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■ Prevalence of leishmaniosis in dogs in Istanbul, Turkey determined by using PCR

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ABSTRACT. *Leishmania infantum* is widespread in Mediterranean countries including Turkey and can cause a serious disease in both humans and dogs. Dogs are considered to be its main reservoirs. The current epidemiological study was carried out in Istanbul for detection of leishmaniosis among dogs. A total of 246 dogs were included in the study. Twenty one (8.54%) blood samples were found to be positive based on PCR diagnosis, using primers specific for the kinetoplast DNA of *Leishmania*. Infection rate was 6.51% in 169 dogs living in shelters and 12.99% in 77 client-owned dogs. The rate was significantly ($p<0.001$) higher in the dogs (37.93%) presenting one or more clinical symptoms which may be attributable to leishmaniosis, than in the asymptomatic dogs (4.61%). Eleven (52.4%) of 21 PCR positive dogs presented clinical symptoms whereas the rest of the dogs (47.62%) were asymptomatic. The major clinical sign in PCR positive animals was dermatological problems. Amongst PCR positive dogs, skin lesions were present in 11, weight loss in 2, lymphadenopathy in 2, epistaxis in 2 and lethargy in 2 dogs.

Keywords: Dogs, *Leishmania*, PCR, Prevalence, Istanbul, Turkey

INTRODUCTION

Leishmaniosis is a zoonotic disease and an increasing public health problem worldwide (Aedo-Sánchez et al., 1996; Fisa et al., 1999; Sideris et al., 1999). *Leishmania* species cause a variety of human and animal diseases, ranging from self-limited cutaneous to the more severe diffuse cutaneous and visceral forms as consequence of the complex host immunological response depending on the invading *Leishmania* species (Grimaldi and Tesh, 1993; Gomes et al., 2008, Gramiccia M, 2011). Dogs are considered to be the

main reservoir of *L. infantum* constituting part of the epidemiological cycle of human transmission (Aedo-Sánchez et al., 1996; Fisa et al., 1999; Gramiccia, 2011). The phlebotomine sand flies are the natural vector of *Leishmania* and play an important role in the transmission (Alonso et al., 2010). The clinical signs of leishmaniosis seen in dogs were classified as: symptomatic, those presenting more than three clinical signs; oligosymptomatic, from one to three clinical signs; and asymptomatic dogs, with no clinical signs (Mancianti et al., 1988). The most

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frequent clinical signs reported were lymphadenomegaly, weight loss, poor appetite, dermatological lesions, onychogryphosis and to a lesser extent ocular lesions, epistaxis, renal failure, lameness, diarrhea and meningitis (Slappendel, 1988; Ciaramella et al., 1997; Vinuelasat al., 2001, Saridomichelakis et al., 2009).

The conventional diagnosis of leishmaniosis consists of serological tests like ELISA and immunofluorescence, direct examination of smears after Giemsa staining, culture of parasite and histopathological techniques (Grimaldi and Tesh, 1993; Gomes et al., 2008). These methods are not used for species identification. Therefore, molecular techniques are used to detect and discriminate the species in recent years (Lachaud et al., 2002; Ferroglio et al., 2006; Gramiccia, 2011). The PCR method has advantages as it is rapid, sensitive and specific (Moreira et al., 2007; Gomes et al., 2008). The aim of this study was to investigate the frequency of canine leishmaniosis in Istanbul (north-western Turkey). PCR was used to detect *Leishmania* in blood after the clinical examination.

MATERIALS AND METHODS

Study population and collection of samples

This study was conducted as a survey of canine leishmaniosis in Istanbul. Dogs were selected according to geographical localization and by willingness of the owner to participate in the study. Dogs were examined clinically and a report containing individual data for dogs was kept. Treatment was started in severely ill dogs.

Dogs of various breeds, age and sex, living in both European and Asiatic part of Istanbul were included in the study. 246 dogs were selected and clinically examined particularly for the presence of skin lesions, enlargement of lymph nodes, loss of appetite and epistaxis. According to clinical findings, dogs were divided into 2 groups as symptomatic and asymptomatic. Blood samples from client-owned dogs and dogs living in shelters were drawn into vacutainer tubes containing EDTA. Pearson's chi-squared test, or when appropriate Fisher's exact test was used to compare groups.

