Molecular detection of *Leptospira* in urine samples of dogs from Kerman City, southeastern Iran

Gonabadi H.[1]; Mohammadi E.[1*]

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Abstract

Leptospirosis is a globally significant zoonotic disease, prevalent in tropical, subtropical, and temperate regions. The causative agents are spiral-shaped aerobic spirochetes of the genus *Leptospira*, classified into saprophytic (*L. biflexa*) and pathogenic (*L. interrogans*) species. Environmental and anthropogenic factors such as climate change, heavy rainfall, flooding, poor sanitation, improper waste management, and high reservoir host populations (e.g., rodents) contribute to transmission. Clinical manifestations in dogs range from mild fever to severe organ damage. Transmission occurs via direct contact with infected animals or through exposure to contaminated soil and water. Due to slow bacterial growth and absence of specific antibodies during early infection, culture and serological tests have limited diagnostic value. Polymerase chain reaction (PCR), with high sensitivity and specificity, offers a reliable alternative. In this study, urine samples from 50 dogs in Kerman, southeastern Iran, were tested for *Leptospira* DNA using PCR targeting the 16S rRNA gene. *Leptospira* DNA was detected in 6 out of 50 samples (12%). These results confirm the presence of *Leptospira* in the canine population of Kerman, highlighting a potential zoonotic risk. Enhanced surveillance and preventive measures are recommended to reduce public health impact.

Keywords: *Leptospira*, Urine samples, PCR, Dog, Kerman City

1-Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

*Corresponding author's Email: E_mohammadi@uk.ac.ir

Introduction

Leptospirosis is one of the zoonotic diseases, prevalent worldwide, particularly in tropical, subtropical, and humid regions (Goarant, 2016). Leptospirosis is an acute febrile illness caused by spirochetes of the genus *Leptospira* (De Brito *et al*., 2018). These bacteria are long, slender, helical organisms with hooked ends, capable of surviving freely in the environment or parasitizing within animal hosts. Generally, *Leptospira* is classified into two groups: pathogenic *Leptospira interrogans* and non-pathogenic *Leptospira biflexa* (Mohammed *et al*., 2011). It is characterized by non-specific clinical signs such as fever, vomiting, muscle weakness, polyuria, polydipsia, jaundice, and renal or hepatic dysfunction (Van de Maele *et al*., 2008). In Iran, considering the prevalence of both acute and chronic forms of this zoonotic disease, it is essential to plan for its treatment or control. Identifying the causative agent is the first step in this regard. The polymerase chain reaction (PCR) method is highly sensitive and accurate, as a suitable method. In other words, since treatment is effective only in the early stages of the disease, accurate and rapid diagnosis of leptospirosis is crucial. Because of the bacterium's slow growth and the lack of specific antibodies during the first week of the disease, culture and serological tests are inefficient respectively. Therefore, PCR is the only method for early detection (Mullan and Panwala, 2016). This technique aids in the rapid diagnosis of the pathogen, allowing early treatment in animals, reducing costs associated with disease transmission within herds, and preventing zoonotic transmission from animals to humans. Recently, dogs have become popular pets and play a significant role in transmitting leptospiral infections to humans (Lau *et al*., 2016). Therefore, dogs can serve as significant reservoirs for zoonotic transmission (López *et al*., 2019). Also, leptospirosis is a serious disease in dogs, with a mortality rate of 10–20%. The disease is typically characterized by acute kidney failure, liver damage, and coagulation disorders. Infected dogs often present with symptoms such as vomiting, anorexia, severe weakness, and depression. Azotemia, indicative of renal insufficiency, may only be detectable through laboratory tests (Ward *et al*., 2002). Dogs are important primary reservoirs of *Leptospira interrogans*. The most common serovars in dogs are *Leptospira canicola*, *Leptospira icterohaemorrhagiae*, and *Leptospira grippotyphosa*. The primary route of transmission in dogs is direct or indirect contact with contaminated water, soil, food, or tissues from infected animals (Delaude *et al*., 2017; Torkan and Momtaz, 2019). Dogs can act as asymptomatic carriers, harboring the bacteria in their renal tubules and shedding it in their urine over extended periods (Sant'Anna *et al*., 2019). Thus, this study aimed to detect *Leptospira* in the urine of dogs in Kerman using the PCR method.

