very similar to those of the MST method (Fig. 5). Most *Leptospira* isolates are in first cluster (A).

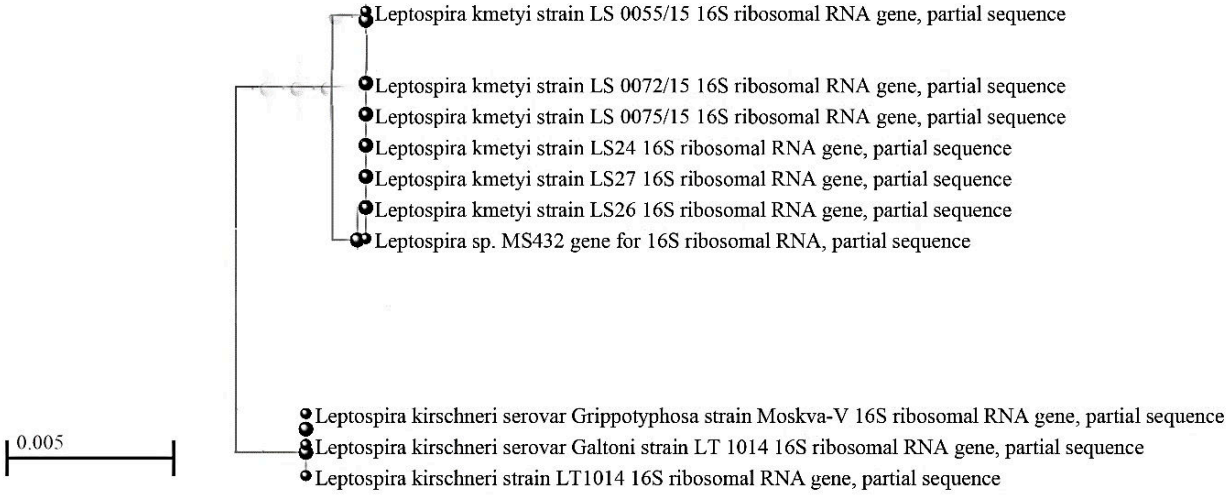
Table 5: *Leptospira* isolates' VNTR characteristics

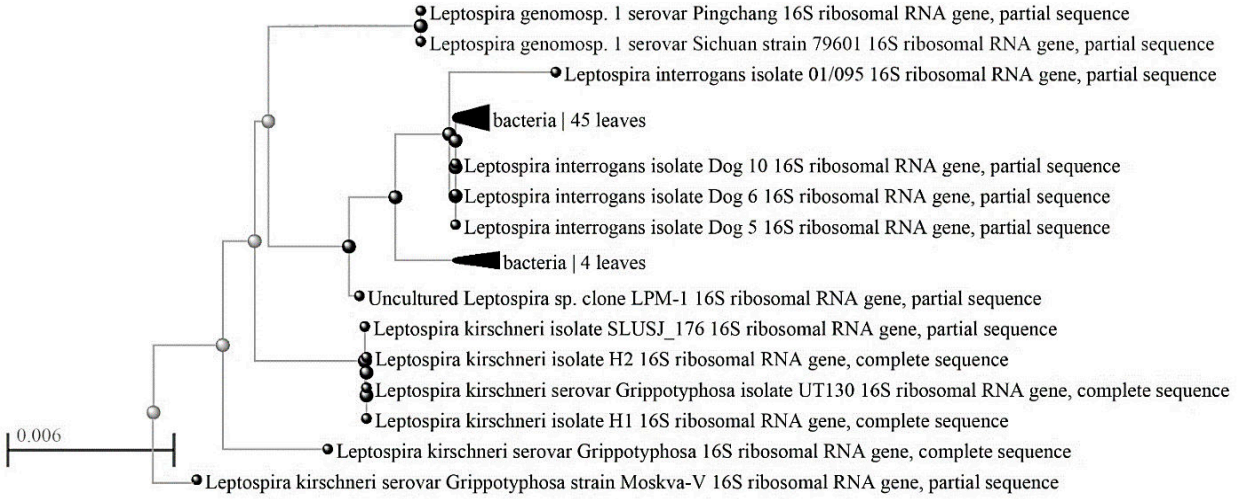
Max(pi)	Variation coefficient of Hunter-Gaston	CI (95%)	K	VNTR locus
1.000	0.012	0.000-0.130	1	A
0.902	0.114	0.033-0.198	2	B
0.941	0.115	0.000-0.234	1	C
0.961	0.097	0.000-0.331	3	VNTR7
0.961	0.147	0.000-0.221	4	VNTR10
0.961	0.222	0.000-0.175	4	VNTRLb5
0.000	0.009	0.000-0.002	1	VNTRLb4

