

Molecular and seroprevalence of canine visceral leishmaniasis in West Anatolia, Turkey

Serkan BAKIRCI^{1*}, Hüseyin Bilgin BİLGİÇ¹, Onur KÖSE¹, Ayca AKSULU¹,
Selin HACILARLIOĞLU¹, Hasan ERDOĞAN², Tülin KARAGENÇ¹

¹Department of Parasitology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey

²Department of Internal Medicine, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey

Received: 27.08.2015 • Accepted/Published Online: 23.03.2016 • Final Version: 02.11.2016

Abstract: Canine leishmaniasis (CanL) is a protozoal disease caused by *Leishmania* species. Dogs play an important role as a reservoir. The aim of this study is to determine the prevalence of CanL by molecular and serological techniques among stray dogs living in kennels in Aydın, Manisa, and İzmir, located in western Turkey. Blood samples were collected from a total of 191 dogs and analyzed using indirect immunofluorescence antibody test (IFAT) and polymerase chain reaction (PCR). The isolates that tested positive using PCR were further analyzed by real-time PCR for species identification and confirmation. A total of 21 (10.99%) dogs were found to be seropositive by IFAT. Molecular analysis revealed that 8 (4.19%) of the examined dogs were positive for *L. infantum/donovani* complex and two were found to be positive only by genus-specific PCR. The findings obtained from the screened animals indicate a higher risk of getting an infection caused by a *Leishmania* spp. in areas where the study was carried out. With this field study, it was obvious that to control the spread of disease into nonendemic areas, preventive measures like application of long-acting topical insecticides to infected and uninfected dogs should be taken. If necessary, positive dogs should be treated according to international guidelines and kept under control.

Key words: *Leishmania*, dog, IFAT, PCR

1. Introduction

Leishmaniasis is a protozoal disease caused by *Leishmania* species and transmitted by infected female phlebotomine sand flies (1). Leishmaniasis has been reported as one of the nine parasitic infections by WHO in 2013 and is commonly seen in 98 countries and three territories on five continents (2,3). There are three main clinical forms of leishmaniasis: visceral (VL), cutaneous (CL), and mucocutaneous (MCL) (4). Within the 1.3 million estimated new cases every year, 300,000 are VL and rest of them are CL or MCL (3). *L. infantum* is an obligate intracellular protozoan belonging to the *L. donovani* complex (1). *L. infantum* is the most important causative agent of canine leishmaniasis (CanL) in the world. The parasite is distributed primarily in the Mediterranean Basin countries, such as Spain, Italy, Portugal, and Turkey (4). Due to its geographical location and/or ecological and climatic features, Turkey plays an important role in the epidemiology of leishmaniasis (4). Two *Leishmania* species (*L. tropica* and *L. infantum*) are commonly seen in Turkey and they cause anthroponotic CL and zoonotic VL, respectively (2,4–6). In addition to these species, there are also some reports about the emergence of *L. donovani* and *L. major* in Southeastern

Anatolia, in Turkey (7,8). Previous studies conducted in different regions of Turkey demonstrated the prevalence of CanL was between 1.45% and 27.5% (6,9).

So far, two zymodemes of *L. infantum* (MON-1 and MON-98) have been isolated from dogs in Turkey (6,9). Dogs can be infected clinically and animals that recovered from acute infection play an important role as an important reservoir of the parasite. On their own, clinical signs of the infection are mostly insufficient for diagnostic purposes; specific laboratory tests are needed for the correct diagnosis of CanL (10). Definitive diagnosis requires the demonstration of parasite in a tissue smear (usually bone marrow or spleen) or in a specific culture (10). In the Mediterranean Basin countries where CanL is endemically seen, the disease is mainly diagnosed either by microscopic examination of biopsy smears or by detecting anti-*Leishmania* antibodies in serum samples using serological methods (9,11–13). However, microscopy and serological methods are unable to discriminate among different *Leishmania* spp. due to morphological similarities and cross-reactivity among *Leishmania* spp., respectively (13,14). In recent years, molecular techniques such as polymerase chain reaction (PCR) targeting different

* Correspondence: serkanbakirci@adu.edu.tr

regions in kinetoplast or nuclear DNA have been shown to be a sensitive and specific method for the detection of different *Leishmania* DNA at species level in tissue or blood samples (4,9,13,15–17).

The aim of the present study was to detect prevalence rates of CanL by molecular (conventional and real-time PCR) and serological (indirect immunofluorescence antibody test; IFAT) techniques among stray dogs living in kennels in three cities located in western Turkey: Aydın, Manisa, and İzmir.

