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Original Article

Molecular Detection of *Leptospira* Species Serotypes in Iranian Stray Dogs

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Background and Aims: Leptospirosis is a spirochetal disease with public health importance globally. This disease affects a wide range of domestic and wild animals. Dogs are one of the species most sensitive to *Leptospira canicola* and *Leptospira icterohaemorrhagiae*. The present study was concluded to evaluate the prevalence rate of *Leptospira* species and *L. canicola* and *L. icterohaemorrhagiae* serovars in Iranian stray dogs.

Materials and Methods: One-hundred and twenty blood samples were first taken from stray dogs. Then the samples were transferred to the laboratory. Sera were extracted from blood samples and genomic DNA was extracted. DNA samples were subjected to conventional polymerase chain reaction. Positive samples for *Leptospira* spp. were analyzed for presence of *L. canicola* and *L. icterohaemorrhagiae* serovars using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: Nine samples out of 120 serum samples (7.5%) were positive for the flagella gene of the *Leptospira* spp. Prevalence of *Leptospira* spp. in serum samples of male and female dogs were 5.4% and 10.86%, respectively. Prevalence of *L. canicola* and *L. icterohaemorrhagiae* serovars were 55.55% and 33.33%, respectively. We found that 11.11% of samples were positive for both serovars. Two to three and 3-4 year old dogs had the highest prevalence of *Leptospira* spp.

Conclusions: The considerable prevalence of leptospirta spp. and also their zoonotic serovars among Iranian stray dogs represented an important public health issue regarding the contact of healthy human with these dogs. Identification of infected dogs and their vaccination can inhibit the distribution of *Leptospira* spp.

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Introduction

Leptospirosis is a common infectious disease with zoonotic impact globally. It causes septicemia, interstitial nephritis, anemia, hemoglobinuria and abortion in human and animal. Leptospirosis is one of the most extensive zoonotic diseases which can affect at least 160 different species of animals. The disease is caused by the pathogenic spirochete bacteria of the genus *Leptospira*. *Leptospira* bacteria include two separate species of interrogans (pathogenic strains) and biflexa (non-pathogenic strains). *Leptospira interrogans* (*L. interrogans*) are divided into 220 different serotypes.

Dogs are one of the most important sources of *L. interrogans* species. *L. canicola*, *L. icterohaemorrhagiae* and *L. grippotyphosa* are the most frequently detected serovars accompanied by dogs. The primary mode of transmission of leptospirosis is through direct or indirect contact of the animals or humans with contaminated water, soil, food and infected tissues of animals. *Leptospira* microorganisms enter the body through floating drops of infected body fluids like urine and feces [1-3].

Culture-based identification of leprospira spp. and using from the traditional techniques like Microscopic Agglutinations Test (MAT) are considered useful for confirmation of the disease. Unfortunately, these traditional methods are time-consuming, expensive and dangerous. Therefore, several serological tests have been developed for identification of the lepotspira spp. Previously published data revealed the occurrence of cross-reaction and also low sensitivity and specificity of serologic tests for diagnosis of the leptospirosis. Polymerase chain reaction (PCR)-based identification of the *Leptospira* specific genes are considered as rapid, safe and accurate technique for diagnosis of *Leptospira* species and their serovars [4-6].

Considering the lack of microbiological and epidemiological investigations about the *Leptospira* spp. in the Iranian stray dogs, the present research was performed to study the prevalence rate and distribution of *L. canicola* and *L. icterohaemorrhagiae* serovars in the Iranian stray dogs.

Materials and Methods

The study was approved by the Ethical Council of Research of the Faculty of Veterinary Medicine, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran (Consent Ref Number 94-29).

From January to March 2016, a total of 120 blood samples were randomly taken from stray dogs of the Charahmahal Va Bakhtiari province, Iran. All dogs were subjected to anesthesia using the acepromazine (Merck, Germany). Ten milliliters blood samples were taken from the cephalic vein of each dog. Samples were transferred to the Microbiology Research Center of the Islamic Azad University, Shahrekord Branch, Shahrekord, Iran using sterile tubes. Serum was obtained by allowing the blood to clot at room temperature for 2 h. The clotted blood was

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then centrifuged for 10 min. at 12,000 *g*. Serum was then collected and stored at -20°C.

