# Identification of Leptospira in Patients With Renal Failure Using Serological, Molecular and Pathological Based Techniques, in Shiraz, Iran

**Mostafa Sayyadi**
Shiraz University
**Saeid Hosseinzadeh** ( hosseinzadeh@shirazu.ac.ir )
Institute of Public Health and Hygiene
**Gholamreza Abdollahpour**
University of Tehran
**Seyed Shahram Shekarforoush**
Shiraz University
**Azadeh Samiei**
Shiraz University
**Masoud Hosseinzadeh**
Shiraz Medical School: Shiraz University of Medical Sciences

**Research Article**

**Keywords:** Leptospira, Serum, Kidney, Nested-PCR, Warthin-Starry silver staining

**Posted Date:** January 6th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1149322/v1

# Abstract

Leptospirosis is a relatively rare bacterial infection that affects people and animals caused by pathogenic species of *Leptospira.* The present study was conducted using nested polymerase chain reaction (PCR), microscopic agglutination test (MAT) and hematological and biochemical tests on 200 blood samples of renal disorders patients in Shiraz, Iran. Also nested-PCR assay and Warthin-Starry (WS) silver staining method was performed on 30 nephrectomised kidney sample. The frequency of pathogenic species of *Leptospira* infection in patients with renal disorders was 20 % and this infection was significantly correlated with BUN, anemia, RDW, MCV, MCH and hemoglobin levels ($P < 0.01$). MAT analysis showed that serum samples had positive titers against *L. Grippotyphosa* (13 samples), *L. Ballum* (6 sample), *L. Pomona* (3 samples), *L. Canicola* (2 samples), *L. Icterohaemorrhagiae* (1 sample) and *L. Hardjo* (1 sample) serovars. Twenty-three percent of the kidney samples from the patients with pyelonephritis were infected with the pathogenic species of *Leptospira*. This study showed that pathogenic *Leptospira* serovars are present in this area and in patients with renal disorders more attention should be paid to this zoonotic disease.


# Highlights

- *Leptospira* infection in patients with renal disorders
- Using different techniques to identify human leptospirosis
- Optimization of nested polymerase chain reaction (PCR), microscopic agglutination (MAT) and hematological and biochemical tests to verify leptospiral infection
- Warthin-Starry silver staining as a specific staining method to detect Leptospira

# Introduction

*Leptospira* is consider as one of the causative agents of acute febrile infection both in human and animals. Urine of the clinically infected and carrier animals are the major sources of human infection (Roger et al., 2006). Currently, the genus *Leptospira* is divided into 35 distinct species classified into three phylogenetic clusters, the classification is which hypothetically associate with the pathogenicity of the bacteria (Thibeaux et al., 2018a; Thibeaux et al., 2018b; Vincent, 2019). *Leptospira* species are considered ubiquitous, as they are usually present in a wide variety of environments such as surface water, soil, with the wide host range including mammals but also in birds, amphibians, and reptiles (Dietrich, et al., 2015; Jobbins et al., 2015). According to the phase of infection, clinical signs of leptospirosis are varied from the acute icteric fatal infection to the chronic anicteric symptoms (Levett et al, 2001). In human, the disease is a career associated infection repeatedly occurred in farmers, veterinarian and abattoir workers (Thaipadungpanit et al., 2007; Sharma, 2008) with the major risk factors such as water sports and travel to the endemic countries (Victoriano et al., 2009) using nested PCR assay and restriction length polymorphism (RFLP) techniques revealed a good correlation between rejected kidneys and infection with *Leptospira*, in Shiraz slaughterhouse (Taghadosi et al., 2016). In Iran, the disease in human was firstly reported by Rafyi and Maghami in 1968. While, in the developed countries, *Leptospira* infection was not frequently associated with the acute renal failure (ARF) (Jayakumar et al., 2006; Regina et al., 2008).

The microscopic agglutination test (MAT) is frequently applied as a standard screening test all around the world (Regina et al., 2008; Abdollahpour et al., 2009). The standard MAT was established according to the reaction of the bacterial cell components and hyper immune antisera. A PCR assay using species-specific primers is also developed for the quick identification of leptospiral DNA (Rahelinirina et al., 2010; Thaipadungpanit et al., 2011; Vital-Brazil et al., 2012; Benacer et al., 2013; De Brito et al., 2018). Various diagnostic techniques such as direct culture, selective staining and immunohistochemistry (IHC) have been used to identify the microorganism in the tissues (Fonseca, et al., 2006;

Ooteman et al., 2006). Due to some limitations, culture of *Leptospira* is not always applicable (Obregón, et al., 2004; Rahim, 2005). Warthin-Starry (WS) silver staining has used to detect *Leptospira* and other spirochetes as a simple method in the tissues, thus, it could be applied in any laboratory (Fornazari, et al., 2012). The aim of this study was to find out whether the Leptospiral infection is a serious zoonotic disease in Shiraz, Southern Iran, using developed nested PCR, MAT and WS silver staining methods.

# Materials And Methods

### Sample collection and preparation

#### Step one

Total of 200 blood samples (113 male and 87 female) were collected from Nemazi Hospital, Shiraz, Iran. From which, 150 blood samples were taken from patients with a history and clinical symptoms suggestive of renal disorders, based on laboratory data including complete blood count (CBC), blood urea nitrogen (BUN), creatinine (Cr), total and direct bilirubin and urine analysis (WHO, 2003), the remaining 50 samples were belonged to healthy persons. The median age of the patients was 48.7 years (range = 13 -78 years). They were divided according to their age into three groups including $\leq 30$, 31 – 60, and $\geq 61$. Blood samples were subjected to various hematological and biochemical tests including hemoglobin, platelet, red cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit, red blood cell, serum creatinine and blood urea nitrogen. Some of the sera separated from blood samples were used for PCR and MAT tests.

#### Step two

In this step, 20 and 10 nephrectomised kidney samples were respectively taken from patients following surgical operation and the rejected transplant kidneys which were apparently healthy. The apparently normal kidneys were considered as negative control. All the samples were subjected to the PCR assays, pathological examination including WS staining.