2. Materials and methods

2.1. Ethical clearance

This study was approved by the Adnan Menderes University Animal Experimentation Ethics Committee according to the ethical principles of animal research (Authorization number: 2012/046 and date: 28 August 2012).

2.2. Study area and selection of dogs

Dogs were selected from the municipal kennels located in Aydın, Manisa, and İzmir cities, where CanL is endemic. Dogs visiting veterinary medicine clinics in Adnan Menderes University were also included in the study. All samples were collected between November 2013 and January 2014. A total of 191 dogs (Manisa n = 42; İzmir n = 108; Aydın n = 31, and faculty clinics n = 10) with different breeds, ages, and sexes were sampled. Blood samples were collected from vena cephalica antebrachii into EDTA tubes for DNA extraction and serum tubes for IFAT. Sera samples were stored at –20 °C until used. DNA was extracted from 300 µL of collected blood using the Promega Wizard Genomic DNA Extraction Kit (Madison, WI, USA), following the manufacturer's instructions. Extracted DNA was resuspended in a 100 µL buffer and stored at –20 °C.

2.3. PCR

A two-step PCR protocol was used to analyze *Leishmania* species in DNA samples. In the first step, DNA samples were used to amplify a 145 bp region of kDNA minicircles using RV1 (5'-CTT TTC TGG TCC CGC GGG TAG G-3') / RV2 (5'-CCA CCT GGC CTA TTT TAC AC-3') primers specific for parasites belonging to the genus *Leishmania*, including *L. infantum*, *L. donovani*, *L. major*, *L. tropica*, and *L. braziliensis* (18,19). Then all genus-positive samples were further analyzed in a second step of PCR using MC1 (5'-GTT AGC CGA TGG TGG TCT TG-3') and MC2 (5'-CAC CCA TTT TTC CGA TTT TG-3') primers amplifying a 447 bp region of *L. infantum/donovani* complex kDNA minicircles (20). PCR reaction was performed in a total volume of 50 µL, containing 10 mM Tris-HCl, (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 200 µM of each deoxynucleotide triphosphate, 1.5 units of Hot start *Taq* DNA polymerase (Solis BioDyne, Tartu, Estonia), 25 µM

of each related primer (RV1/RV2 or MC1/MC2), and 2 µL of template DNA (10–20 ng of DNA). The reactions were performed in an automated thermal cycler (Veriti; Applied Biosystems, Foster City, CA, USA) and consisted of an initial denaturation step at 94 °C for 10 min, followed by 45 cycles of denaturation (95 °C for 1 min), primer annealing (62 °C for 1.5 min), with an exception for MC1/MC2 primers carried out at 50 °C for 1.5 min and primer extension (72 °C for 30 s). A final extension at 72 °C for 10 min was performed and the samples held at 4 °C. Aliquots of 10 µL PCR product was electrophoresed on a 1.5% agarose gel containing 10 µL/mL Safe View in Tris-acetate-EDTA buffer at 100 V for 1 h and visualized under UV light.

2.4. Real-time PCR

Additionally, all *Leishmania* genus-positive samples were also analyzed with ITS-1 real-time PCR for species identification. Real-time PCR was performed using species-specific primers and probes for detecting *L. tropica*, *L. major*, and the *L. infantum/donovani* complex (4). Ready-to-use real-time PCR master mix (QuantiTect Probe PCR master mix; Qiagen, Hilden, Germany) was used with 10 pmol of each primer, 200 nM of each probe, and 10–20 ng of DNA. The amplification conditions were as follows: 95 °C 10 min, followed by 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at 50 °C for 10 s, and extension at 72 °C for 20 s. After the cooling step, melting analysis was performed, starting at 50 °C and increasing by 1 °C each minute to 80 °C. Melting curves were analyzed using channels 2 and 3.

2.5. IFAT

The diagnostic efficacy of PCR was tested in comparison to IFAT. IFAT was performed as previously described (21), using promastigotes of a local *L. infantum* (MON-1) stocks as an antigen. Antigen slides were prepared as described in a previous study (21) and were stored at –20 °C until used. Each antigen slide included positive and negative control serum samples diluted at 1:16, 1:32, 1:64, and 1:128 in PBS and PBS alone as a conjugate control. Rabbit anti-dog IgG conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO, USA) at a dilution of 1:250 in PBS was used as second antibody. PBS containing 0.01% of Evans Blue (Sigma) was used for counterstaining. Slides were examined using an Olympus BX51 fluorescence microscope (Tokyo, Japan). The degree of fluorescence was recorded as strong fluorescence, weak, or no fluorescence. Only strong fluorescence was accepted as positive (+). Weak fluorescence and no fluorescence at all were considered negative (–). The IFAT titer of 1:64 was used as cut-off value for the positivity of serum samples.