Materials and methods

Sample collection

Fifty urine samples were collected via catheterization or cystocentesis of dogs referred to the Veterinary Hospital of Shahid Bahonar University in Kerman. The collected samples were stored at -20°C for further molecular analysis. All relevant information regarding the samples is provided in Table 1.

Table 1: Characteristics of the collected urine samples.

Attribute	Details
Gender	24 male dogs, 26 female dogs
Neutering status	11 spayed dogs, 39 intact dogs
Vaccination status	49 unvaccinated dogs, 1 vaccinated dog
Living environment	25 household dogs, 25 shelter dogs
Urine appearance	34 yellow urine, 11 dark yellow urine (concentrated), 5 orange to red urine (bloody)
Urine clarity	48 clear urine samples, 2 cloudy urine samples with suspended particles
Method of collection	23 samples via catheterization, 27 samples via cystocentesis
Season of collection	Year 2023: 18 samples in autumn, 2 samples in winter Year 2024: 2 samples in winter, 17 samples in spring, 5 samples in summer, 6 samples in autumn
Age	2 months to 10 years

DNA Extraction

DNA extraction from the samples was performed using the DNA Extraction Kit (50T-EX6071) from Sinaclon company, Iran. The process was carried out according to the manufacturer's instructions, as follows: The lysis buffer was placed in a 37°C incubator for 10 minutes. Samples were removed from the freezer and thawed at room temperature. After vortexing the microtubes containing the urine samples, 100 µL of urine was mixed with 100 µL protease buffer solution and 5 µL proteinase K in a sterile microtube. The mixture was incubated in a thermomixer at 55°C for 30 minutes. The 100 µL solution from the previous step was mixed with 400 µL lysis buffer and vortexed for 15–20 seconds to ensure complete suspension. 300 µL of precipitation solution was added to the mixture and vortexed for 5 seconds. The samples were centrifuged at 12,000 rpm for 10 minutes, and the supernatant was discarded. The microtubes were placed upside down on individual filter papers to absorb any remaining liquid. To the sediment in each microtube, 1 mL wash buffer was added, vortexed for 3–5 seconds, and centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded. The remaining sediment was placed in a thermomixer at 65°C for 5 minutes (longer if necessary) to ensure complete drying of the samples. To the

dried sediment in each microtube, 30 µL solvent buffer was added. The microtubes were incubated in a thermomixer at 65°C for 5 minutes to completely dissolve the sediment. The microtubes containing the extracted DNA were stored at -20°C until the PCR analysis.

Conventional PCR

The PCR reaction was carried out in a final volume of 25 µL, containing 12.5 µL of PCR master mix (Sinaclon, Iran), 1 µL of each primer LP (0.4µM) (Pishgam biotech company, Iran), 5.5 µL of nuclease-free water and 5 µL of template DNA. The information on primers is described in Table 2. PCR reactions were performed using a Thermal Cycler (Bio-Rad, USA). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min, with final extension at 72°C for 10 min. Distilled water and *Leptospira* DNA (Intron Biotechnology, South Korea) were used as negative and positive controls, respectively. The PCR products were visualized on a 2% agarose gel stained with 6 µL of DNA gel stain (Sinaclon, Iran) and the amplicon size was compared with a 50 bp DNA ladder (Pishgam Biotech, Iran).

Table 2: Primers used for amplifying the 16S rRNA gene.

target	primer	Sequence	Product Size (bp)
16S rRNA	LP1-F	5'-GCGCGTCTTAAACATGCAAG -3'	306
	LP2-R	5'-CTTAACTGCTGCCTC CCGTAG-3'	

Categorical variables, including sex, age, housing location, urine characteristics, vaccination status, neutering status, and sampling season, were analyzed to assess their association with positive test results. Two statistical methods were employed: the Chi-squared test and Fisher's exact test. The Chi-squared test was applied where all expected cell counts in contingency tables were ≥5. In cases where expected frequencies were less than 5 in any cell, or where more than 20% of the cells fell below this threshold, Fisher's exact test was used instead to ensure the reliability of the analysis. P-values were evaluated at a significance level of 0.05. Variables with p-values less than 0.05 were considered significantly associated with positive outcomes.

