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Serological and molecular studies on visceral leishmaniosis in stray dogs in Torbat-e-Heidareih area

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Keywords

Leishmania infantum, stray dogs, Torbat-e- Heidareih area

Abstract

Visceral leishmaniosis is an important zoonotic disease in Iran. The agent, vector and reservoir are *Leishmania infantum*, Phlebetomous major and dogs, respectively. Thus far, no data has been reported about the prevalence of visceral leishmaniosis in human and dog in Torbat-e- Heidareah area and the present study has been performed to determine the frequency of *L. infantum* infection in stray dogs. One hundred blood and skin samples were collected from 2014 to 2015. The sera samples were examined by Indirect Immunofluorescent Antibody Test (IFAT). The skin samples of seropositive and seronegative in association with skin lesions were examined by PCR method. In this study, antibodies against *L. infantum* were detected in 3 (3%) sera samples of dogs and kDNA *Leishmania spp.* was detected in 4 (4%) of seronegative dogs by PCR. *L. infantum* was detected in positive samples by semi nested PCR. Based on serological and molecular results, 7% of asymptomatic stray dogs of Torbat-e- Heidareih area were infected with *L. infantum* and may be acted as reservoirs of visceral leishmaniosis.

Abbreviations

IFAT: Indirect Immunofluorescent Antibody Test
kDNA: Kinetoplast DNA
L. infantum: Leishmania infantum

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Introduction

Visceral leishmaniosis is known as a zoonotic disease. Dogs are reservoirs of infection for people [1, 2]. *Leishmania infantum* and *Leishmania chagasi* are the main causes of zoonotic visceral leishmaniosis, while the anthroponitic infection is caused by *L. donovani* in India and East Africa. In the Middle East, zoonotic visceral leishmaniosis is caused by *L. infantum* with domestic dogs asits major reservoir host [1, 2]. The prevalence rates of canine leishmaniosis are different in endemic areas and depend on the climatic conditions. The prevalence rate of disease was reported between 10 to 70% in the Mediterranean area. [3, 4]. So far, visceral leishmaniosis have been reported in dogs and wild carnivores as the main reservoirs from different parts of Iran [5-8]. Canine leishmaniosis is a chronic disease and characterized clinically by lymphadenopathy, cutaneous signs, weight loss and anemia. In addition, many infected dogs are asymptomatic and serve as reservoir hosts of disease in endemic areas [9-11].

The molecular methods have high sensitivity and specificity for the demonstration of every pathogen in human and animal. The PCR assay has been significantly improved the diagnosis of visceral leishmaniosis in man and animal [12]. The range of frequency of visceral leishmaniosis has been determined to be 7 -8.5% in dogs of Mashhad area [13-15]. Due to the proximity and similarity of climatic condition between Mashhad and Torbat –e-Heidareih and lack of data about visceral leishmaniosis in this area, the objective of this study was to determine the prevalence of zoonotic visceral leishmaniosis in asymptomatic stray dogs in Torbat-e-Heidareih area by IFAT and PCR methods.

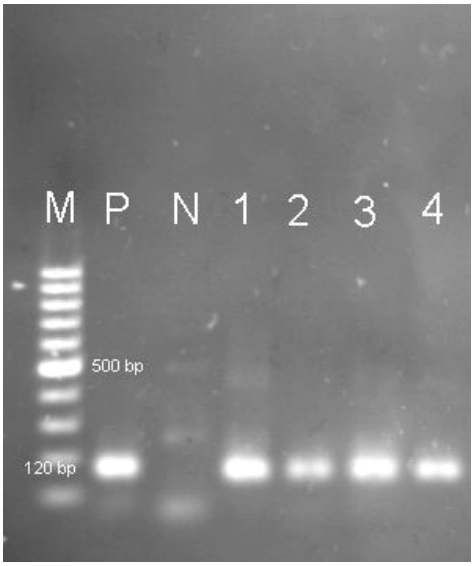

Figure 1
PCR amplification products of *Leishmania spp.* in skin samples. M: molecular weight marker (between 1000 and 100bp); P: positive control; N: negative control; 1, 2, 3, 4: positive samples.

Results

A total of 100 sera of 85 male and 15 female asymptomatic stray dogs were examined by IFAT.