2.6. Data analysis

The chi-squared test was used to test for association among seropositivity determined by IFAT relative to other

variables like sex, breed, age, and origin. Analyses were performed with SPSS (SPSS Inc., Chicago, IL, USA, version 22.0) and $P < 0.05$ was considered statistically significant.

3. Results

The IFAT and PCR results of the analyzed samples are shown in Table 1. According to IFAT results, 21 out of 191 dogs (10.99%) were found to have anti-*Leishmania* antibodies at a titer of $\geq 1/64$, which was considered seropositive. Seroprevalence of CanL ranged from 4.65% to 30% among different kennels located in the study area. The highest (21.95%) and lowest (7.4%) seropositivity rates were found in dogs from kennels in Aydın and İzmir, respectively. Seropositivity was 9.52% in dogs from kennels in Manisa.

Results obtained from PCR using the RV1/RV2 primer set showed that 10 (5.23%) out of 191 samples were positive for the genus *Leishmania* (Figure 1A). One out of 42 dogs was found to be infected with *Leishmania* spp. from kennels in Manisa, while 4 out of 41 and 5 out of 108 were found to be positive in Aydın and İzmir, respectively. Further PCR analysis using MC1/MC2 primers revealed that 8 out of 10 *Leishmania*-genus-positive samples were found to be infected with the *L. infantum/donovani* complex (Figure 1B). MC1/MC2 PCR results were further confirmed by ITS-1 real-time PCR. According to the species-specific melting curve analysis of the real-time PCR, 8 out of 10 *Leishmania*-genus-positive samples were positive for the *L. infantum/donovani* complex (Figure 2A). The remaining

two samples were considered *Leishmania* spp. with genus-specific kDNA PCR. *L. tropica* and/or *L. major* (Figures 2A and 2B) were not identified in our study group according to real-time PCR and species-specific kDNA PCR analysis.

Data analysis showed that there was no significant difference in the prevalence determined using IFA among the age, sex, and origin groups (Table 2). However, there were significant differences in the overall seropositivity obtained by IFAT ($P = 0.003$) among different breeds of dogs (Table 2).

4. Discussion

CanL, caused by *L. infantum*, is endemic throughout the Mediterranean and Aegean regions of Turkey (4,9,11). Canine hosts mainly serve as reservoirs and play an important role in the transmission of the parasite to humans by sand flies (22). Diagnosis of CanL is crucial for the assessment of preventive control measures and for estimating the prevalence of the current disease (9,11). Epidemiological surveys of CanL are mostly based on microscopic examination, in vitro cultivation, and serological and molecular diagnosis (4,23). However, conventional methods like microscopic examination have low and/or moderate sensitivity in carrier animals and are incapable of discriminating among different *Leishmania* species (4,22).

Techniques such as IFAT, ELISA, C-ELISA, Dot-ELISA, and DAT have usually been used for serodiagnosis of CanL (13, 22). IFA is most commonly used test to detect

Table 1. Cross-tabulation of IFAT and RV1/RV2 PCR results.

Region			IFAT		Total	
			Positive	Negative		
İzmir	RV1/RV2 PCR	Positive	3	2	5	
		Negative	5	98	103	
	Total		8	100	108	
Aydın	RV1/RV2 PCR	Positive	3	1	4	
		Negative	6	31	37	
	Total		9	32	41	
Manisa	RV1/RV2 PCR	Positive	1	0	1	
		Negative	3	38	41	
	Total		4	38	42	
				IFAT		Total
				Positive	Negative	
Overall	RV1/RV2 PCR	Positive	7	3	10	
		Negative	14	167	181	
		Total	21	170	191	