Results

This study investigated the frequency of *Leptospira* in the urine of dogs in Kerman using the PCR method. Among the 50 samples tested, six were found to contain the *Leptospira* specific 16S rRNA gene fragment measuring 306 base pairs (Fig. 1). The frequency of *Leptospira* in this study was confirmed to be approximately 12% (Fig. 2). All positive samples had a history of non-vaccination. Additional characteristics of the positive samples are presented (Table 3).

According to the statistical analyses we conducted, significant associations were

identified between positive test outcomes and age (χ^2=7.6364, p=0.0057), urine color (χ^2=10.667, p=0.0011), and housing location (χ^2=6.0, p=0.0143). Fisher's exact test confirmed these findings with p-values of 0.0047, 0.0092, and 0.0223, respectively. Other variables, including sex, vaccination status, neutering status, urine clarity, and sampling season, showed no significant association with positivity (p>0.05). The results highlight public health concerns regarding leptospirosis in shelter dogs and emphasize the importance of preventive measures. Observations during the investigation of shelter conditions suggested that poor sanitary conditions, inadequate ventilation, contact with rodents and carrier animals' urine, and close interactions with other animal species likely contributed to the 12% prevalence of *Leptospira* in urban dogs in Kerman city.

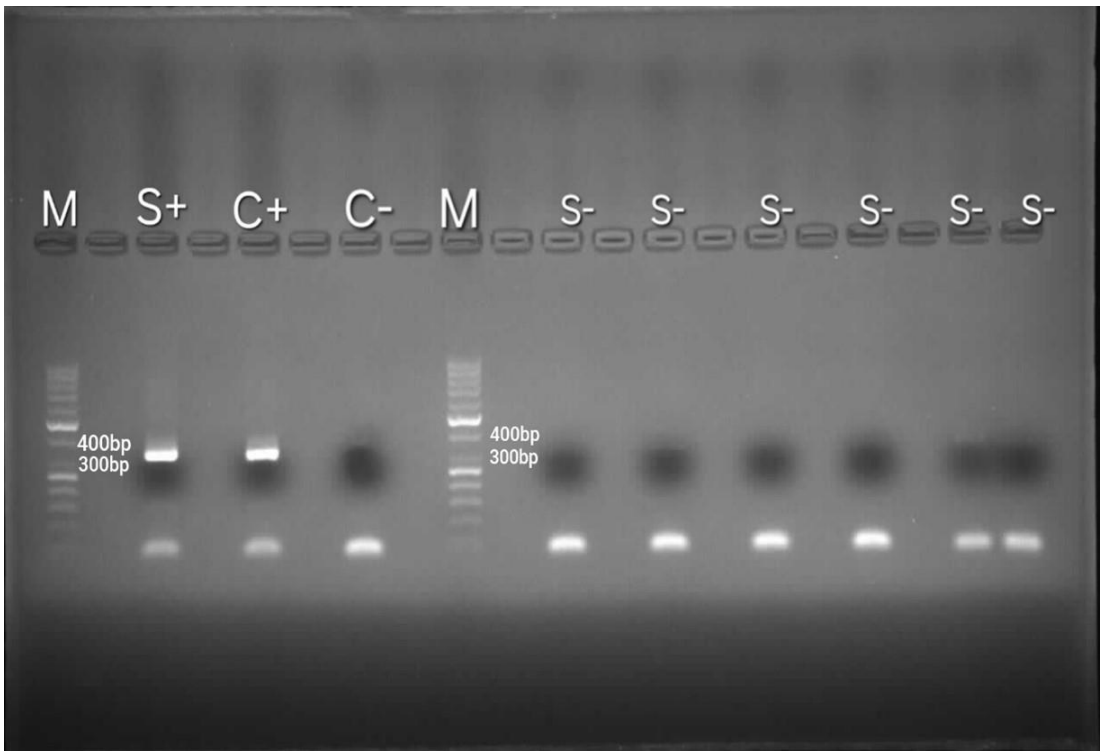

Figure 1: The electrophoresis of PCR products for the 16S rRNA gene of *Leptospira* (306 bp) in canine urine; M: Marker, S^+: Positive Sample, C^+: Positive Control, C^-: Negative Control, S^-: Negative Sample.