### DNA extraction of serum and kidney samples

250 µl buffer I and buffer II containing RNase were initially added to the samples were pelleted. The mixture was then centrifuged at 10000 ×g for 10 min. A 550 µl saturated phenol was then added and pellets centrifuged at 10000 ×g for 5 min, the clear phase was then collected into new eppendorf tube. Previous stage was repeated twice to exclude cell debris. A 0.1 volume of 3 M sodium acetate was then added to the preparations, washed twice by absolute and 80 % ethanol, respectively. The tubes were centrifuged at 12000 ×g for 15 min., the final pellet was dried at room temperature and re-suspended in 30 µl TE buffer, kept at – 20 °C until further use.

### Conventional and nested PCR to amplify 16s rRNA gene in the samples

A conventional PCR assay was used to differentiate pathogenic and saprophyte species of *Leptospira* using separate specific primers (Table 1). The PCR was conducted using a 25 µl reaction mixture consisted of 2.5 µl of 10× PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 25 mM $MgCl_2$, 1.0 U of Taq DNA polymerase, 1 µl (25 pmol) of each primer and 50 ng of template DNA. Particular region of 16s rRNA gene of *Leptospira* spp was selected to design the primers (Table 1). All the pathogenic and non-pathogenic species of *Leptospira* were identified using a species-specific nested PCR assay. The second amplification (nested-2) was amplified a 289 bp region within the 525 bp sequence from 16s rRNA gene. 1.5 µl of DNA was used to amplify both primary and secondary PCR amplification reactions in both reactions. The reaction was performed in a total volume of 25 µl of reaction mixture containing 1 mmol/L $MgCl_2$, 1mmol/ml

Deoxyribonucleotide triphosphate (dNTP) mixture (Sinagen, Iran) and 5 U/µl of Taq polymerase for both nested PCR reactions. The PCR reaction was conducted in a volume of 25 uL. A cycle was started with initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, extension at 72 °C for 1.5 min and a final extension at 72 °C for 15 min. The conditions for secondary reaction were the same except for the annealing at 59.5 °C for 1 min, extension time for 1 min and final extension time for 10 min. The amplified DNA fragments were run on 2% agarose gels electrophoresis stained with Safe mode staining and visualized on an ultraviolet transilluminator.

**Table 1. List of primers employed in this study.**

| Ref. | Sequences (5´– 3´) | Primers |
|---|---|---|
| Djadid et al., 2009 | F: 5´-GGCGGCGCGTCTTAAACATG-3´ | Primary |
| | R: 5´-GTCCGCCTACGCACCCTTTACG-3´ | |
| Djadid et al., 2009 | F: 5´-CAAGTCAAGCGGAGTAGCAA-3´ | Nested |
| | R: 5´-CTTAACCTGCTGCCTCCCGTA-3´ | |
| Bexfield et al., 2011 | F: 5´-TGAGTAACACGTGGGTAATCTTCC-3´ | Pathogen |
| | R: 5´-CAGGTACCATCATCACATCGCTGC-3´ | |

### MAT

MAT has been widely used as the reference test for antibody detection. This test was performed in the *Leptospira* Research Laboratory (http://leptolab.ut.ac.ir/en.htm) of Veterinary Research and Teaching Hospital Faculty of Veterinary Medicine at the University of Tehran, on serum samples that had been stored at -20 °C. To perform the test, a 7-10 days culture of *L. interrogans* in liquid medium (GRA-Sina) was used as antigen. A counting chamber (Petroff-Hauser USA) was used to adjust the organism to $2 \times 10^8$ *leptospires*/mL. The leptospiral standard antigen was prepared from six reference strains of *Leptospira interrogans* including *Hardjo, Pomona*, *Icterohaemorrhagiae, Grippotyphosa, Canicola* and *Ballum*. Serum samples were then serially diluted in phosphate buffer solution (PBS) in a microtiter plate (Greiner Bio-One, Kremsmünster, Austria). Recent infection is confirmed by very high antibody titers (≥ 400), but paired serum titers produce more reliable prognostic information. A current leptospirosis infection is suggested by a 4-fold rise in titer, or a seroconversion to ≥ 400. Numerous low-level titers most likely to indicate vaccination reactions, however this test should not be used to monitor protective vaccination status.

### WS staining method

The staining method was performed using the WS stain kit (Asia Chem. Co., Tehran, Iran) as was described by the manufacturer. Briefly, the kidney sections were deparaffinised and rehydrated using milli-Q distilled water followed by immersing in a 1% silver nitrate solution incubated at 43 °C for 30 min. The sections were subsequently rinsed in distilled water and flooded in a fresh developer solution until turn to light brownish yellow, washed in hot water (56 °C) rinsed in distilled water, dehydrated, clean and mounted. The stain sections were finally examined under light microscopy (Mag. X 1000).

### Statistical analysis

Spearman's rho correlation coefficient test was applied to assess the correlation between variables and chi square test (or Fisher's exact test) was used to analyze data using SPSS (version 18.0 for Windows, SPSS, Inc., Chicago, IL, USA) software. P values below 0.05 were considered as significant.

# Results

### Nested PCR analysis of clinical samples in suspected cases

The nested PCR assay was confirmed that 30 out of 150 samples (20 %) were infected to the DNA of pathogenic species of *Leptospira* (Table 2). Seventeen out of 30 (56.7 %) positive patients by PCR were men. The ages of 31 to 60 years were the most affected age groups, though this was not statistically significant between men and women (P = 0.153) (Table 2 and 3). The pathogenic species of *Leptospira* were differentiated according to the size of DNA amplicon (Figure 1). To develop a sensitive and specific PCR assay for detection of all pathogenic and nonpathogenic *Leptospira* spp., two pair of primers were employed (Table 1). These primers were designed based on a particular region of 16S rRNA gene of *Leptospira* spp (Fornazari et al., 2012). According to the clinical pathological findings, 28.5 % of patients showed anemia. The mean level of serum creatinine and blood urea nitrogen of positive patients were 3.6 and 46.9 mg/dl, respectively (Table 3). All the negative samples collected from healthy persons were shown negative PCR.