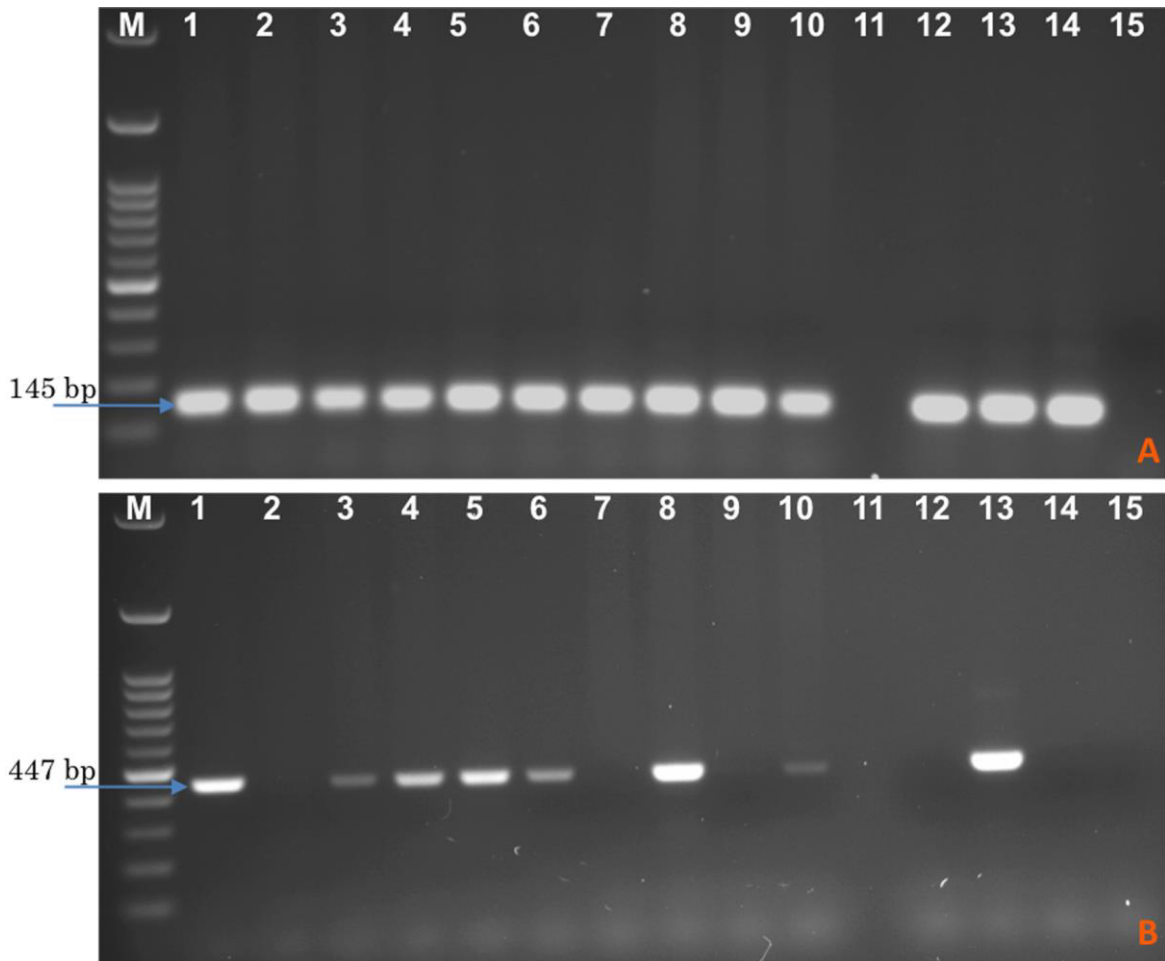


Figure 1. Agarose gel electrophoresis of amplified DNA from collected samples using (A) RV1/RV2 and (B) MC1/MC2 primer sets. M, 100 bp molecular size marker (Invitrogen, Carlsbad, CA, USA); lanes 1–10, template DNA isolated from stray dog blood samples from kennels located in Aydın, Manisa, and İzmir; 11, empty; 12, *L. major*-positive control DNA; 13, *L. infantum*-positive control DNA; 14, *L. tropica*-positive control DNA; 15, negative PCR control (water). Arrows indicate the 145 and 447 bp amplicons specifically generated using RV1/RV2 and MC1/MC2 primer sets, respectively. A very faint band was observed in lane 9 at 447 bp.

anti-*Leishmania* antibodies, but it can cross-react among different *Leishmania* species (22,24,25). In previous studies carried out in Turkey, the seroprevalence of CanL ranged between 2.5% and 23% in the Aegean Region, where infection is endemically seen (11,26–28). In this study, seroprevalence of *Leishmania* species in stray dogs from kennels located in Aydın, Manisa, and İzmir were screened with IFAT using promastigotes of local *L. infantum* MON1 strain as an antigen. Overall, IFAT results showed that 21 out of 191 dogs (10.99%) were serologically positive and the results obtained in this study support the previous studies based on the detection of seroprevalence of CanL in Turkey.