For DNA isolation, total DNA was extracted from 400 µL serum using the DBA isolation and extraction Kit according to the manufacturer's instructions (Fermentas, Germany).The quality and concentration of extracted DNA samples were measured using thespectrophotometer [7].

Flagella gene of the *Leptospira* spp. was used as a target gene for PCR amplification [8]. All runs were performed using a programmable thermal cycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device. The 10 ml bacterial DNA extract and controls were amplified with 0.5 mM primers

Forward: 5'-TCTCACCGTTCTCTAAAGTTCAAC-3'
Reverse: 5'-CTGAATTCGGTTTCATATTTGCC-3')
(793 bp), 200 mM of each dNTP (Fermentas, Germany), 2 mM MgCl2, 10 mM KCl PCR buffer and 1.0 U Taq polymerase (Fermentas, Germany). The DNA was amplified in a programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device using the following protocol: 94ºC for 6 min, 34 cycles of 94ºC for 50 s, 58ºC for 5 min, 72ºC for 45 s, and final 72ºC for 10 min. Fifteen microliters of amplified PCR products were subjected to electrophoresis in a 1.5% agarose gel. Gels were stained using the SYBR Green (Fermentas, Germany). PCR grade water and positive DNA samples were used as negative and positive controls, respectively.

Restriction fragment length polymorphism (RFLP) analysis was used for identification of the *Leptospira* serovars. Enzymatic digestion of the PCR products was conducted using the *Hae*III restriction enzyme (Fermentas, Germany) (GGCC/CCGG restriction site). The reagents in the enzyme digestion reaction were included 2 µl of Buffer 10X, 10 µl PCR product, 1 µL *Hae*III restriction enzyme, and 7 µl sterile distilled water. The incubation of the above mixture was carried out at 80°C for 20 min. and electrophoresed with 1.5% agarose gel. The serovars were identified according to the obtained banding pattern and compared with *Leptospira* serovar patterns.

Statistical analysis

Statistical analysis was performed using SPSS/21.0 software for significant relationship for prevalence of *Leptospira* spp. and serovars betweendifferent stray dogs. Chi-square test was performed and differences were considered significant at $p<0.05$.

Results

A total of 120 blood samples were collected from male and female stray dogs of different ages. Of 120 samples studied, 74 samples (61.66%) were male and 46 samples (38.33%) were female. Figure 1 shows the results of the gel electrophoresis of the PCR amplification of the flagella gene of the *Leptospira* spp. Table 1 shows the prevalence of *Leptospira* spp. in male and female dogs. Nine out of 120 serum samples (7.50%) were positive for the *Leptospira* spp. Prevalence of *Leptospira* spp. in male and female dogs were 5.40% and 10.86%, respectively. None of the studied dogs show the clinical signs of the leptospirosis. Statistically significant difference was observed for the prevalence of

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Leptospira spp. between the male and female dogs ($p<0.05$).

Total prevalence of *L. canicola* and *L. icteroha-emorrhagiae* serovars were 33.33% and 55.55%, respectively. Both serovars were more prevalent in male dogs. We also found that 11.11% of the samples were simultaneously positive for both serovars.

Figure 2 shows the prevalence of *Leptospira* spp. amongst different age groups of stray dogs. We found that 2-3 and 3-4 years old stray dogs had the highest prevalence of *Leptospira* spp. (30%). Statistically significant difference was also identified for the prevalence of *Leptospira* spp. between different age groups ($p<0.05$).