**Table 2. Nested PCR results of Pathogenic *Leptospira* infections in different sex and age groups of patients with renal function disorders.**

| Age groups | Sex | No. of blood samples | Pathogenic *Leptospira* species infections (by nested PCR assay) | | P value |
|---|---|---|---|---|---|
| | | | No. | % | |
| ≤ 30 | Male | 17 | 4 | 23.5 | 0.673 |
| | Female | 13 | 2 | 15.4 | |
| 31-60 | Male | 41 | 10 | 24.4 | 1 |
| | Female | 39 | 10 | 25.6 | |
| ≥ 61 | Male | 30 | 3 | 10.0 | 1 |
| | Female | 10 | 1 | 10.0 | |
| Total | Male | 88 | 17 | 19.3 | 0.838 |
| | Female | 62 | 13 | 21.0 | |

There was no significant difference between the different age groups (P = 0.153)

**Table 3. Mean values of serum creatinine and BUN levels in the *Leptospira* positive and negative blood samples in the patients with renal disorders.**

| Factors | Pathogenic *Leptospira* species infections (by nested PCR assay) | | P value |
|---|---|---|---|
| | Negative (n = 120) | Positive (n = 30) | |
| Creatinine ± S.D. (mg/dl) | 2.5 ± 2.2 | 3.6 ± 2.0 | 0.257 |
| Blood urea nitrogen ± S.D. (mg/dl) | 33.5 ± 18.3 | 46.9 ± 22.8 | < 0.001 |

### Laboratory findings of blood samples

The ratio of hemoglobin in the positive PCR cases were significantly lower than the normal values ($P < 0.001$). Moreover, RDW was higher in all positive PCR patients ($P < 0.001$). A marked anemia was shown in the pathogenic strains PCR positives ($P < 0.001$) (Table 4). Microcytic, normocytic and macrocytic anemia were respectively shown in 56.7 %, 33.3 % and 10 % of the anemic patients.

**Table 4. Correlation between some blood parameters and pathogenic *Leptospira* infections detected by nested PCR.**

| Parameters | Criteria | No. | Pathogenic *Leptospira* species infections | | Correlation coefficient | P value |
|---|---|---|---|---|---|---|
| | | | No. | % | | |
| Anemia | + | 93 | 30 | 32.3 | 0.391 | < 0.001 |
| | - | 57 | 0 | 0.0 | | |
| Hemoglobin (g/L) | N (98 -160) | 55 | 0 | 0.0 | 0.371 | < 0.001 |
| | L (< 98) | 94 | 30 | 31.9 | | |
| | H (> 160) | 1 | 0 | 0.0 | | |
| Platelet ($\times 10^9$/L) | N (89-380) | 129 | 25 | 19.4 | 0.039 | 0.636 |
| | L (<98) | 13 | 3 | 23.1 | | |
| | H (>380) | 8 | 2 | 25.0 | | |
| RDW (%) | N (11.5-16.7) | 99 | 1 | 1.0 | 0.679 | < 0.001 |
| | L (<11.5) | 3 | 0 | 0.0 | | |
| | H (>16.7) | 48 | 29 | 60.4 | | |
| MCV (fl) | N (72-97) | 121 | 20 | 16.5 | 0.185 | 0.024 |
| | L (<72) | 26 | 8 | 30.8 | | |
| | H (>97) | 3 | 2 | 66.7 | | |
| MCH (pg) | N (22.6-33.5) | 76 | 8 | 10.5 | 0.229 | 0.005 |
| | L (<22.6) | 72 | 22 | 30.6 | | |
| | H (>33.5) | 2 | 0 | 0.0 | | |
| MCHC (g/dL) | N (30.5-36.2) | 64 | 9 | 14.1 | 0.128 | 0.118 |
| | L (<30.5) | 86 | 21 | 24.4 | | |
| | H (>36.2) | 0 | 0 | 0.0 | | |
| Hematocrit (%) | N (28.9- | 62 | 15 | 24.2 | -0.088 | 0.284 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 48.7) | | | | | |
| | L (<28.9) | 88 | 15 | 17.0 | | |
| | H (>48.7) | 0 | 0 | 0.0 | | |
| RBC ($\times 10^{12}$) | N (3.39-5.83) | 78 | 16 | 20.5 | -0.016 | 0.842 |
| | L (<3.39) | 71 | 14 | 19.7 | | |
| | H (>5.83) | 1 | 0 | 0.0 | | |

N: normal, H: high, L: low, RDW: Red Cell Distribution Width, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration. Hematology reference values are taken from Dosoo et al., 2012

### Laboratory findings of kidney samples

Thirty kidney sample biopsies, were taken from Pathology Lab., Nemazi Hospital, Shiraz University of Medical School. 60 % and 40 % of the patients were respectively males and females with the average of 40 years old. The incorporated kidney lesions were hyperemia, petechial hemorrhage, urinary cysts, shrinkage, pathological congestion, hydronephrosis and paleness, change in color and consistency and blood accumulation in the pelvis (Table 5). Three of the 20 kidney samples of patients using nested PCR assay were positive for the pathogenic species of *Leptospira* (15%) (Figure 1) and all 10 samples of control group were negative. Positive samples were belonged to the male patients over 40 years old. No association was observed between kidney lesions and presence of leptospiral DNA. The *Leptospira* pathogenic species were confirmed in all the nested PCR positive kidney samples, however, no evidence of infection with the saprophytic species was observed.

**Table 5. Association between kidneys lesions and *Leptospira* positive and negative samples.**

| Pathological lesions | Number | % | pathogenic species of *Leptospira* (using the nested PCR assay) + | - |
|---|---|---|---|---|
| Pyelonephritis | 13 | 43.3 | 3 | 10 |
| Cyst | 2 | 6.7 | 0 | 2 |
| Tumor | 15 | 50.0 | 0 | 15 |
| Total | 30 | 100 | 3 | 27 |

### MAT

The MAT was conducted in 50 blood samples that were available. The results of this test showed that 26 samples had positive reactions against different serovar of *Leptospira interrogans* as shown in Table 6 and 7. According to these results, positive titers were reported against *L. Grippotyphosa* (13 samples), *L. Ballum* (6 sample), *L. Pomona* (3 samples), *L. Canicola* (2 samples), *L. Icterohaemorrhagiae* (1 sample) and *L. Hardjo* (1 sample) serovars.