In recent years, different molecular techniques, such as PCR, multiplex PCR, RFLP, and nested PCR, have successively been evaluated for the differential diagnosis of

Leishmania species causing CanL (4,22). Today, researchers are able to specifically identify *Leishmania* species by PCR via targeting different regions of kinetoplast and nuclear DNA (1,18,20). kDNA minicircles were considered good candidates for detecting *Leishmania* spp. because of their high copy numbers (13). In this study, RV1/RV2 and MC1/MC2 primer pairs were used to PCR amplify 145 and 447 bp regions of LT1 fragment of kDNA minicircles of *Leishmania* spp. and the *L. infantum*/*donovani* complex, respectively. In addition to conventional PCR targeting kDNA, all genus-positive samples were also screened for the presence of *L. tropica*, *L. major*, and *L. infantum*/*donovani* complex by real-time PCR targeting the ITS-1 region between the SSU and 5.8S rRNA genes (4).

During the past years, *L. infantum* DNA has been detected in samples collected with molecular tests from dogs

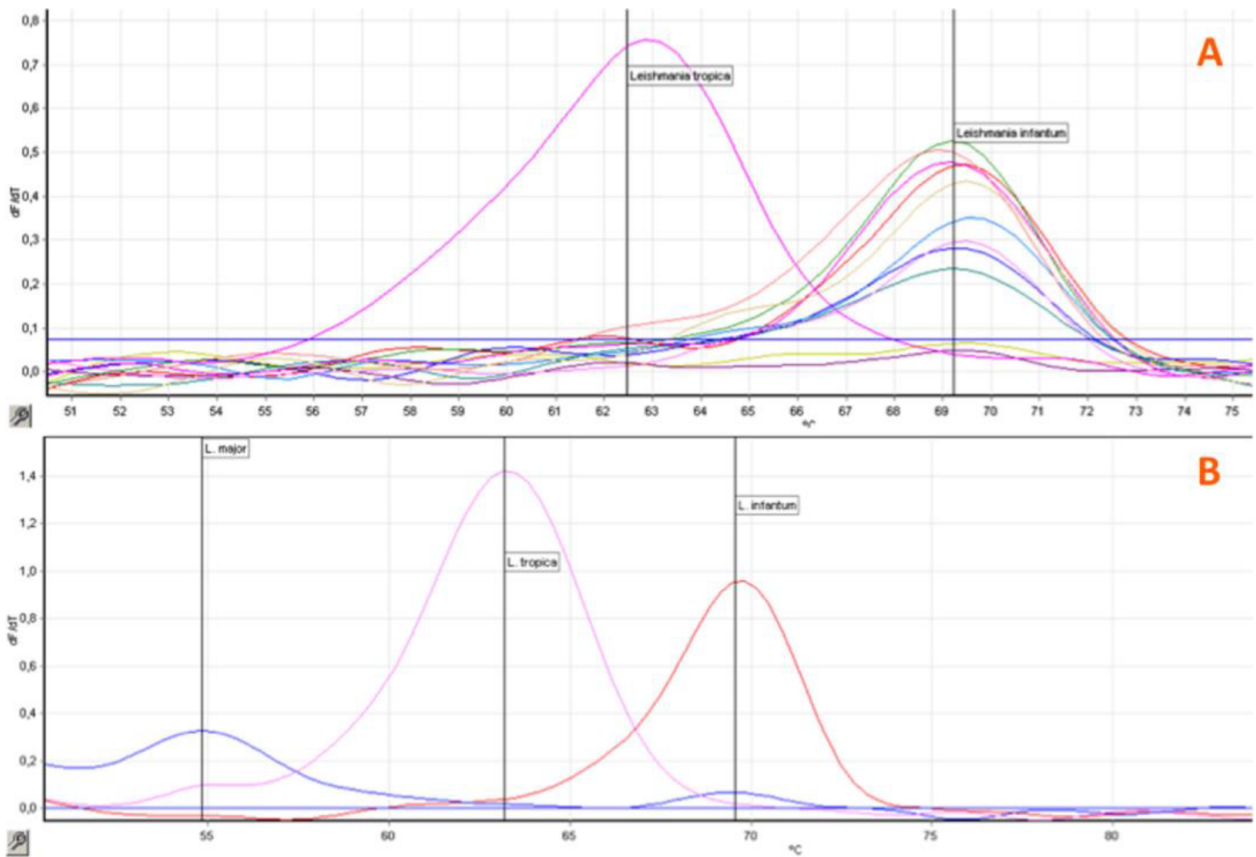


Figure 2. A melting curve analysis of ITS-1 real-time PCR showing species-specific melting peaks for (A) *L. infantum* (MHOM/TN/80/IPT1), *L. tropica* (MHOM/SU/74/SAF-K27) internal control DNA and DNA samples from 10 *Leishmania*-genus-positive dogs; (B) *L. major* (MHOM/SU/73/5ASKH) internal control DNA.