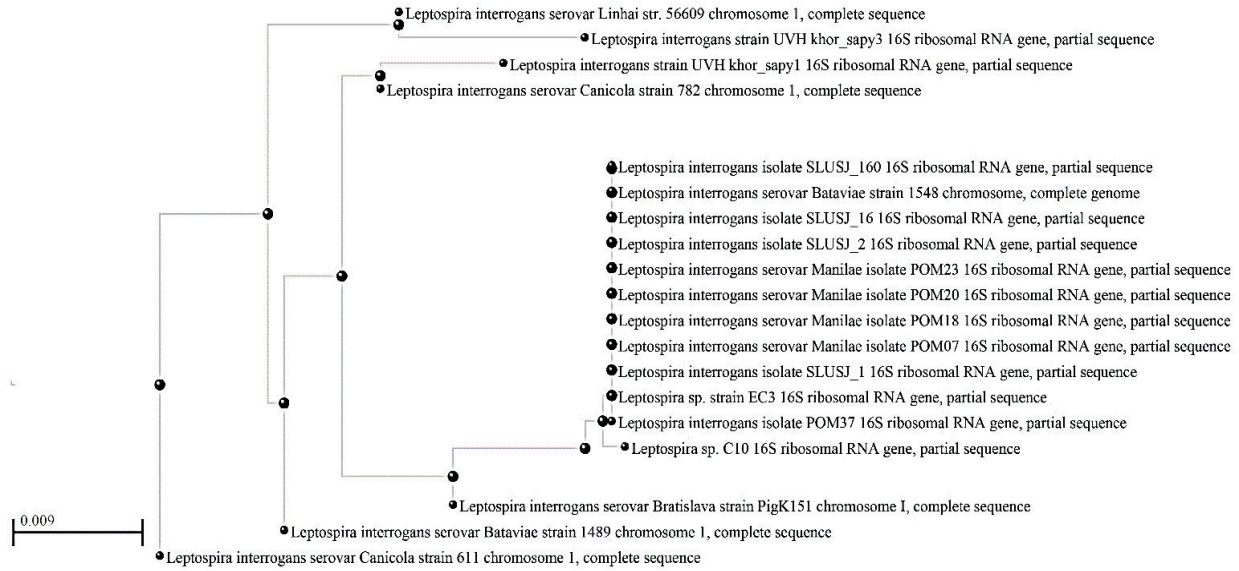
Each locus' Simpson's coefficient of variation is indicated by the 0.95 confidence interval. At each locus, K means the number of different alleles. Max (pi) contains the most allelic diversity among isolates