**Table 6. Number and frequency (%) of positive different *Leptospira* serovars by MAT.**

| Sex | No. of serum samples | Leptospira serovars | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Grippotyphosa* | *Ballum* | *Pomona* | *Canicola* | *Icterohaemorrhagiae* | *Hardjo* | Total |
| Male | 25 | 8 (32 %) | 3 (12 %) | 2 (8 %) | 2 (8 %) | 1 (4 %) | 1 (4 %) | 17 (68%) |
| Female | 25 | 5 (20 %) | 3 (12 %) | 1 (4 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) | 9 (36%) |
| Total | 50 | 13 (26 %) | 6 (12 %) | 3 (6 %) | 2 (4 %) | 1 (1 %) | 1 (1 %) | 26 (52%) |

**Table 7. Number and frequency (%) of serum samples with positive titer against each serovar, at different dilution.**

| | Serum dilutions | | |
|---|---|---|---|
| Serovar | >1: 100 | 1:100 | 1: 200 |
| *Grippotyphosa* | 10 (34.5) | 10 (34.5) | 3 (10.3) |
| *Pomona* | 1 (3.4) | 2 (6.9) | 1 (3.4) |
| *Icterohaemorrhagiae* | 0 (0.0) | 0 (0.0) | 1 (3.4) |
| *Canicola* | 1 (3.4) | 1 (3.4) | 1 (3.4) |
| *Ballum* | 1 (3.4) | 3 (10.3) | 3 (10.3) |
| *Hardjo* | 0 (0.0) | 1 (3.4) | 0 (0.0) |

### WS staining

*Leptospira* was observed in 2 of the 20 biopsy specimens of the patients (10%) by WS staining. All two positive samples in the nested PCR assay were also positive. *Leptospira* was not observed in any of the kidney biopsies of the control group. The spiral or filiform objects in the lumens of cortical tubules are suggesting the presence of *Spirochaetes* (Figure 2).

## Discussion

Leptospirosis is a widespread zoonotic infection worldwide, especially in the tropical and subtropical regions with the warm and humid climate which make favorite conditions for the survival of microorganism. Based on the information given by WHO, the infection is considered as a neglected disease with the estimation median of 1.5 cases per 100,000 each year in the endemic areas (WHO, 2003; Lau et al., 2012). Similar to other regions, the incidence of human leptospirosis is not well documented in the Middle East. Besides, the underestimating incidence, the complexity of diagnosis of infection is problematic (Murdoch et al, 2004). Little information is currently available on the occurrence of infection in human, in south of Iran. The present study was the first molecular, serological and pathological work focused on the human leptospirosis in our country. Surprisingly, the disease was confirmed despite of the dry and hot climate summer with average temperature of 28.1 °C (19.3 to 37.0 °C), and cold winter with the average temperature of 6.0 °C (- 0.3 to 12.4 °C) in this geographical area. The recently developed nested PCR, MAT methods and WS silver staining were employed. This study was also showed a numerical relation of the most affected age group which was between the ages of 31 to 60 years in both genders. A comparison between the occurrences of infection in different age groups in the former studies showed a higher prevalence in the patients above 50 years old (Honarmand, 2009). A higher rate of occurrence in Iran was also confirmed in the ages above 40 years old (Esmaeili et al., 2009). Nested PCR confirmed that 30 (20 %) of 150 serum samples infected to the *Leptospira* infections. Additionally, the conventional

PCR assay was approved that all positive cases had a pathogenic *Leptospira* species. The sensitivity of nested PCR to identify *Leptospira* spp. was approved by Djadid et al. (2009) and Ahmed et al. (2012). The primers were designed based on 16S rDNA gene sequence, which is able to identify all pathogenic and non-pathogenic species of *Leptospira*, to detect 1 to 2 pg of DNA in a sample (Djadid et al. 2009). The ratio of hemoglobin in the positive cases were significantly lower than the normal values (P = 0.05). Moreover, RDW was higher in all positive PCR patients (P < 0.001). RDW is an approved index to assess anemia. Increase in the proportion of large, young cells with older and smaller red blood cells should directly associate with anemia. In the immune-mediated hemolytic anemia there are often immature cells mixed with normal ones. The RDW should be particularly high with immune-mediated hemolytic anemia (IMHA) where there are often immature cells mixed with cells that are small due to losing pieces of their membranes (spherocytes). Unfortunately, the RDW does not always reflect the degree of anisocytosis seen on peripheral blood examination, which emphasizes the importance of smear evaluation (Stockham, and Scott, 2013). A marked anemia was shown in the pathogenic strains PCR positives patients (P < 0.001). Low serum creatinine, severe anemia and low hematocrit values were observed in acute form of leptospirosis (Reis et al., 2013). In our study, the most prevalent species was *L. grippotyphosa* and the least prevalent were *L. hardjo* and *L. icterohaemorrhagiae*. The results from 261 suspected cases tested by different tests including PCR, MAT, ELISA on 261 suspected cases have shown 171 (65.5 %) positive outcomes using MAT, 185 (70.8 %), IgM-ELISA, 200 (76.6 %), IgG-ELISA and 244 (93.8 %) by PCR( Natarajaseenivasan et al., 2011).-Moreover, positive titers of leptospiral antibody in 26.3 % of patients were detected by MAT. Javid et al. (2012) reported serovar *L. Icterohaemorrhagiae* (31 samples), *L. Hardjo* (26 samples), *L. Grippotyphosa* (7 samples), *L. Pomona* (5 samples), *L. Canicola* (4 samples) and *L. Ballum* (2 sample). The overall incidence in the Northeast province of Iran were 27.29 % and 28 % in men and women, respectively (Sakhaee et al., 2010). MAT assay was employed to confirm *leptospira* in 400 samples, 194 (48.5 %) were found positive, of which, the highest and lowest titers were respectively recorded against *L. gripotyphosa* and *L. hardjo*. In addition, a higher prevalence of infection in women was noticeable (Ebrahimi et al., 2015). Occurrence of various leptospira Spp. in different climates of Iran is mainly associated with the presence of appropriate reservoir, sensitivity of hosts and methods of leptospira surveillances in our country. Survival of microorganism in wet areas made it endemic in the north of the country (Ko et al., 2009; Sakhaee and Abdollahpour, 2011). Here, we have also shown the occurrence of infection in the dried and semi-dried regions which confirmed that the epidemic is less dependent on the ambient humidity. The formalin-fixed tissues was employed to apply on the WS staining. The effectiveness of WS for detecting the microorganism have been already approved by some previous studies (Sebastian et al., 2005; Léon et al., 2006; Ortega-Pacheco et al., 2008). However, the positive cases confirmed by WS technique, were fewer than PCR. The low efficacy of WS to detect *Leptospira* was possibly associated with low burden of microorganism in tissues. Un-specified staining of reticulin fibers to mis-interpreting results of WS makes it problematic to properly differentiate the infection (Szeredi and Haake, 2006). In the current study, pyelonephritis was more prevalent than other lesions because the microorganism is a nephrophilic and can generate many renal lesions (Visith and Kearkiat 2005). White spotted lesions are repeatedly observed as an important signs of chronic leptospirosis, whilst, inspection of 329 healthy cattle's kidneys, showed that both Leptospira spp. and active infection caused by other bacteria are associated with the "white spotted kidneys" (Uzal et al. 2002a; Uzal et al. 2002b).