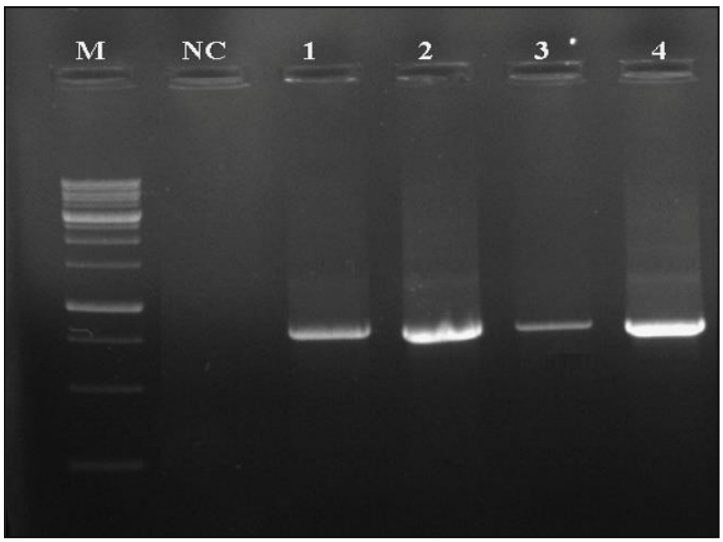

Fig. 1. Results of the gel electrophoresis of PCR products used for amplification of flagellagene of *Leptospira* spp. M: 1 kb ladder, 1: Positive control, 2-4: Positive samples for the flagella gene (793 bp) and NC: Negative control.

Table 1. The frequency distribution of various *Leptospira* serovars in blood samples of stray dogs of Chaharmahal Va Bakhtiari province

Type of samples	No. samples	No. *Leptospira* (%)	Distribution of serovars (%)		
			L. icterohaemorrhagiae	*L. canicola*	Both serovars
Female dogs serum	46	5 (10.86)	2 (40)	1 (20)	-
Male dogs serum	74	4 (5.40)	3 (75)	2 (50)	1 (25)
Total	120	9 (7.50)	5 (55.55)	3 (33.33)	1 (11.11)

Discussion

Leptospirosis is a zoonotic disease with a public health importance.This disease has worldwide distribution, affecting both wild and domestic mammals, human and even birds. Leptospirosis is associated with jaundice, anemia, hemoglobinuria, septicemia, petechial hemorrhages in different tissues, interstitial nephritis, abortion and mastitis [1-3]. Therefore, it is important to find a rapid, safe, accurate, sensitive and specific assay for its diagnosis.

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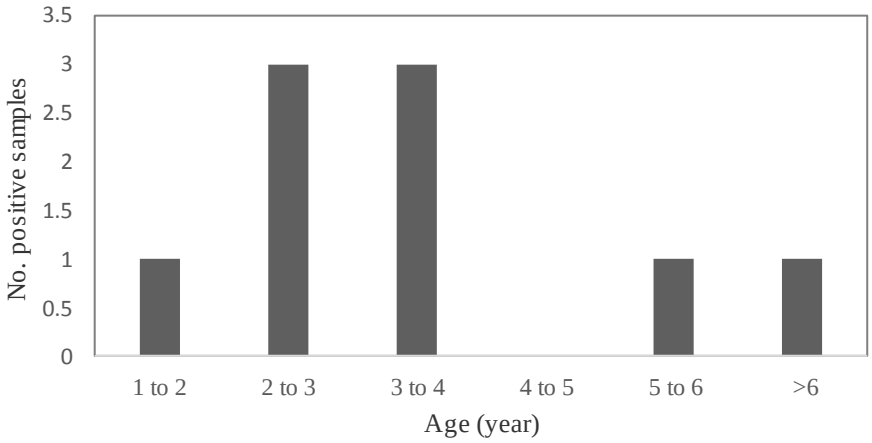

Fig. 2. Frequency of *Leptospira* spp. in different age groups of the stray dogs

The present study indicated that *Leptospira* spp. has a significant prevalence rate in the serum samples of stray dogs. As is shown, none of the studied dogs showed the clinical signs of the leptospirosis, they were positive, however, in the PCR amplification of the flagella gene of the *Leptospira* spp. One possible explanation for this finding is the fact that the PCR test can only detect the DNA of the target gene. Therefore, it is not possible to differ healthy and sick dogs. On the other hand, it is likely that the detected DNA was related to a dead *Leptospira* bacterium. Another explanation for this finding is that perhaps all the studied dogs were at the primary stages of leptospirosis. Therefore, they did not show the symptoms of leptospirosis at that time. Considering the asymptomatic infection in all dogs, this can suggest three basic conditions. First, the dogs in the present study might have recently been infected with *Leptospira* spp., therefore, the bacteria did not produce any symptoms at the moment. Second, the detected genome might have been associated with inactivated or dead bacteria. Third, dogs could be at the primary stages of the leptospirosis and thus did not show any symptoms at the time of research. Therefore, other tissues and also their secretions could have alive *Leptospira*. In keeping with this, the present research showed an important public health issue regarding the contact of household dogs with strays and their infection. In addition, it found that infected stray dogs can disseminate bacteria into human environment.