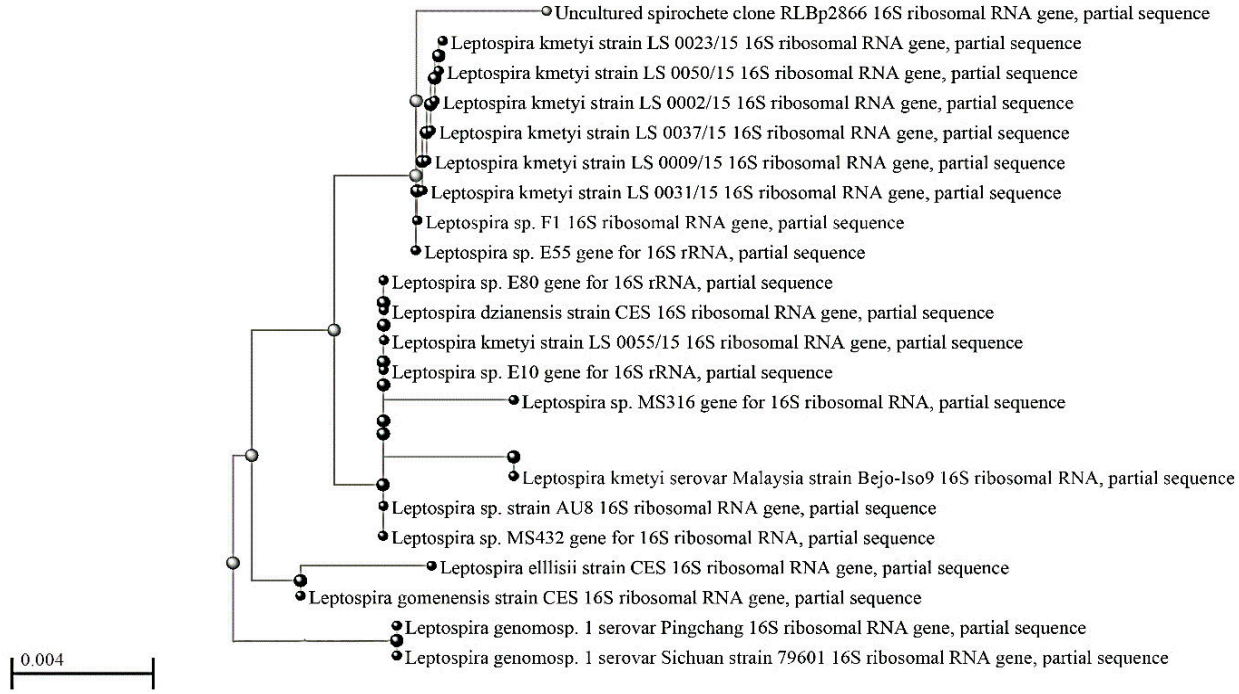
Discussion

It is common for people and animals to contract *Leptospira* spp. from a variety of wild and domestic animals and rodents in humid tropical, subtropical, or temperate climates. The Caspian Sea planes in northern Iran have a mild climate and humid conditions which are ideal for *Leptospira* infection. In addition, the environment is ripe for the outbreak of human leptospirosis, since many farmers grow rice and maintain livestock, horses, and dogs (Zamanmirabadi *et al*., 2022). During 1379, the Moghan agricultural and industrial sectors experienced an epidemic of this disease following heavy rains. While this city is quite similar to the northern provinces of Iran and is somewhat adjacent to Gilan province, it has a mild and humid climate, and on the other hand, the surveys were conducted during a time when it was extremely rainy. The outbreak of leptospirosis has occurred in this region, so we can conclude that it is associated with the milder weather and increased rainfall in this region. In a study on Urmia buffaloes, it was found that keeping these animals in swampy and humid areas increases the risk of disease transmission (Dalir-Naghadeh *et al*., 2006). An understanding of leptospirosis epidemiology can be




Leptospira genomosp. 1 serovar Pingchang 16S ribosomal RNA gene, partial sequence
Leptospira genomosp. 1 serovar Sichuan strain 79601 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate 01/095 16S ribosomal RNA gene, partial sequence
bacteria | 45 leaves
Leptospira interrogans isolate Dog 10 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate Dog 6 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate Dog 5 16S ribosomal RNA gene, partial sequence
bacteria | 4 leaves
Uncultured Leptospira sp. clone LPM-1 16S ribosomal RNA gene, partial sequence
Leptospira kirschneri isolate SLUSJ_176 16S ribosomal RNA gene, partial sequence
Leptospira kirschneri isolate H2 16S ribosomal RNA gene, complete sequence
Leptospira kirschneri serovar Grippotyphosa isolate UT130 16S ribosomal RNA gene, complete sequence
Leptospira kirschneri isolate H1 16S ribosomal RNA gene, complete sequence
0.006
Leptospira kirschneri serovar Grippotyphosa 16S ribosomal RNA gene, complete sequence
Leptospira kirschneri serovar Grippotyphosa strain Moskva-V 16S ribosomal RNA gene, partial sequence