## Conclusion

The clinical, serological and epidemiological findings from our studies in Fars province, Iran were implied on the presence of pathogenic species in human. However, overall information on the dynamic of transmission of *leptospira* was suggesting an emerge focus on the occurrence of disease especially in the global travelers. The results of present study highlight the urgent need to achieve further details about the possible role of the bacteria to induce renal failure in various climate conditions, in Iran.

# Declarations

**Funding:** Not applicable.

**Competing Interests:** The authors declare no financial and non-financial competing interests.

### Ethic approval

These experiments were permitted by the members of state ethic committee, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). It is confirming that informed consent was obtained from all subjects.

**Availability of data:** Data produced during this study are available from corresponding author upon reasonable request.

**Code availability:** Not applicable

**Conflict of interest:** Authors declare that they have no conflict of interest

### Author Contributions

S.S.S., S.H. designed the experiments and performed the statistical analysis, M.S. wrote the drafts of manuscript and performed PCR assay, G.A. performed the serological tests, M.H. performed the pathological experiments and A.S. revised the manuscript and checked the English writing of final version. All authors reviewed the article.

### Acknowledgments

We would like to thank all the staffs at the Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University. This research was supported by Shiraz University, Shiraz, Iran, which is gratefully appreciated.

# References

Abdollahpour, G., Shafighi, S.T., Sattari Tabrizi, S., 2009. Serodiagnosis of Leptospirosis in cattle in north of Iran, Guilan. Iran. J. Vet. Res. 3, 7-10.20. http://10.22059/ijvm.2009.19603.

Ahmed, A., Grobusch, M.P., Klatser, P.R., Hartskeerl, R.A., 2012. Molecular approaches in the detection and characterization of *Leptospira*. J Bacteriol. Parasitol. 3(2), p.e133. doi: 10.4172/2155-9597.1000133.

Benacer, D., Woh, P. Y., Mohd Zain, S. N., Amran, F., Thong, K. L., 2013. Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in peninsular Malaysia. Microbes. Environ. 28(1). 135-140. http:// 10.1264/jsme2.me12154.

Bexfield, N.H., Andres-Abdo, C., Scase, T.J., Constantino-Casas, F., Watson, P.J., 2011. Chronic hepatitis in the English springer spaniel: clinical presentation, histological description and outcome. Vet. Record. 169(16), pp.415-415. https://doi.org/10.1136/vr.d4665

De Brito, T., Silva, A.M.G.D., Abreu, P.A.E., 2018. Pathology and pathogenesis of human leptospirosis: a commented review. Revista do Instituto de Medicina Tropical de São Paulo. 60. https://doi.org/10.1590/s1678-9946201860023.

Dietrich, M., Mühldorfer, K., Tortosa, P., Markotter, W., 2015. *Leptospira* and bats: story of an emerging friendship. PLoS Pathog. 11(e1005176). https://doi.org/10.1371/journal.ppat.1005176. PMID: 26562435.

Djadid, N.D., Ganji, Z.F., Gouya, M.M., Rezvani, M., Zakeri, S., 2009. A simple and rapid nested polymerase chain reaction–restriction fragment length polymorphism technique for differentiation of pathogenic and nonpathogenic *Leptospira* spp. Diagn. Micr. Infec. Dis. 63, 251–256. doi: 10.1016/j.diagmicrobio.2008.10.017.

Dosoo, D.K., Kayan, K., Adu-Gyasi, D., Kwara, E., Ocran, J., Osei-Kwakye, K., Mahama, E., Amenga-Etego, S., Bilson, P., Asante, K.P., Kor Yousefi am, K.A., 2012. Haematological and biochemical reference values for healthy adults in the middle belt of Ghana. PloS one. 7(4), e36308.

Ebrahimi, A., Alijani, L., Abdollahpour, G. R., 2015. Serological survey of human *leptospirosis* in tribal areas of west central Iran. Iran. J. Medic. Sci., 28(2), 93-95.

Esmaeili, R., Hesamzadeh, A., Navaei, R.A., Haghshenas, M.H., 2009. Incidence of leptospirosis in Mazandaran province, North of Iran: A one year survey. Pak. J. Biol. Sci. 12: 1330-1333. http//: 10.3923/pjbs.2009.1330.1333.