Their results of the present study indicated that 2 to 3 and 3 to 4 year old female dogs had the highest prevalence of *Leptospira* spp. Although the main reason for this finding is unknown but the weaker immunity of female dogs in this age maybe its probable reason. Two to four year old dogs are more social, more playful and more warlike than those of other ages. Therefore, they are more prone to being infected with leptospirosis.

Several investigations have been conducted in this regard. Hernandez- Rodrigues et al. (2011) compared PCR with microscopic method to diagnose *Leptospira* bacteria in the urine

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samples of cows [9]. They identified the sensitivity and specificity of the PCR assay to be 100% and 90%, respectively which was higher than those of MAT. Higher sensitivity and specificity of the PCR method have been reported by Khairani Bejo et al. (2004) [10], Villumsen et al. (2012) [11] and Xu et al. (2014). [12] Senthil et al. (2013) [13] reported that 97 out of 460 Indian stray dogs (41%) were infected with *Leptospira* spp. They also showed that male dogs and younger than one year old dogs had the higher prevalence of Leptospira spp. Prevalence of *Leptospira* spp, in stray dogs of Iran [14], United States [15], Brazil [16], Chile [17] and India [18] were 8.20%, 9.30%, 25.10%, 41% and 71.20%, respectively. High difference in the prevalence of *Leptospira* spp. found in different studies can be attributed to the differences in the type of samples, method of sampling, study experiment, geographical region and even climate of region from which the samples collected.

Ayral et al. (2014) [19] reported 63% of dogs in France to be infected with *Leptospira* spp. and the prevalence of *grippotyphosa* and *sejroe* serovars amounted to 9% and 6%, respectively. They also showed the prevalence of the *icterohaemorrhagiae* serovar to be 3%. Mayer-Scholl et al. (2013) [20] reported a total of 18% of the dogs in Germany with clinical leptospirosis; here the most prevalent serovars were *australis* (28%), *grippotyphosa* (18%), and *pomona* (14%). Samir et al. (2015) [21] reported the prevalence of *Leptospira* spp. based on the culture, MAT and PCR methods in dogs in Egypt being 11.30%, 11.30% and 58.30%, respectively. They explored the prevalence of *L. canicola* and *L. icterohae-morrhagiae* serovars to be 52.63% and 47.36%, respectively. Majetić et al. (2012) [22] reported that of 151 dog sera, 57 (37.7%) were seropositive for *Leptospira* spp. They showed *pomona* (8/26, 30.8%), *grippotyphosa* (5/26, 19.2%), *icterohaemorrhagiae* (4/26, 15.4%), *australis* (4/26, 15.4%), *saxkoebing* (1/26, 3.8%) and *hardjo* (1/26, 3.8%) to be the most commonly detected serovars.

Conclusion

We identified the prevalence rate of the 7.50% of the *Leptospira* spp. in stray dogs. High prevalence of *Leptospira* spp. in female 2-3 and 3-4 year old dogs represents their higher risk. Prevalence of *L. canicola* and *L. icterohaemorrhagiae* serovars were 33.33% and 55.55%, respectively. Simultaneous presence of both pathogenic serovars in the serum samples of stray dogs poses an important public health threat regarding the close contact of the stray dogs with household dogs, domestic animals and also human environment. High prevalence of these two serovars in stray dogs of our research maybe due to close contact of these dogs with rodents. Vaccination of stray dogs by polyvalent vaccines or putting the rodents to death humanely can prevent the spread and transmission of *Leptospira* spp. to human.

Conflict of Interest

The authors did not declare any conflict of interests.

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References

[1].Goldstein RE. Canine leptospirosis. Vet Clin North Am Small Anim Pract. 2010; 40(6): 1091-101.

[2]. Schuller S, Francey T, Hartmann K, Hugonnard M, Kohn B, Nally JE, et al. European consensus statement on leptospirosis in dogs and cats. J Small Anim Pract. 2015; 56(3): 159-79.

[3]. Van de Maele I, Claus A, Haesebrouck F, Daminet S. Leptospirosis in dogs: a review with emphasis on clinical aspects. Vet Rec. 2008; 163(14): 409-13.