Leptospira interrogans serovar Linhai str. 56609 chromosome 1, complete sequence
Leptospira interrogans strain UVH khor_sapy3 16S ribosomal RNA gene, partial sequence
Leptospira interrogans strain UVH khor_sapy1 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Canicola strain 782 chromosome 1, complete sequence
Leptospira interrogans isolate SLUSJ_160 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Bataviae strain 1548 chromosome, complete genome
Leptospira interrogans isolate SLUSJ_16 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate SLUSJ_2 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Manilae isolate POM23 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Manilae isolate POM20 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Manilae isolate POM18 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Manilae isolate POM07 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate SLUSJ_1 16S ribosomal RNA gene, partial sequence
Leptospira sp. strain EC3 16S ribosomal RNA gene, partial sequence
Leptospira interrogans isolate POM37 16S ribosomal RNA gene, partial sequence
Leptospira sp. C10 16S ribosomal RNA gene, partial sequence
Leptospira interrogans serovar Bratislava strain PigK151 chromosome I, complete sequence
0.009
Leptospira interrogans serovar Bataviae strain 1489 chromosome 1, complete sequence
Leptospira interrogans serovar Canicola strain 611 chromosome 1, complete sequence


Uncultured spirochete clone RLBp2866 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0023/15 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0050/15 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0002/15 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0037/15 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0009/15 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0031/15 16S ribosomal RNA gene, partial sequence
Leptospira sp. F1 16S ribosomal RNA gene, partial sequence
Leptospira sp. E55 gene for 16S rRNA, partial sequence
Leptospira sp. E80 gene for 16S rRNA, partial sequence
Leptospira dzianensis strain CES 16S ribosomal RNA gene, partial sequence
Leptospira kmetyi strain LS 0055/15 16S ribosomal RNA gene, partial sequence
Leptospira sp. E10 gene for 16S rRNA, partial sequence
Leptospira sp. MS316 gene for 16S ribosomal RNA, partial sequence
Leptospira kmetyi serovar Malaysia strain Bejo-Iso9 16S ribosomal RNA, partial sequence
Leptospira sp. strain AU8 16S ribosomal RNA gene, partial sequence
Leptospira sp. MS432 gene for 16S ribosomal RNA, partial sequence
Leptospira elllisii strain CES 16S ribosomal RNA gene, partial sequence
Leptospira gomenensis strain CES 16S ribosomal RNA gene, partial sequence
Leptospira genomosp. 1 serovar Pingchang 16S ribosomal RNA gene, partial sequence
0.004
Leptospira genomosp. 1 serovar Sichuan strain 79601 16S ribosomal RNA gene, partial sequence