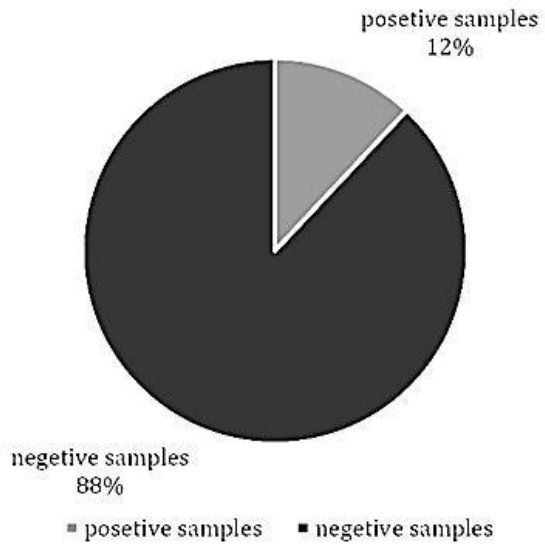

Figure 2: Frequency percentage of positive and negative canine urine samples based on PCR results.

Table 3: Characteristics of *Leptospira* positive canine urine samples.

Number of positive samples	Gender	Neutering status	Age	Method of urine collection	Color and appearance of Urine	Season of sampling	Storage location of dogs
1	Male	spayed	3-3.5 years	Catheterization	Orange to red, Clear	Fall 2023	Shelter
2	Female	spayed	5-6 years	Catheterization	Yellow, clear	Fall 2023	Shelter
3	Male	intact	4 years	Catheterization	Orange to red, clear	Fall 2023	Shelter
30	Female	intact	4 years	Catheterization	Yellow, Clear	Spring 2024	Shelter
46	Female	intact	2 years	Cystocentesis	Orange to red, Clear	Fall 2024	Shelter
47	Male	intact	3 years	Catheterization	Yellow, Clear	Fall 2024	Shelter

Discussion

Leptospirosis is a zoonotic disease that affects both humans and animals, involving a wide range of wild and domestic species. In animals, the infection is typically acquired through direct contact with urine contaminated with *Leptospira* or indirectly through exposure to contaminated water and soil. These spirochetes predominantly proliferate in the central nervous system, kidneys, and liver. While they are cleared from the blood and most tissues through immune responses, they persist and replicate in renal tubules, being excreted via urine for extended periods (Delaude *et al*., 2017; Johnson and Faine, 1984). Infected dogs pose a significant zoonotic risk to their owners, veterinarians, and animal care workers by exposing them to infected urine. In dogs, the infection can be subclinical, making it difficult to detect, or it can manifest with mild, nonspecific clinical symptoms. One of the diagnostic methods for leptospirosis is polymerase chain reaction (PCR), which can be performed on whole blood or urine

samples (Iverson *et al*., 2021). . PCR is a highly sensitive and relatively rapid diagnostic technique that facilitates early detection, particularly when serological methods fail to identify antibodies during the initial stages of infection or in severe cases where death occurs before antibody formation (Pinto *et al*., 2022). Additionally, antibiotic treatment does not interfere with the results of PCR on urine samples (Martin *et al*., 2022). The city of Kerman is located in southeastern Iran in the province of Kerman. It is situated in a flat plain at an elevation of 1,753.8 meters. The region has a semi-arid to arid climate, characterized by hot summers and cold winters, with temperatures ranging from -8°C to 37°C throughout the year (Aboubakri *et al*., 2020). Globally, including in Iran, studies have been conducted to evaluate the presence of *Leptospira* bacteria in dogs. The reported prevalence rates vary, with some findings aligning with the results of the present study (0–25%) and others indicating significantly higher prevalence rates (>25%). Some of these studies are mentioned below.