[4]. Budihal SV, Perwez K. Leptospirosis diagnosis: competancy of various laboratory tests. J Clin Diagn Res. 2014; 8(1): 199.

[5]. Ahmad SN, Shah S, Ahmad FH. Laboratory diagnosis of leptospirosis. J Postgrad Med. 2005; 51(3): 195.

[6]. Waggoner JJ, Pinsky BA. Molecular diagnostics for human leptospirosis. Curr Opin Infect Dis. 2016; 29(5): 440.

[7]. Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th edition, New York: Cold Spring Harbor Laboratory Press; 2012. pp. 20-28.

[8]. Bandara KK, Weerasekera M, Gunasekara CP, Ranasinghe N, Marasinghe C, Fernando N. Molecular characterisation and disease severity of leptospirosis in Sri Lanka. Memórias do Instituto Oswaldo Cruz. 2015; 110(4): 485-91.

[9]. Hernández-Rodríguez P, Díaz CA, Dalmau EA, Quintero GM. A comparison between polymerase chain reaction (PCR) and traditional techniques for the diagnosis of leptospirosis in bovines. J Microbiol Methods 2011; 84(1): 1-7.

[10]. Khairani-Bejo S, Oii SS, Bahaman AR. Rats: leptospirosis reservoir in Serdang Selangor residential area. J Anim Vet Adv. 2004; 3(2): 66-9.

[11]. Villumsen S, Pedersen R, Borre MB, Ahrens P, Jensen JS, Krogfelt KA. Novel TaqMan® PCR for detection of Leptospira species in urine and blood: pit-falls of in silico validation. J Microbiol Methods. 2012; 91(1): 184-90.

[12]. Xu C, Loftis A, Ahluwalia SK, Gao D, Verma A, Wang C, et al. Diagnosis of canine leptospirosis by a highly sensitive FRET-PCR targeting the lig genes. PloS one 2014; 9(2): e89507.

[13]. Senthil NR, Palanivel KM, Rishikesavan R. Seroprevalence of Leptospiral antibodies in canine population in and around Namakkal. J Vet Med. 2013; 2013: 971810.

[14]. Avizeh R, Ghorbanpoor M, Hatami S, Abdollahpor G. Seroepidemiology of canine leptospirosis in Ahvaz, Iran. Iranian J Vet Med. 2008; 2(2): 75-9.

[15]. Harkin KR, Roshto YM, Sullivan JT, Purvis TJ, Chengappa MM. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. J Am Vet Med Assoc. 2003; 222(9): 1230-233.

[16]. Morikawa VM, Bier D, Pellizzaro M, Ullmann LS, Paploski IA, Kikuti M, et al. Seroprevalence and seroincidence of Leptospira infection in dogs during a one-year period in an endemic urban area in Southern Brazil. Rev Soc Brasil Med Trop. 2015; 48(1): 50-5.

[17]. Lelu M, Muñoz-Zanzi C, Higgins B, Galloway R. Seroepidemiology of leptospirosis in dogs from rural and slum communities of Los Rios Region, Chile. BMC Vet Res. 2015; 11(1): 31.

[18]. Ambily R, Mini M, Joseph S, Krishna SV, Abhinay G. Canine leptospirosis-a seroprevalence study from Kerala, India. Vet World. 2013; 6(1): 42-4.

[19]. Ayral FC, Bicout DJ, Pereira H, Artois M, Kodjo A. Distribution of Leptospira serogroups in cattle herds and dogs in France. Am J Trop Med Hyg. 2014; 91(4): 756-59.

[20]. Mayer-Scholl A, Luge E, Draeger A, Nöckler K, Kohn B. Distribution of Leptospira serogroups in dogs from Berlin, Germany. Vector-Borne Zoonotic Dis. 2013; 13(3): 200-202.

[21]. Samir A, Soliman R, El-Hariri M, Abdel-Moein K, Hatem ME. Leptospirosis in animals and human contacts in Egypt: broad range surveillance. Rev Soc Brasil Med Trop. 2015; 48(3): 272-77.

[22]. Štritof Majetić Z, Habuš J, Milas Z, Mojčec Perko V, Starešina V, Turk N. A serological survey of canine leptospirosis in Croatia-the changing epizootiology of the disease. Vet Arhiv. 2012; 82(2): 183-91.

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