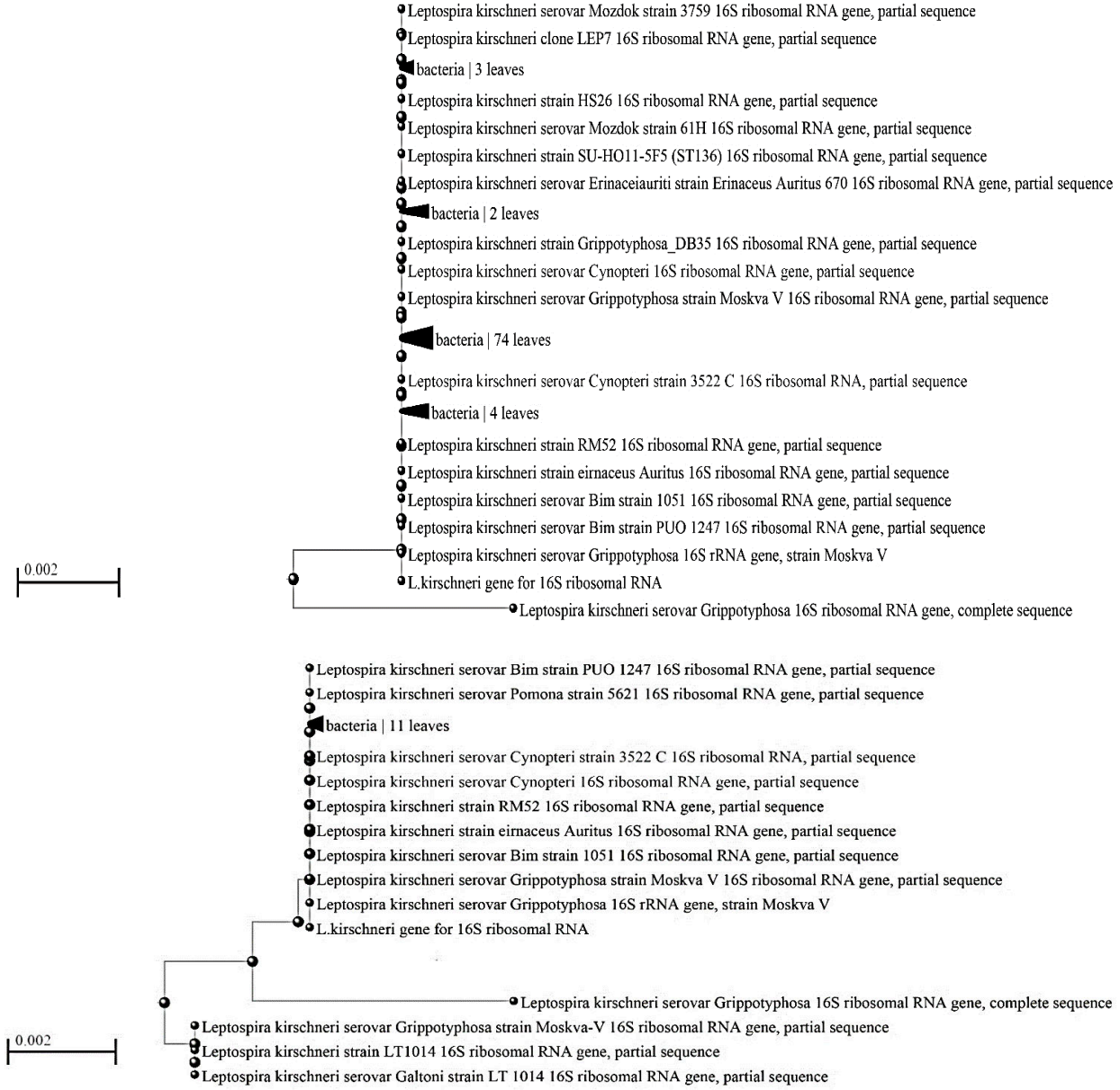


Fig 4: Data clustering in all four strains shows the differences and similarities between VNTR synonyms from the four main and sub-branches based on NJ analysis for *Leptospira* isolates

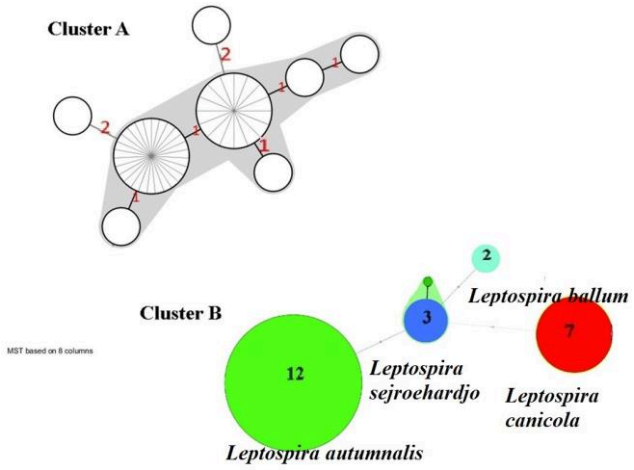


Fig. 5: Analysis of VNTR data using minimum spanning tree (MST) algorithm. **Cluster A:** Each circle represents a specific genotype. The gray shades around the circles represent clonal complexes. The numbers above the lines indicate how many loci are different between them. **Cluster B:** MST analysis of *Leptospira* strains in domestic animals. The circle size indicates how many strains are present and the color indicates which serogroups are present. A line between the two circles indicates the number of STs. In STs, shading connects them to the same clonal complex (CC)

gained by identifying common serovars and their host. Identification of indigenous serovars is important for preventive measures and leptospirosis control (Loffler *et al*., 2017). Based on our data, *Leptospira* Autumnalis was mainly found in horses, and the bacteria were shed in urine, suggesting that animals could transmit leptospirosis. *Leptospira* Autumnalis dominated at 50%, followed by *Leptospira* Canicola, Hardjo, and Ballum at

29.17%, 12.50%, and 8.33%, respectively. The maintenance hosts of Autumnalis and Ballum serovars are rodents, especially rats and bandicoots (Saravanan *et al*., 2000). In Japan, *L. interrogans* serovar Autumnalis isolated from humans, dogs and mice (Koizumi *et al*., 2020). One of the important reasons for the infection of horses with *L. interrogans* serovar Autumnalis is their improper storage where rodents can easily enter this place. The maintenance host of Canicola serovar is dog, although it has been isolated from cows, pigs and rodents. In 2009, serodiagnosis of leptospirosis in cattle in Gilan province revealed that *L. interrogans* serovar Canicola is the dominant serovar in this region (Abdollahpour *et al*., 2009).