Fonseca, C.A., Teixeira, M. M., Romero, E.C., Tengan, F.M., Silva, M.V., Shikanai-Yasuda M.A., 2006. *Leptospira* DNA detection for the diagnosis of human *leptospirosis*. J. Infec., 52(1): 15-22. https://doi.org/10.1111/j.1365-3156.2006.01727.x.

Fornazari, F., Da Silva, R.C., Richini-Pereira, V.B., Beserra, H.E., Luvizotto, M.C., Langoni, H., 2012. Comparison of conventional PCR, quantitative PCR, bacteriological culture and the Warthin Starry technique to detect *Leptospira* spp. in kidney and liver samples from naturally infected sheep from Brazil'. J. Microbiol. Methods. 90, 321–326. http://dx.doi.org/10.1016/j.mimet.2012.06.005.

Honarmand, H., 2009. A decade, the incidence of *leptospirosis* in Guilan. Iran. J. Infec. Dis., 47: 47-53. (Abstract in English)

Javid, N., Dadgar, T., Khodabakhshi, B., Bazouri, M., Sedaghat, M., Bakhshandeh-nosrat, S., Rahimi, S., Allah Ghaemi, E., 2012. Seroepidemiology of anti-leptospira antibody in Golestan province, north of Iran. Int. J. Mol. Clin. Microbiol. 2, 124-127. http://www.ijmcm.ir/article_513250.html

Jayakumar, M., Ram Prabahar, M., Fernando, E.M., Manorajan, R., Venkatraman, R., Balaraman, V., 2006. Epidemiologic trend changes in acute renal failure—a tertiary center experience from South India. Ren. Fail. 28:405–410. https://doi.org/10.1080/08860220600689034.

Jobbins, S.E., Alexander, K.A., 2015. Evidence of *Leptospira* sp. infection among a diversity of African wildlife species: beyond the usual suspects. Trans R Soc Trop Med Hyg. 109:349–51. https://doi.org/10. 1093/trstmh/trv007. PMID: 25669841.

Ko, A.I., Goarant, C., Picardeau, M., 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat. Rev. Microbiol. 7(10), pp.736-747. doi: 10.1038/nrmicro2208.

Lau, CL., Dobson, A., Smythe, L., Fearnley, E., Skelly, C., Clement, S A., 2012. *Leptospirosis* in American Samoa 2010—epidemiology, environmental drivers, and the management of emergence. Am. J. Trop. Med. Hyg. 86:309–19. https://doi.org/10.4269/ajtmh.2012.11-0398.

Léon, A., Pronost, S., Tapprest, J., Foucher, N., Blanchard, B., André-Fontaine, G., Laugier, C., Fortier, G., Leclercq, R., 2006. Identification of pathogenic *Leptospira* strains in tissues of a premature foal by use of polymerase chain reaction analysis. J. Vet. Diagn. Invest. 18, 218–221. http://dx.doi.org/10.1177/104063870601800216.

Levett, P.N., 2001. *Leptospirosis*. Clin. Microbiol. Rev. 14:296–326. 10.1128/CMR.14.2.296-326.2001.

Murdoch, D.R., Woods, C.W., Zimmerman, M.D., Dull, P.M., Belbase, R.H., Keenan, A.J., Scott, R.M., Basnyat, B., Archibald, L.K., Reller L.B., 2004. The etiology of febrile illness in adults presenting to Patan Hospital in Kathmandu. Nepal. Am. J. Trop. Med. Hyg. 70: 670-675. https://doi.org/10.4269/ajtmh.2004.70.670.

Natarajaseenivasan, K., Raja, V., Narayanan, R., 2011. Rapid diagnosis of *leptospirosis* in patients with different clinical manifestations by 16S rRNA gene based nested PCR. Saudi J. Biol. Sci., 19:151-155. https://doi.org/10.1016/j.sjbs.2011.11.005.

Obregón, A. M., Fernández, C., Rodríguez, I., Balbis, Y., Martínez, B., Rodríguez, J., 2004. Latex agglutination system for the rapid diagnosis of leptospirosis in Cuba. Rev. Panam. Salud. Pública. 16(4), 259-265 http:// 10.1590/s1020-49892004001000005.

Ooteman, M.C., Vago, A.R., Koury, M.C., 2006. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J. Microbiol. Methods. 65: 247-57. https://doi.org/10.1016/j.mimet.2005.07.015.

Ortega-Pacheco, A., Colin-Flores, R.F., Gutiérrez-Blanco, E., Jiménez-Coello, M., 2008. Frequency and type of renal lesions in dogs naturally infected with *Leptospira* species', Ann. NU. Acad. Sci., 1149, 270–274. http://dx.doi.org/10.1196/annals.1428.088.

Rafyi, A., Magami, G.H., 1968. *Leptospirose* ovine et caprine. Arch. Inst. Razi. 20: 2 –38.

Rahelinirina, S., Léon, A., Harstskeerl, R.A., Sertour, N., Ahmed, A., Raharimanana, C., Ferquel, E., Garnier, M., Chartier, L., Duplantier, J.M., Rahalison, L., 2010. First isolation and direct evidence for the existence of large small-mammal reservoirs of *Leptospira* sp. in Madagascar. PLoS One. 5:e14111. https://doi.org/10.1371/journal.pone.0014111.

Rahim, H., Gorbanpour, M., Haidari, M., 2005. 'Comparison of *leptospiral* infection in the horse and donkey'. B. Vet. Pulawy. 49, 175–178.

Regina, C., Abdulkader, M., Silva, M. V., 2008. The kidney in *leptospirosis*. Pediatr. Nephrol. 23(12), 2111 9. https://link.springer.com/article/10.1007/s00467-008-0811-4

Reis, E. A. G., Hagan, J. E., Ribeiro, G. S., Teixeira-Carvalho, A., Martins-Filho, O. A., Montgomery, R. R., Shaw, A. C., Ko, A. I., Reis, M. G., 2013. Cytokine response signatures in disease progression and development of severe clinical outcomes for leptospirosis. PLoS Negl. Trop. Dis. 7:e2457. https://doi.org/10.1371/journal.pntd.0002457.