Studies with findings similar to the present research include: A 2024 study conducted in Colombia using PCR on blood samples from 140 dogs reported that 15% of the extracted DNA contained the LipL32 gene of *Leptospira* bacteria (Caballero Méndez *et al*., 2024). Another 2024 study in Bosnia and Herzegovina found that 3.87% (156 of 4,028) of blood samples from stray dogs with unknown vaccination status tested positive for leptospirosis using the microscopic agglutination test (MAT) (Maksimović *et al*., 2024). In Sweden, a serological study reported a seroprevalence of 7.3% using MAT and 0.9% using ELISA in healthy dogs (Scahill *et al*., 2022). A Canadian study analyzed 10,437 blood and urine samples from dogs collected between 2009 and 2018, finding an annual prevalence ranging from 4.8% to 14.0%, attributed to differences in sampling locations (Stull *et al*., 2022). In 2021, Khaki and colleagues reported a seroprevalence of 21.84% using MAT in stray dogs in Alborz Province, Iran (Fahimipour and Khaki, 2021). Similarly, Shabestari Asl *et al*. (2020) found a 6.7% seroprevalence against the Canicola serovar in 45 household and 45 stray dogs in Iran using MAT (Asl *et al*., 2020). In a molecular study conducted in 2019 in Shahrekord, Iran, Momtaz *et al*. (??) used RFLP-PCR to analyze 120 blood samples from stray dogs and reported a 7.5% positivity rate (Torkan and Momtaz, 2019). Khamisipour *et al*. (2013) reported a prevalence of 19% in blood samples from dogs in Shahrekord and Isfahan using PCR (Khamesipour *et al*., 2014). Avizeh *et al*. (2008) found a seroprevalence of 5.4% in 149 serum samples from dogs in Ahvaz, Iran, using MAT (Avizeh *et al*., 2008). Hayatroohi *et al*. (2014) in Urmia *et al*. (2003) in Razavi Khorasan reported seroprevalences of 6.4% and 14.3%, respectively, using MAT (Hayatrohi *et al*., 2014, Kamrani and Sardari, 2003).

Studies with different findings from the present research include: In 2013, a study in India found seroprevalence rates of 57% among 42 vaccinated dogs

and 35% among 34 stray dogs using MAT (Senthil *et al*., 2013). Between 1998 and 2000, Rad *et al*. (2004) reported seroprevalence rates of approximately 31% using MAT and 34.3% using indirect fluorescent antibody tests in Tehran, Iran (Rad *et al*., 2004). A 2015 study in Chile reported a 45.1% seroprevalence among 71 dogs from urban slums using MAT (Lelu *et al*., 2015). In Mexico, a 2008 study reported a seroprevalence of 35% among 400 stray dogs using MAT and ELISA (Jimenez-Coello *et al*., 2008). A 2017 study in the Caribbean Islands reported a 73% seroprevalence among 101 domestic dogs, with the highest prevalence linked to the autumnalis serovar (Pratt *et al*., 2017). A 2018 study in Colombia found a seroprevalence of 36.46% among 192 owned dogs using MAT, with the high prevalence attributed to unrestricted access to water sources and rodent hunting (Cárdenas *et al*., 2018). A 2014 study in three tropical African countries reported a 95% seroprevalence using MAT with a titer of 1:40 (Roqueplo *et al*., 2015). In Brazil, studies conducted between 2018 and 2019 reported a seroprevalence of 64.5% and a molecular prevalence of 38.7% using PCR among 31 suspected cases of acute leptospirosis (Santos *et al*., 2021). Another 2018 study in Brazil divided 64 dogs into two groups (A: dogs with chronic kidney failure; B: healthy dogs) and found that 100% of group A and 25% of group B tested serologically positive, while 75% of group A and 20.8% of group B were PCR-positive (Sant'Anna *et al*., 2019). In 2013, a study in Pakistan found a seroprevalence of 36.1% among 429 dogs using IgG ELISA kits (Saleem *et al*., 2013).

The discrepancies between the findings of the present study and the above studies can be attributed to several factors: 1. The present study employed a molecular method (PCR), while some of the referenced studies used serological techniques such as MAT. 2. The referenced studies primarily analyzed blood or serum samples, whereas this study focused on urine samples. 3. Environmental factors such as rainfall, flooding, seasonal variations, and climatic conditions can influence the transmission and prevalence of leptospirosis. 4. The hygiene conditions in the sampling area significantly impact disease prevalence. For example, rural, suburban, and impoverished areas report higher rates of leptospirosis. 5. The dogs' living conditions—ownership status, access to stagnant water, rodent hunting, exposure to rodent urine, overcrowding, or residence in high-risk areas such as farms—also play crucial roles in disease prevalence. In summary, all the mentioned studies confirm the presence of *Leptospira* bacteria in dogs. Considering the serious effects on dogs and the fact that the disease can spread to humans, this is a worrying issue. It shows the need for the World Health Organization to take effective steps to control and prevent leptospirosis.

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