DNA extraction and PCR

DNA was extracted from the blood using a commercial kit as described by the manufacturer (EZ-10 Spin Column Blood Genomic DNA Minipreps Kit Biotechnology Department Bio Basic Inc, Canada) DNA extracts were stored at -20 °C until required. Samples were tested for the presence of kinetoplast DNA of *Leishmania* by PCR. The method of PCR used in this study was similar to those published by others (Ferroglio et al., 2006; Lachaud et al., 2002). Nucleotide sequences of primer pairs specific for *Leishmania* were same as in the study published previously (Lachaud et al., 2002) and primers were purchased from MWG (Germany). The forward and reverse primers for kinetoplast DNA were as follows: F:5'-CTTTCTGGTCCCGCGGGTAGG-3' R:5'-CCACCTGGCCTATTTACACCA-3' A 145 bp PCR product was expected by using these primers. Different amount of primers (by doing doubling dilutions), template DNA and Master Mix (Fermentas) containing dNTPs, magnesium and tag polymerase were used in different temperatures to optimize PCR conditions. A total volume of 25 µl standardized PCR reaction consisted of 25 µl of Master Mix, 2.5 µl of template DNA and 25 pmol of each primers and the mixture was put into the thermocycler (Hybaid). Distilled water as negative control without DNA and the positive control DNA kindly supplied by Professor Ezio Ferroglio (University of Torino, Department of Animal Production, Epidemiology and Ecology, Via Leonardo da Vinci 44, 10095 Grugliasco, Italy) were used as controls.

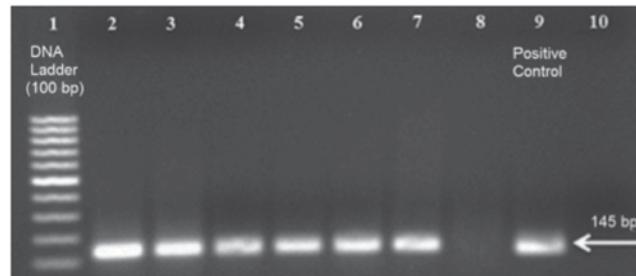


Figure 1. Products seen on gel electrophoresis after PCR performed for the presence of leishmania kinetoplast DNA. **1:** DNA ladder (100 bp); **2, 3, 4, 5, 6 and 7:** ~145 bp products amplified from the dog samples; **8:** Negative control; **9:** Positive control (~145 bp); **10:** A negative dog sample

The amplification was performed using the conditions as follows: 1 cycle of 94 °C for 2 min followed by 45 cycles of 94 °C for 1 min, 62 °C for 1 min 30 s, 70 °C for 45 s (increasing 5 s in each cycle) and, finally, 1 cycle of 70 °C for 10 min. Products were analysed by 1.5% agarose gel electrophoresis and visualised using ethidium bromide under UV light.

The sensitivity of PCR was assessed using DNA extracted from positive controls. Serial 10-fold dilutions of DNA (positive control DNA) were made in TE buffer and 2.5 µl of aliquots was tested in PCR as described above. The DNA extracted from positive and negative control was used for specificity.

RESULTS

A PCR product of 145 bp for kinetoplast DNA of *Leishmania* was detected in the positive control DNA and in 21 blood samples tested from the dogs (Figure 1). The PCR product was not seen in the negative control and negative samples (Figure 1). The PCR results and individual data about the positive dogs are shown in Table 1. The results of PCR have shown that kinetoplast DNA of *Leishmania* was detected in the blood of 21 (8.54%) amongst 246 dogs. The number of PCR positives among male and female

were evenly distributed. Percentage of positive dogs living in the shelters and owned dogs were 6.51% and 12.99%, respectively and this difference was not statistically significant (Table 1). Infection rate was significantly ($p<0.001$) higher in the dogs (37.93%) presented one or more clinical symptoms which may be attributable to leishmaniosis than the asymptomatic dogs (4.61%) (Table 1).

Out of 21 PCR positive dogs, 11 (52.38%) presented one or more clinical symptoms which may be attributable to leishmaniosis where as the rest of the dogs (47.62%) were asymptomatic. Dermatological problems were the most prominent clinical sign in PCR positive animals since skin lesions were present in all 11 symptomatic PCR positive dogs. Among these dogs, weight loss in 2, lymphadenopathy in 2, epistaxis in 2 and lethargy in 2 dogs were also observed.