Roger, E. Morey, R. L., Galloway, S. L., Bragg, A.G., Steigerwalt, Mayer, L., Levett, P. N., 2006. Species-specific identification of *leptospiraceae* by 16S rRNA gene sequencing. J. Clin. Microbiol. 44(10):3415-3416. http:// 10.1128/JCM.00670-06.

Sakhaee, E., Abdollahpour, G., 2011. Detection of Leptospiral antibodies by microscopic agglutination test in north–east of Iran. Asian Pac. J. Trop. Biomed. 1(3), pp.227-229. https://doi.org/10.1016/S2221-1691(11)60032-4

Sakhaee, E., Abdollahpour, G., Bolourchi, M., Sattari Tabrizi, S., 2010. Comparison between microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) for detection of leptospiral antibodies in cattle. Comp. Clin. Pathol. 19, 5-9. http:// 10.1007/s00580-009-0907-7.

Sebastian, M., Giles, R., Roberts, J., Poonacha, K., Harrison, L., Donahue, J., Benirschke, K., 2005. Funisitis associated with leptospiral abortion in an equine placenta. Vet. Pathol. 42, 659–662. http://dx.doi.org/10.1354/vp.42-5-659.

Sharma, M., 2008. *Leptospirosis*: epidemiology, diagnosis, and control. J. Infect. Dis. Antimicrob. Agents. 25:93–103.

Stockham, S. L., Scott, M. A., 2013. Fundamentals of Veterinary Clinical Pathology. John Wiley & Sons, London, 2nd edition, 120-123.

Szeredi, L., Haake, D.A., 2006. Immunohistochemical identification and pathologic findings in natural cases of equine abortion caused by leptospiral infection. Vet. Pathol. 43(5), 755–761. https://doi.org/10.1354/vp.43-5-755.

Taghadosi, V., Hosseinzadeh, S., Shekarforoush, S.S., Samiei, A., 2016. Prevalence of renal lesions in slaughtered cattle in Shiraz. Trop. Anim. Health Prod. 48: 1961. DOI: 10.1007/s11250-016-1145-z.

Thaipadungpanit, J., Wuthiekanun, V., Chierakul, W., Smythe, L.D., Petkanchanapong, W., Limpaiboon, R., Apiwatanaporn, A., Slack, A.T., Suputtamongkol, Y., White, N.J., Feil, E.J., 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. PLoS Negl. Trop. Dis. 1:e56. https://doi.org/10.1371/journal.pntd.0000056.

Thaipadunpanit, J., Chierakul, W., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Boonslip, S., Smythe, L.D., Limpaiboon, R., Hoffmaster, A.R., Day, N.P., Peacock, S.J., 2011. Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: a case-control study. PLoS ONE. 6:e16236. https://doi.org/10.1371/journal.pone.0016236.

Thibeaux, R., Girault, D., Bierque, E., Soupé-Gilbert, M.E., Rettinger, A., Douyère, A., Meyer, M., Iraola, G., Picardeau, M., Goarant, C., 2018a. Biodiversity of environmental *Leptospira*: improving identification and revisiting the diagnosis. Front Microbiol. 9:816. https://doi.org/10.3389/fmicb.2018.00816.

Thibeaux, R., Iraola, G., Ferrés, I., Bierque, E., Girault, D., Soupé-Gilbert, M.E., Picardeau, M., Goarant, C., 2018b. Deciphering the unexplored *Leptospira* diversity from soils uncovers genomic evolution to virulence. Microb Genom. https://doi: 10.1099/mgen.0.000144.

Uzal F.A., Dobrenov B., Smythe L., Norris M., Dohnt M., Symonds M., O'Boyle D., Schouten F., Kelly W.R., 2002a. A study of white spotted kidneys^ in cattle. Vet. Microbiol. 86, 369–375 https://doi.org/10.1016/S0378-1135(02)00021-4.

Uzal F.A., More S.J., Dobrenov B., Kelly W.R., 2002b. Assessment of organoleptic postmortem inspection techniques for bovine offal. Aust. Vet. J. 80, 70–74. https://doi.org/10.1111/j.1751-0813.2002.tb12841.x.

Victoriano, A.F.B., Smythe, L.D., Gloriani-Barzaga, N., Cavinta, L.L., Kasai, T., Limpakarnjanarat, K., Ong, B.L., Gongal, G., Hall, J., Coulombe, C.A., Yanagihara, Y., 2009. Leptospirosis in the Asia Pacific region. BMC Infect. Dis. 9:147–156. https://bmcinfectdis.biomedcentral.com/articles/10.1186/1471-2334-9-147.

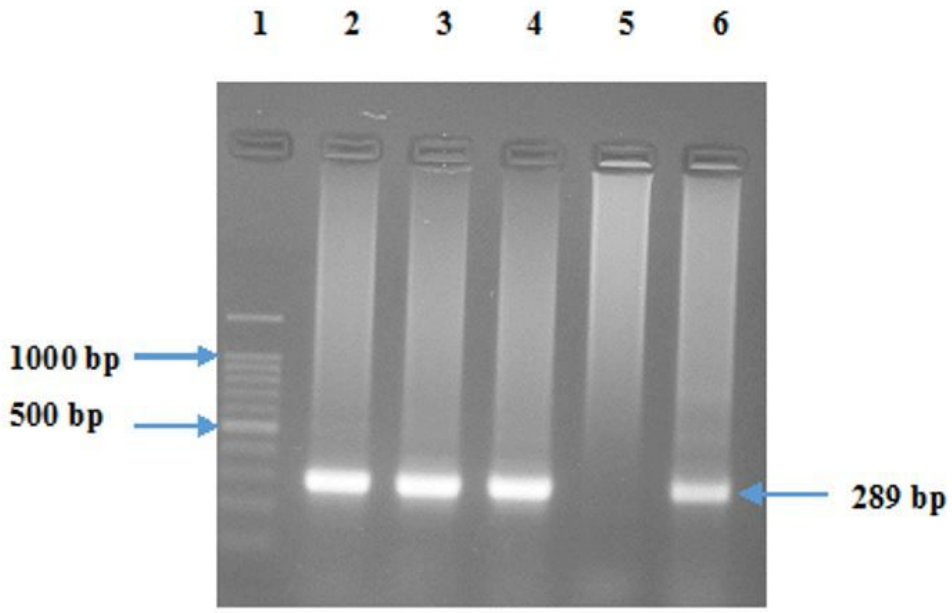
Vincent, A.T., Schiettekatte, O., Goarant, C., Neela, V.K., Bernet, E., Thibeaux, R., Ismail, N., Mohd Khalid, M.K.N., Amran, F., Masuzawa, T., Nakao, R., 2019. Revisiting the taxonomy and evolution of pathogenicity of the genus *Leptospira* through the prism of genomics. PLoS Negl Trop Dis 13(5): e0007270. https://doi.org/10.1371/journal. pntd.0007270