in Aydın and İzmir provinces (4,9). In Manisa Province, molecular studies based on the detection of *Leishmania* species with PCR indicated a low level (5.3%) of infection rate among dogs (11). Overall, the PCR results obtained in this study showed that 10 out of 191 dogs (5.23%) were *Leishmania*-genus-positive by RV1/RV2 PCR. Further PCR analysis indicated that 8 out of 10 *Leishmania*-genus-positive samples were found to be infected with the *L. infantum/donovani* complex, and that the remaining two samples were only found to be positive by genus-specific PCR. The results obtained in this study indicate that CanL continues to be an important anthroponotic disease in different regions of the Western Aegean Region. It is obvious that the extent of CanL in this region needs to be further determined by larger epidemiological studies. *L. tropica* and *L. major*, the etiological agents of CL, were also reported in Turkey (4–8). Besides *L. infantum*, Toz et al. (2013) detected *L. tropica* as a causative agent of CanVL in Turkey (4). In the present study, PCR results revealed a lower prevalence of the disease than that obtained by IFAT (Table 1). In dogs, PCR sensitivity is higher in samples collected from bone marrow, lymph nodes, spleen, and

culture isolates than in blood (4). In this study, PCR was performed using blood samples; this can explain the higher levels positivity detected by IFAT. It is obvious that the importance of sampling size needs to be considered in studies aiming to determine the prevalence of CanL in areas where the disease is endemically seen.

Except three dogs (two from İzmir and one from Aydın), 7 out of the 10 dogs who were RV1/RV2-positive according to PCR were also found to be seropositive according to IFAT (Table 1). Diagnostic tools, such as demonstration of specific antibodies, antigens, or parasite DNA in peripheral blood, depend on the clinical and/or immunological status of the animal, the methods employed, and laboratory experience (10). These seronegative but PCR positive dogs either had a prior, acute infection or they were long-term carriers with undetectable antibody levels. In both cases, antibody titers were under detectable levels. However, none of these three dogs showed any detectable clinical symptoms during the sampling. Geographic origin and antigen repertoire of the parasite also play significant roles in the serodiagnosis of the disease (10) and these three positive PCR samples

Table 2. Epidemiological data and serological results of the 191 dogs tested for *Leishmania* spp. in the Western Aegean Region.

İzmir, Aydın, Manisa	No. of dogs	Results IFAT (%)	Chi-squared P-values*
Breed			
Pit bull	1	1(100%)	
Mixed breed	116	12(12.12%)	
Presa canario	2	2(100%)	
Bull shepherd	2	1(50%)	
Doberman	5	2(40%)	
Kangal	10	2(20%)	
Golden retriever	12	1(8.3%)	
Pointer	6	-	
Cane corso	13	-	
Doga canario	15	-	
Cavalier King Charles spaniel	1	-	
German shepherd	2	-	
Bull terrier	6	-	
Total	191	21 (10.99%)	0.003*
Sex			
Male	78	7 (9%)	
Female	113	14 (12.4%)	
Total	191	21 (10.99%)	0.458
Age			
<2 years	11	2(18.2%)	
2-4	118	14(11.9%)	
4-7	51	3(5.9%)	
>7	11	2(18.2%)	
Total	191	21(10.99%)	0.455

(*) indicates statistically significant P-values (<0.05) based on the chi-squared test.

may originate from a different region with a distinct and/or highly polymorphic antigenic structure; however, this prediction needs to be further investigated. In 14 out of 21 seropositive dogs, RV1/RV2 PCR was unable to detect and/or amplify the target DNA (Table 1). The level of parasitemia should drop to undetectable levels after a certain period of time and generally remain very low during the late phase of the infection (1). These 14 animals were either sampled when the level of parasitemia was not sufficient to be detected by PCR, or the sensitivity of PCR was not enough to detect the parasite in such a low level of parasitemia. Of the dogs in Manisa, Aydın, and İzmir that tested seropositive according to IFAT, 75%, 67%, and 62.5% were found to be negative according to PCR, respectively. Such high numbers of seropositivity should be related to

immune status and origin of the sampled animals; however, statistical analysis revealed no significant difference among the age, sex, and origins groups (Table 2). Cringoli et al. indicated that a correlation exists between dog breeds and *L. infantum* seroprevalence (29). They observed that setters and pit bulls had the highest rates of infection, and that Yorkshires had the lowest (29). In another study, foxhound breeds were found to have a higher rate of seroprevalence than other breeds (30). In the present study, the mixed-breed dogs were found to have significantly higher levels of infection. This situation is closely associated with the biology of the vector. The conditions of municipal kennels are also possibly involved in this correlation.