DISCUSSION

Leishmaniosis is an important zoonosis mainly seen in the Mediterranean countries (Acedo-Sánchez et al., 1996; Uysal et al., 2003; Ferroglio et al., 2006; Athanasiou et al., 2012). Dogs are the main reservoirs for *Leishmania infantum* (Gomes et al., 2008; Gramiccia, 2011). Therefore, the frequency of the

Table 1. PCR results of 246 dogs, with respect to their gender, lifestyle and clinical status.

	Total number tested	Gender		Lifestyle		Clinical status	
		Male	Female	Shelter dogs	Owned dogs	Asymptomatic	Symptomatic
Total Tested	246	112	134	169	77	217	29
PCR positive	21	11	10	11	10	10	11
Percentage in Total	8.54	9.82	7.46	6.51	12.99	4.61	37.93
Significance			$(\chi^2=0.435, p=0.510)^*$		$(\chi^2=2.843, p=0.092)^*$		$(p<0.001)^{**}$

*Pearson's chi-squared test, **Fisher's exact test.

infection in dogs needs to be investigated in order to establish control programs. The aim of this study was to detect *Leishmania* in dogs by using molecular technique and to investigate the frequency in dogs in Istanbul. This is the first comprehensive study in Istanbul investigating leishmaniosis. During investigation PCR was used since it is easy, quick, sensitive and specific compare to other tests used for the diagnosis of leishmaniosis (Moreira et al., 2007; Gomes et al., 2008). This test overcomes the problems of for instance cross-reactions in serology, nonspecific appearance in microscopy since several problems reported in the specificity and sensitivity of other tests. The PCR is also suitable for investigating genetic differences in *Leishmania* by allowing sequence analysis (Lachaud et al., 2002; Ferroglio et al., 2006; Moreira et al., 2007).

The prevalence of leishmaniosis was investigated in the central Europe and Mediterranean bordering countries by using different diagnostic techniques as mentioned below. In central Europe, from February to October 2003, 291 dogs imported or returning from Southern Europe (Spain, Italy, Greece, Turkey, France, Malta, Portugal and others) were analysed for the presence of antibodies to *Leishmania*. 111 (38%) dogs were found to be positive; 103 being imported and eight travelling dogs. The majority of seropositive dogs were originated from Spain (67%) (Mettler et al., 2005). In a predictive study which included Spain, France, Portugal and Italy, the prevalence was estimated up to 23.2 % (Franco et al., 2011). Low and high prevalence has been reported in the Mediterranean countries where the disease is endemic. Canine leishmaniosis is considered to be endemic in northern and southern Italy with high prevalence reaching up to 30.3% (Zaffaroni et al., 1999; Paradies et al., 2006). However, lower prevalence (2.1% and 2.5%) was also reported in the northern Italy (Maroli et al., 2008; Baldelli et al., 2011).

In France, a study performed using the PCR and western-blotting techniques indicated that a high number of dogs in southern France (Berrahal et al., 1996) and up to 50 % in southeastern France have been exposed to *Leishmania* (Aoun et al., 2009). In another study in France, 79.8% and 29.6% positivity were found in 263 dogs by PCR and serology,

respectively (Lachaud et al., 2002).

Similar results were obtained in studies in Portugal (65%) and in Mallorca-Spain (67%) (Cabral et al., 1998; Solano-Gallego et al., 2001). In a studies in southern Spain, the prevalences were reported as 5.3% (Acedo-Sánchez et al., 1996) and 22% in dogs older than 1 year old (Alonso et al., 2010), whereas, in Catalonia, the seroprevalence was reported as 10.2% (Fisa et al., 1999). The seroprevalence in Madrid region found in 1,803 dogs analysed was 7.8% (Miró et al., 2007) and 8.1 % in another study in the same region (Gálvez et al., 2010). However, the seroprevalence was lower (3.7%) in the northwestern Spain (Amusategui et al., 2004).