Visith S., Kearkiat P., 2005. Nephropathy in leptospirosis. J. Postgrad. Med. 51, 184–188. https://www.jpgmonline.com/text.asp?2005/51/3/184/19018.

Vital-Brazil, J.M., Balassiano, I.T., Oliveira, F.S.D., Costa, A.D.D.S., Hillen, L., Pereira, M.M., 2010. Multiplex PCR-based detection of *Leptospira* in environmental water samples obtained from a slum settlement. Mem. I. Oswaldo Cruz. 105:353–355. https://doi.org/10.1590/S0074-02762010000300020.

WHO, 2003. Human *leptospirosis*: guidance for diagnosis, surveillance and control. Available in: http://apps.who.int/iris/bitstream/10665/42667/1/WHO_CDS_CSR_EPH_2002.23.pdf.

## Figures




**Lane 1: DNA Ladder (100 bp), 2: Kidney tissue positive sample, 3 & 4: Positive serum samples, 5: Negative control (no template), 6: Positive control.**

Figure 1

Gel electrophoresis analysis of pathogenic species of *Leptospira* from serum and kidney tissue samples.

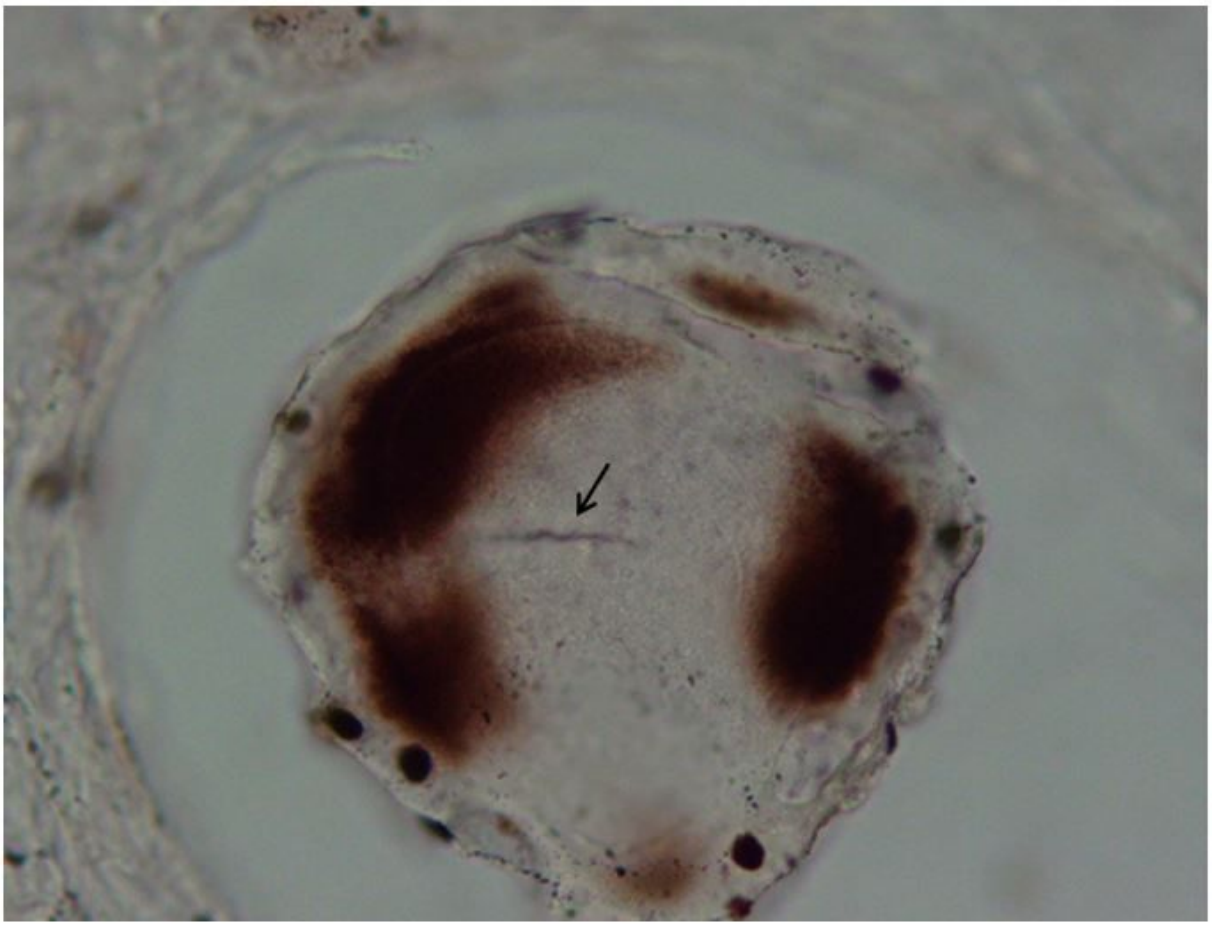


Figure 2

Warthin-Starry (WS) staining of kidney. Presence of filiform, dark brown *Leptospira* (arrows) in a secretion in the lumens of cortical tubules.