

Original Article

A Molecular and Serological Study on Visceral Leishmaniasis in Asymptomatic Stray Dogs in Mashhad, Iran

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ABSTRACT

Zoonotic visceral leishmaniasis is caused by *Leishmania infantum* (*L. infantum*), and its major reservoir hosts are domestic dogs, most of which are asymptomatic. This study aimed to detect *L. infantum* spp. in asymptomatic stray dogs by molecular and serological methods in Mashhad, Iran, during 2011-12. In this study, 94 asymptomatic stray dogs were randomly selected and their blood samples were collected for indirect fluorescent antibody testing. Furthermore, tissue samples from all the *L. infantum* seropositive stray dogs were examined using semi-nested polymerase chain reaction (PCR). According to the results, 11.7 % (11/94) of the dogs were *L. infantum* seropositive. The PCR positivity rate of *L. infantum* was 63.6% (7/11) in at least one of the collected specimens of the seropositive dogs. The *L. infantum* kinetoplast DNA (kDNA) was detected in the liver of 36% (4/11), the spleen of 27% (3/11), and the skin of 54.5 % (6/11) of the stray dogs. In this study, based on the molecular and serological examinations, visceral leishmaniasis infection among the stray dogs in Mashhad was confirmed.

Keywords: *Leishmania infantum*, Stray dogs, Mashhad

Analyse sérologique et moléculaire de la leishmaniose viscérale chez les chiens errants asymptomatiques de la ville de Mechhed

Résumé: Les chiens errants ont été identifiés comme la source principale de la leishmaniose viscérale provoquée par l'espèce *Leishmania infantum*. Un nombre important de chiens infectés ne présente aucun signe clinique et sont considérés comme des vecteurs potentiels de cette maladie. Cette étude a pour objectif d'évaluer par le biais d'examens sérologiques et moléculaires, le taux d'infection par la leishmaniose viscérale chez les chiens errants de la ville de Mechhed. Entre 2011 et 2013, 94 chiens ont été sélectionnés au hasard et des échantillons de sang ont été prélevés pour mener des tests d'immunofluorescence indirecte. De plus, une analyse moléculaire par semi-nested-PCR a été effectuée sur des échantillons de tissus provenant des chiens errants séropositifs. Ces analyses ont révélé que 11 chiens (11,7%) présentaient un sérum positif contre *leishmania infantum*. La méthode semi-nested-PCR a permis de confirmer l'infection par le *Leishmania infantum* chez 7 des 11 chiens errants séropositifs (63,6%). La présence d'ADN appartenant au *Leishmania infantum* a été détectée dans 36% des tissus hépatiques, 27% des tissus de rate et 54,5% des tissus de peau prélevés à partir de chiens errants séropositifs. Nos analyses sérologiques et moléculaires ont donc confirmés la présence de l'infection au *Leishmania Infantum* chez les chiens errants de la ville de Mechhed ne présentant aucun symptôme apparent.

Mots-clés: *Leishmania Infantum*, chien errant, ville de Mechhed

INTRODUCTION

Visceral leishmaniasis (VL) is known as a zoonotic and anthroponotic disease, and dogs and humans are the reservoirs of the infection (Baneth, 2005). *L. infantum* and *L. chagasi* are the main causes of zoonotic VL, while anthroponitic infection is caused by *L. donovani* in India and East Africa. In the Middle East, zoonotic VL is caused by *L. infantum*, and domestic dogs are the major reservoir hosts (Baneth, 2005). The prevalence of canine leishmaniasis varies in endemic areas (between 10% and 70% in the Mediterranean region) and is assumed to be hinged upon the climate (Mancianti et al., 1986; Kirmse et al., 1987). Therefore, the main reservoirs of VL caused by *L. infantum* in Iran are dogs and wild carnivores (Nadim et al., 1978; Hamidi et al., 1982; Bokai et al., 1998; Mohebali et al., 2001). Canine leishmaniasis is a chronic disease clinically characterized by lymphadenopathy, cutaneous manifestations, weight loss, and anemia. In addition, many infected dogs are asymptomatic and are reservoir hosts of the disease in endemic areas (Dantas-Torres, 2007; Moshfe et al., 2009; Hosseinijad et al., 2012). The molecular methods have high sensitivity and specificity to discriminate between various pathogens in humans and animals. The polymerase chain reaction (PCR) assay significantly improved the diagnosis of VL in men and animals (Lachaud et al., 2002). This study sought to investigate zoonotic VL in asymptomatic stray dogs by indirect immunofluorescent antibody test (IFAT) and semi-nested PCR.

MATERIALS AND METHODS

Study area. This study was performed during February 2011-12 in Mashhad, Iran. This city is located at 36.20° latitude and 59.35° east longitude. The mean annual temperature and precipitation are 26.7 °C and 255.7 mm, respectively. In Mashhad, summers are typically hot and dry and sometimes the temperature exceeds 35 °C (95 °F), and winters are typically cool to

cold and somewhat damper, with overnight lows routinely dropping below freezing.

Sampling. In this study, asymptomatic stray dogs were randomly selected during a zoonosis control program performed by Mashhad Municipality from February 2011-12. The blood samples were collected from jugular vein and kept in plain tubes. The blood tubes were transported to laboratory under cool condition. The blood clots were centrifuged for 5-10 min at 800 × g, the sera were stored at -20 °C prior to the serological examination. After a euthanasia procedure, tissue samples were collected from the spleen, liver, and skin of the stray dogs and stored at -20 °C for DNA extraction.