In Greece (Sideris et al., 1999) and Iran (Moshfe et al., 2012), both neighboring countries to Turkey, *Leishmania* antibodies were detected in 22.4% and 10%, respectively. In another study in Greece, 2,620 sera belongs to clinically healthy dogs were analysed and the overall seropositivity was found as 20% (Athanasio et al., 2012). The overall prevalence found in Greece is higher than what was found in the present study (8.54%) and in some studies performed in Turkey as mentioned below.

Leishmaniosis in dogs have also been investigated in various locations of Turkey. Low and high prevalences were reported. Seropositivity to *Leishmania* in dogs was reported 4.3 % in Bursa, 33.3% in Muğla (Coskun et al., 1997), 1% (2/50) in Kırıkkale (Aydenizöz et al., 2010), 7.95% (14/176) in Antalya (Balcioğlu et al., 2009), 20.7 % in Denizli (Ozensoy Töz et al., 2009) and 2.58 % (3/116) in Ankara (Aslantaş et al., 2005). In addition, 5.3 % of 490 dogs in Manisa, (Ozbel et al., 2000), 3.2 % of 158 dogs in Izmir and Aydin (Voyvoda et al., 2004) and 9.1% of 109 dogs and 4.7% of 85 dogs in Kusadasi (Ozensoy et al., 2005) were found to be seropositive. Antibodies to *Leishmania* in 13.51% of 111 dogs from Eskisehir, Afyon and Bilecik (Dogan et al., 2005), 5.9% of 432 dogs from Istanbul, Bursa and Çorlu (Uysal et al., 2003) and 3.07% of 65 dogs in Kocaeli (Tamer et al., 2008) were also detected. However, no antibodies to *Leishmania* were detected in other studies in Turkey performed in Canakkale (Tok et al., 2009), Erzurum (Aktaş et al., 2010) and

Diyarbakir (İçen et al., 201).

Beside serological studies, there are few studies on direct detection of *Leishmania* in dogs, either by using antigen or DNA detection assays in Turkey. In one study (Ozbel et al., 2000), PCR was used to confirm leishmaniosis in seropositive dogs and the confirmation rate was found to be 13 (76.4%) in 17 seropositive dogs. A more recent study conducted in Kayseri province based on PCR detection of parasite revealed that none of the 300 dogs examined were positive for leishmaniosis (İça et al., 2008). Therefore, this study was focused on the detection of kinetoplast DNA of *Leishmania* in order to get specific results to avoid cross-reactions occurs in the serological tests.

In this study, 246 dogs were investigated by PCR and 8.54 % of positivity was detected. This result is close but a bit higher than those obtained by others as mentioned above indicating that a very densely populated city (Istanbul) is in the risk of leishmaniosis through dogs. However, it appears that Mediterranean countries in Europe have a high background of canine leishmaniosis which is likely to be a risk factor for the emergence of human leishmaniosis. As a result of those studies mentioned above, leishmaniosis in dogs in the regions investigated in this study is lower than what was found in the majority of studies performed in other Mediterranean countries. However, the cities included in this study are not located in the Mediterranean coast and therefore lower frequency can be expected.

In the present study, 11 (52.4%) of 21 PCR positive dogs were symptomatic and all of the dogs had skin problems. The results of this study indicate the clinical signs of the symptomatic dogs were similar to

those reported in other studies (Slappendel, 1988; Ciaramella et al., 1997, Saridomichelakis et al., 2009). However, the result of dogs' life style in this study contrast with other studies (Acedo-Sánchez et al., 1996; Zaffaroni et al., 1999) since the prevalence is higher in this study in the owned dog group. This may be associated with a traditional habit of dog owners in Turkey. In summer, the owners usually take their pets with them to the Mediterranean coast where the number of sand flies is higher compared to places from where the dogs living in the shelters and owned dogs in this study. The positivity in dogs living in the shelters in this study was 6.5 %. However, in other studies, it was found higher in outdoor group of dogs as 8.3% (Acedo-Sánchez et al., 1996) and 20.2% (Zaffaroni et al., 1999).

CONCLUDING REMARKS

Results of this study shows that *Leishmania* exist in Istanbul in a considerable frequency in dogs indicating there is risk of leishmaniosis to people. Further research on vectors present in Istanbul is needed to investigate the zoonotic cycle of *Leishmania*.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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