In this study, seroprevalence of *L. infantum* infection was determined % 3 (3/100) in asymptomatic stray dogs. The kDNA of *L. infantum* was detected in 4 (4 /100) of the skin samples in seronegative stray dogs (Table 1) (Figures 1 and 2). All positive samples belonged to male stray dogs, but the frequency rate in male and female dogs was not significantly different ($p < 0.05$) There was not any agreement between IFAT and PCR methods. (Kappa= -0.036)

Discussion

In the present study, the antibodies against *L.*

Table 1
Results of IFAT and PCR examination of sera and skin samples of symptomatic stray dogs in Torbat-e- Hyeidreih area

Gender	IFAT		PCR		Total
	Positive	Negative	Positive	Negative	
Male	3	82	4	81	85
Female	0	15	0	15	15
Total	3	97	4	96	100

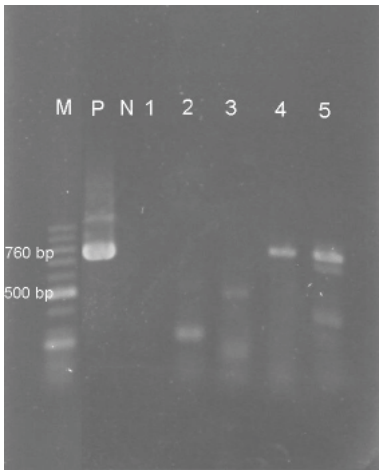

Figure 2
Semi-nested PCR amplification products of L. infantum in skin samples. M: molecular weight marker (between 1000 and 100bp); P: positive control; N: negative control; 1, 2, 3, negative samples; 4, 5, positive samples

infantum was detected in 3 serum samples (3%) of asymptomatic stray dogs. This rate was lower than the seroprevalence of canine leishmaniosis that has been reported in Mashhad area [13-15]. The seroprevalence range of canine leishmaniosis has been determined from 4.4% to 18.2% in other provinces of Iran [11, 16-18]. Difference in the prevalence of canine leishmaniosis in above studies may be due to the difference in climate of sampling areas and various serologic methods.

In the present study, *Leishmania* DNA was detected in 4% of skin samples in asymptomatic seronegative dogs by a genus-specific PCR [19]. This finding is in agreement with other studies in the world [20-24]. Two reasons may be caused this results: 1) the seronegative /PCR positive dogs belong to the resistance group that is characterized by strong cell-mediated immunity with low antibody production, 2) the dogs may have been recently infected and the antibodies titer is non-detectable against *L. infantum*.

Our study showed that the frequency of infection in male and female dogs was not significant. Similar results have been reported by other researchers in Iran [8, 11, 15, 16, 17], although some studies showed that the frequency of visceral leishmaniosis in males is higher than female dogs. [22-24].

In this study, there was not any agreement between PCR and IFAT methods. Previous studies have also reported a poor agreement between PCR and serology methods [25-26]. The *leishmania*l species of all PCR –positive sample were identified as *L. infantum* by a semi nested PCR [27]. This results supports the results of many epidemiological studies that *L. infantum* is the main causative agent of canine leishmaniosis in Iran [7, 22]. Based on the serological and molecular examination, it seems that asymptomatic stray dogs could act as reservoirs of visceral leishmaniosis in this area. Further epidemiological studies are recommended to determine the prevalence of visceral leishmaniosis in human population in this area.

Materials and Methods

Study area

The study was performed from February 2013 to February 2014 in Torbat-e-Heidreih that is located at 35.17˚ north latitude and 59.12˚ east longitude, in Khorasan Razavi Province, Iran. The average annual temperatures and precipitation are 21 °C and 250 mm, respectively. Summers are typically hot and dry, with high temperatures sometimes exceeding 35°C (95 °F). Winters are typically cool to cold and somewhat damper, with overnight lows routinely dropping below freezing.

Sample size

The prevalence of *leishmania*l infection was estimated 7% in stray dogs of Mashhad area. Based on this prevalence, the desired sample size was 100 stray dogs, using a 95% level confidence and 5% desired absolute precision (28). One hundred asymptomatic stray dogs were randomly selected during a zoonotic disease control program that was done by Torbat-e- Heidareih Municipality. The blood and skin samples were collected. The blood tubes were transferred to laboratory in cool condition. The clotted blood was centrifuged at 800 ×g for 5–10 min. The sera and skin samples were stored at -20˚C before serological and molecular examination.