The prevalence of CanL is directly related to the presence and/or prevalence of vector population,

ecological conditions such as moisture and climate, immunological status, and/or reservoir population (28). The results from dogs in this study and the known coexistence of vector populations within the sampling areas (27) indicate an increased possibility of infection caused by a *Leishmania* sp. Fortunately, it was shown that seropositivity detected in dogs does not always overlap with the number of human VL cases (27). The possible role of dogs in the epidemiology of human VL cases is still not very clear. These seropositive dogs (27) indicate a possible carrier status or a past infection without any parasitemia in the circulating blood that can be transmitted to sand flies. In previous studies, the number of human VL cases was quite low in Aegean Region, Turkey (27). The application of PCR and serology not only helps to detect the extension of subclinical infections but also allows the estimation of the number of dogs to be targeted for the control measures in areas where CanL is endemically seen (11,15). In this study, the results obtained from screened animals indicate an ongoing higher risk of infection caused by *L. infantum* and/or *Leishmania* spp.

In conclusion, in areas where CanL is endemically seen, preventive measures like application of long-acting topical insecticides to dogs should reduce the risk of new infections by reducing the number of sand fly bites on infected dogs. In necessary cases, seropositive and/or PCR positive dogs should be treated according to international guidelines and protected against sand fly bites by using repellent dog collars and kept under control for the rest of their lives. With this preliminary field study, it was obvious that to prevent the spread of the disease into nonendemic areas, dogs should be screened periodically for the presence of the disease.

Acknowledgments

This study was supported by Adnan Menderes University Research Fund (grant number: VTF-13004, primary investigator: Serkan Bakırcı). The authors are grateful to Prof Sema ERTUĞ and Prof Hatice Ertabaklar for providing *Leishmania*-positive controls. The authors would like to thank Dr Mehmet Karakuş for his collaboration during the real-time PCR.

References

1. Ready PD. Epidemiology of visceral leishmaniasis. *Clin Epidemiol* 2014; 6: 147-154.
2. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, the WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 2012; 7: e35671.
3. WHO. Sustaining the drive to overcome the global impact of neglected tropical diseases. Second Who Report on Neglected Tropical Diseases 2013; 67-71.
4. Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarli AT, Gunduz C, Ozbel Y. A real-time ITS1-PCR based method in the diagnosis and species identification of leishmania parasite from human and dog clinical samples in Turkey. *PLoS Negl Trop Dis* 2013; 7: e2205.
5. Ok UZ, Balcioglu IC, Ozkan AT, Ozensoy S, Ozbel Y. Leishmaniasis in Turkey. *Acta Trop* 2002; 84: 43-48.
6. Ozbel Y. The infections transmitted by sand flies in Turkey. *Ankara Üniv Vet Fak Derg* 2013; 60: 225-228.
7. Koltas IS, Eroglu F, Alabaz D, Uzun S. The emergence of *Leishmania major* and *Leishmania donovani* in southern Turkey. *Trans R Soc Trop Med Hyg* 2014; 108: 154-158.
8. Zeyrek FY, Gürses G, Uluca N, Doni NY, Toprak Ş, Yeşilova Y, Çulha G. Is the agent of cutaneous leishmaniasis in Sanliurfa changing? First cases of *Leishmania major*. *Türkiye Parasitol Derg* 2014; 38: 270-274 (article in Turkish with an English abstract).
9. Toz SO, Nasereddin A, Ozbel Y, Ertabaklar H, Culha G, Sevil N, Alkan MZ, Jaffe CL. Leishmaniasis in Turkey: molecular characterization of leishmania from human and canine clinical samples. *Trop Med Int Health* 2009; 14: 1401-1406.
10. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol* 2002; 9: 951-958.
11. Ozbel Y, Oskam L, Ozensoy S, Turgay N, Alkan MZ, Jaffe CL, Ozcel MA. A survey on canine leishmaniasis in western Turkey by parasite, DNA and antibody detection assays. *Acta Trop* 2000; 74: 1-6.
12. Ertabaklar H, Toz SO, Ozkan AT, Rastgeldi S, Balcioglu IC, Ozbel Y. Serological and entomological survey in a zoonotic visceral leishmaniasis focus of North Central Anatolia, Turkey: Corum province. *Acta Trop* 2005; 93: 239-246.
13. Ozerdem D, Eroglu F, Genc A, Demirkazik M, Koltas IS. Comparison of microscopic examination, rK39, and PCR for visceral leishmaniasis diagnosis in Turkey. *Parasitol Res* 2009; 106: 197-200.
14. Eroglu F, Koltas IS, Genc A. Identification of causative species in cutaneous leishmaniasis patients using PCR-RFLP. *J Bacteriol Parasitol* 2011; 2: 1000113.
15. Andrade HM, Reis AB, Santos SL, Volpini AC, Marques MJ, Romanha AJ. Use of PCR-RFLP to identify *leishmania* species in naturally-infected dogs. *Vet Parasitol* 2006; 140: 231-238.
16. Baneth G, Aroch I. Canine leishmaniasis: A diagnostic and clinical challenge. *Vet J* 2008; 175: 14-15.
17. Hernández L, Montoya A, Checa R, Dado D, Gálvez R, Otranto D, Latrofa MS, Baneth G, Miró G. Course of experimental infection of canine leishmaniasis: Follow-up and utility of noninvasive diagnostic techniques. *Vet Parasitol* 2015; 207: 149-155.