There was no significant difference in contamination between the regions where blood samples were collected. The prevalence of leptospirosis was not statistically significant in relation to the type of animal (P>0.05). Horses were the most contaminated, followed by cattle and sheep. In this study, the MAT test was used as the gold standard method for the serological diagnosis of leptospirosis. There is a strong correlation between leptospirosis and a moderate climate in Iran; thus, this disease can be classified as a seasonal one. In Gilan, characterized by its humid temperature, *Leptospira* species can survive and remain infectious for several weeks in an environment where animals excrete bacteria. In 2016, it was found that 24.6% of incidences among livestock farms around Tehran were related to cows (Beer, 2016), which was consistent with our findings. According to the research conducted in 2013 on Urmia goats, female goats suffer from greater conflict than male goats, and their incidences are significantly different than those of male goats (Ramin and Azizzadeh, 2013). According to Kakita's report in Okinara, Japan, cats were more likely to have anti-leptospiral antibodies in rural areas (Kakita *et al*., 2021). In Okinawa, there are urban areas in the south, and rural areas in the north due to recreational activities in rivers. There is no regional difference in the risk of cats contracting Leptospiral infection in Okinawa (Kakita *et al*., 2021). Seven VNTR alleles were detected in our study, all located within two clonal complexes. Since the variety of *Leptospira* strains in Gilan province was relatively low, it appears that the clones causing disease in domestic animals are limited. *Leptospira* spp. has a low number of VNTR alleles due to its monophyletic nature. *Bacillus anthracis*, *Shigella Sonnei*, and *Salmonella enterica* serotypes are monophyletic bacteria, and therefore high-resolution methods are required for genotyping their strains. It should be noted that our study was conducted in a limited geographical area (Gilan province), while other studies collected samples from different geographic areas, which could be why our results differ from previous reports. The number of samples was small and loci with larger repeat sizes were used, resulting in a lower diversity, whereas in other studies loci with both large and small repeat sizes were used. This study indicates that *Leptospira* strains exhibit very little diversity. MLVA can replace expensive methods such as MLST, PFGE, and ribotyping. Since this method only requires a thermocycler and unlike other techniques, does not require expensive devices and equipment, it is apparent that it will be extremely helpful to microbiologists and epidemiologists in remote laboratories. The results of the NJ method were almost similar to the results of the MST method. Software search with the help of primers used in this study, for the amplification of VNTRLb5 and VNTR10 locus in 4 strains, was able to identify the position of the locus and had the most repetition among the 24 isolated *Leptospira* strains. Based on the drawing of the genotypic tree, the most similarity among the 4 *Leptospira* strains is in cluster A, and in cluster B, the relationship between several common markers and the distance between them shows the difference in the number of repetitions. This issue can indicate the occurrence of deletion of a part or parts of this locus in the genome of the studied strains. The occurrence of genetic deletion in VNTR loci is a phenomenon that has been previously observed and reported among many other bacteria. In the present study, it was shown that the using of MLVA technique can distinguish strains from each other with high speed and accuracy. Furthermore, due to the use of a serology-based leptospiral taxonomy system, several serovars belong to more than one genome and species; thus, it is doubtful that the MLVA typing described in this paper will be useful in determining the species genomes of these related serovars. It is necessary to compare the results of the MLVA technique with the results of one or more other techniques. As there are limited clones, it is necessary to collect more strains in different geographical areas and for longer periods of time in order to compare the results of the MLVA technique to those of other techniques. The MLVA technique should also be applied to bacteria that are hard to grow and high-risk, such as *Leptospira* strains.

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Conflict of interest

The authors declare no conflicts of interest.

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