The indirect immunofluorescent antibody test (IFAT)

In the present study, IFAT was used as sero-diagnostic tool. The *L. infantum* antigen (Megacor Co, Austria MegaScreen FLUOLEISH) has been used for this study. For each reaction, 10 µl of serum dilution (1:50) in PBS was added over the slide holes. The slides were incubated in a humid chamber at 37°C for 30 min. The slides were washed for 5 min in PBS. The conjugate was diluted in 1:200 in PBS with 0.025% Evan's Blue and then 15 µl of this solution were placed over the slide holes. The incubation and washing steps were repeated once, as outlined above. Slides were mounted with buffered glycerin, covered with a cover slip and read under an Olympus BX-FLA fluorescent microscope equipped with a 100W mercury lamp with 400× magnifying power. The diluted serum showing an evident yellow-green fluorescent signal upon microscopic examination was accepted as positive IFAT titer.

DNA extraction

Genomic DNA of skin samples of seropositive dogs and

the seronegative dogs were extracted using MBST Genomic DNA kit (Institute of Molecular and Biological transmission systems, Tehran, Iran) as per manufacturer's recommendations. The extracted DNA was stored at -20°C for examination of *leishmania* kDNA detection.

PCR assay

Leishmania genus-specific oligonucleotide primers K13A (5'-dGTGG GGGAGGGGCGTTCT-3') and K13B (5'-dATTTTACACCAACCCCCAGTT-3') described by Rodgers et al. (19) were used to amplify a 120 bp fragment in the conserved region of *Leishmania* kDNA mini-circles

To identify *Leishmania* species, an assay based on the semi nested PCR was used for amplification of variable area of the mini-circle kDNA (with a slight modification) as described by Aransay, et al. [27]. Negative (H_2O) and positive controls were used for each experiment. DNA of positive control was prepared from *Leishmania* culture [29]. After amplification, the PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Statistical analysis

The Seroprevalence rate of *L. infantum* in male and female dogs were statistically analyzed using Chi square. The agreement between IFAT and Semi nested PCR were assessed by Cohen's kappa test. The agreement between the different tests is shown as k value. The agreement is poor if k values are between 0.2 and 0.4, moderate if k values are between 0.4 and 0.6, substantial if 0.6 and 0.8, and good if it exceeds 0.8 [30].

Ethical consideration

Study protocols and methodologies were revised and approved by the Ethical committee of Ferdowsi University of Mashhad.

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Author Contributions

Collected samples: M.P. Performed IFAT and PCR: M.P. and S.S. Designed the study, and set up IFAT and PCR, wrote, and edited the manuscript: G.R.

Conflict of Interest

The authors declare that they have no competing interests.

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Abstracts (In Persian)

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بررسی سرولوژی و ملکولی لیشمانیوز احشایی در سگ های ولگرد در شهرستان تربت حیدریه

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چکیده

لیشمانیوز احشایی یک بیماری مهم مشترک بین انسان و دام در ایران است که عامل، ناقل و مخزن آن به ترتیب لیشمانیا اینفانتوم، فلبتوموس میجور و سگ می‌باشند. تاکنون هیچگونه اطلاعاتی در باره میزان شیوع لیشمانیور احشایی درانسان و سگ شهرستان تربت‌حیدریه گزارش نشده است. مطالعه حاضر جهت بررسی آلودگی لیشمانیا اینفانتوم در سگ‌های ولگرد این شهرستان انجام شد. از بهمن ماه ۱۳۹۳تا خرداد ۱۳۹۴تعداد ۱۰۰نمونه خون و پوست از سگ‌های ولگرد شهرستان تربت‌حیدریه، جمع آوری شد. نمونه‌های سرمی خون با روش ایمنوفلورسانس غیرمستقیم (IFAT) و نمونه‌های پوست سگ‌های سرم مثبت و سرم منفی واحد ضایعه جلدی جهت شناسایی جنس و گونه لیشمانیا مورد آزمایش PCR قرارگرفتند. در این مطالعه، سرم های ۳ قلاده سگ (۳٪) در روش ایمنو فلورسانس غیرمستقیم واجد عیار سرمی مثبت بر علیه لیشمانیا اینفانتوم بودند و DNA لیشمانیا در تعداد ۴نمونه (۴٪) پوست سگ‌های سرم منفی تعیین گردید. با روش Semi-nested PCR آلودگی نمونه‌های پوست به گونه لیشمانیا اینفانتوم تایید شد. با توجه به نتایج به دست آمده۷٪ از سگ‌های ولگرد شهرستان تربت حیدریه آلوده به لیشمانیا اینفانتوم بوده که می تواند به عنوان مخازن بیماری لیشمانیوز احشایی عمل نماید.

واژگان کلیدی: لیشمانیا اینفانتوم، سگ ولگرد، شهرستان تربت‌حیدریه