18. Fichoux YL, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D, Kubar J. Occurrence of *Leishmania infantum* parasitemia in symptomatic blood donors living in an area of endemicity in Southern France. *J Clin Microbiol* 1999; 37: 1953-1957.
19. Gao CH, Ding D, Wang JY, Steverding D, Wang X, Yang YT, Shi F. Development of a LAMP assay for detection of *Leishmania infantum* infection in dogs using conjunctival swab samples. *Parasit Vectors* 2015; 8: 370.
20. Cortes S, Rolão N, Ramada J, Campino L. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* *s.l.*-specific kinetoplastid primers. *Trans R Soc Trop Med Hyg* 2004; 98: 12-17.
21. Abranches P, Silva-Pereira MCD, Conceiao-Silva FM, Sontos-Gomes GM, Janz JG. Canine leishmaniasis: pathological and ecological factors influencing transmission of infections. *J Parasitol* 1991; 77: 557-561.
22. Ica A, Inci A, Yıldırım A, Atalay O, Duzlu O. Investigation of canine leishmaniasis by Nested-PCR in Kayseri and vicinity. *Türkiye Parasitol Derg* 2008; 32: 187-191 (article in Turkish with an English abstract).
23. Aydenizöz M, Yağcı BB, Taylan Özkan A, Duru SY, Gazyağcı AN. Investigation of the prevalence of visceral leishmaniasis by the microculture method and IFAT in dogs in Kırıkkale. *Türkiye Parasitol Derg* 2010; 34: 1-5 (article in Turkish with an English abstract).
24. Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniasis. *Vet Parasitol* 2009; 165: 1-18.
25. Bourdeau P, Saridomichelakis MN, Oliveria A, Oliva G, Kotnik T, Galvez R, Manzillo VE, Koutinas AF, Fonseca IF, Miró G. Management of canine leishmaniasis in endemic SW European regions: a questionnaire-based multinational survey. *Parasit Vectors* 2014; 7: 110.
26. Voyvoda H, Pasa S, Toz SO, Ozbel Y, Ertabaklar H. Prevalence of *Leishmania infantum* and *Dirofilaria immitis* infection in dogs in Aydın province and the town of Selçuk, İzmir, Turkey. *Turk J Vet Anim Sci* 2004; 28: 1105-1111 (article in Turkish with an English abstract).
27. Toz SO, Sakru N, Ertabaklar H, Demir S, Sengul M, Ozbel Y. Serological and entomological survey of zoonotic visceral leishmaniasis in Denizli Province, Aegean Region, Turkey. *New Microbiol* 2009; 32: 93-100.
28. Atasoy A, Pasa S, Toz SO, Ertabaklar H. Seroprevalence of canine visceral leishmaniasis around the Aegean coast of Turkey. *Kafkas Univ Vet Fak Derg* 2010; 16: 1-6.
29. Cringoli G, Rinaldi L, Capuano F, Baldi L, Veneziano V, Capelli G. Serological survey of *Neospora caninum* and *Leishmania infantum* co-infection in dogs. *Vet Parasitol* 2002; 106: 307-313.
30. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, Rowton ED, Schantz PM. Canine visceral leishmaniasis, United States and Canada, 2000-2003. *Emerg Infect Dis* 2006; 12: 440-446.