The Indirect Immunofluorescent Antibody Test (IFAT). In the present study, IFAT and *L. infantum* antigen (MegaCor Co., Austria MegaScreen FLUOLEISH) were used as the tools for serodiagnosis. For each reaction, 10 µl of a serum dilution (1:50) in phosphate-buffered saline (PBS) were added over the slide holes. The slides were incubated at 37°C and humidity of 70% for 30 min and rinsed with PBS for 5 min. The conjugate was diluted to 1:200 in PBS with 0.025% Evans Blue, and then 15 µl of this solution was placed over the slide holes. The incubation and washing steps were repeated once again as outlined above. The slides were mounted in buffered glycerin covered with a coverslip and read under an Olympus BX-FLA fluorescence microscope equipped with a 100W Mercury Apo lamp with 400 × magnification. The IFAT result was considered as positive if the dilution of serum gave an evident yellow-green fluorescent signal upon microscopic examination.

DNA extraction. DNA extraction from the obtained samples was carried out by DNA-plus Extraction Kit (CinnaGen Co., Iran) according to the manufacturer's instructions. The extracted DNA was stored at -20 °C for the detection of *Leishmania* kDNA detection.

PCR assay. As described by Aransay et al. in 2000, semi-nested PCR was employed for the amplification of the variable regions of the minicircle kDNA (slightly modified). Negative (tube without DNA) and positive

(DNA from *Leishmania*) controls were performed for each experiment. After amplification

, the PCR products were visualized on a 2% agarose gel electrophoresis stained with ethidium bromide under ultraviolet light.

Ethical consideration. Study protocols and methodologies were revised and approved by the Ethics Committee of Ferdowsi University of Mashhad, Mashhad, Iran.

Table 1. The results of seminested-polymerase chain reaction in the 11 seropositive stray dogs

No	Semi nested PCR		
	Skin	Spleen	Liver
1	-	+	-
2	-	-	-
3	+	-	+
4	+	-	+
5	-	-	-
6	+	+	+
7	-	-	-
8	+	+	-
9	+	-	-
10	+	+	-
11	-	-	-
Total	6 (54.5%)	4 (36%)	3 (27%)

RESULTS

Out of the 94 serum samples, 11.7 (11/94) of them were positive for *Leishmania*-specific antibodies with the titer of 1:50. The PCR positivity rate was 63.6 (7/11) in the tissue samples of the stray dogs. The kDNA of *L. infantum* was detected in 27% (3/11) of the liver, 36% (4/11) of the spleen, and 54% (6/11) of skin samples in asymptomatic stray dogs (Table 1). According to the results, four seropositive dogs were PCR negative.

DISCUSSION

Given the results of the present study, 11.7% of the serum samples collected from the asymptomatic stray dogs were positive for antibodies against *L. infantum*. In Iran, the sero-prevalence of canine leishmaniasis was reported to be 18.2% in Ardabil, 12.2% in Chaharmahal-and-Bakhtiari and Qom, and 4.4 % in Khuzestan and Bushehr provinces by using direct

agglutination test (DAT) (Mohebbali et al., 2005), 7.6% in Alborz Province by DAT (Haddadzade et al., 2013), 7.6% and 8% in Khorasan Razavi Province by DAT and IFAT, respectively (Adinezhadeh et al., 2013; Sabzevari et al., 2013; Heidarpoor et al., 2014), 8.9% in Tehran Province, 10% in Khuzestan Province, and 16% in Chaharmahal- and- Bakhtiari Province by DAT (Hosseininejad et al., 2012), and 15.4% in Kerman and Sistan-and-Baluchestan provinces by enzyme-linked immunosorbent assay (ELISA) (Mahshid et al., 2014). These serological studies confirmed that canine leishmaniasis is a common infection in different parts of Iran. The inconsistency between the results might be attributable to differences in sensitivity and specificity of the serological methods or the impact of climate change. In the present study, semi-nested PCR was used to detect the kDNA of *Leishmania* spp. in tissue samples of seropositive stray dogs (Aransay et al., 2000). Regarding the results of this study, semi-nested PCR is a useful method to detect *Leishmania* in reservoirs and vectors (Lachaud et al., 2002; Azizi et al., 2006; Rassi et al., 2007). Moreover, the kDNA of *L. infantum* was detected in 50% of the skin, 35.5% of the spleen, and 28.5% of the liver specimens of the dogs. According to the literature, the kDNA of *Leishmania* spp. was detected in the skin tissue more than the other tissues (Manna et al., 2004; de Andrade et al., 2006; Xavier et al., 2006; Solano-Gallego et al., 2009; de Queiroz et al., 2011; Reis et al., 2013). Although PCR method has high sensitivity, 36% of the seropositive dogs were PCR negative due to insufficient concentration of *Leishmania* kDNA in the tissue samples or the presence of PCR inhibitor. Our findings were in line with the results obtained by (de Andrade et al., 2006; Maia et al., 2009; Moshfe et al., 2009; Camargo et al., 2010; Hamarshah et al., 2012).

According to the results of the present study, a multitude of asymptomatic stray dogs were infected by *L. infantum* and the skin samples were better than the other tissues for the detection of *Leishmania* kDNA by PCR. Therefore, the results of PCR or serological data

alone are not sufficient for the diagnosis of leishmaniasis and both of them should be used to detect this parasite.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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