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Research Article / Araştırma Makalesi

Molecular Prevalence of Canine Leishmaniasis in Burdur, Türkiye

Burudur'da Kanin Leishmaniazisin Moleküler Prevalansı

Önder ÖZEN[1], Onur KÖSE[2*]

[1]Ortaca District Directorate of Agriculture and Forestry, Muğla, Türkiye
[2]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Parasitology, Burdur, Türkiye

Abstract: Canine leishmaniasis (CanL) is among the most important vector-borne parasitic diseases in dogs worldwide, except for Oceania, and can be fatal if left untreated. The present study aimed to investigate the molecular prevalence of *L. infantum*, the causative agent of CanL, in Burdur province of Türkiye. Blood samples collected from 120 dogs were first examined for *Leishmania* spp. by genus-specific PCR and then positives were examined for *L. infantum/donovani* complex by species-specific PCR. As a result, *Leishmania* DNA was detected in five out of the 120 samples in the first-stage PCR, resulting in a molecular positivity rate of 4.16% at the genus level. Among these five samples, four were positive for *L. infantum* in the second-stage PCR, leading to a prevalence of 3.33% for *L. infantum*. No statistically significant differences were found in terms of gender and age concerning *L. infantum* positivity. Additionally, blood smears were examined under a microscope, but no *Leishmania* amastigotes were observed in any of the samples. With this obtained data, the presence of *Leishmania* spp. and *L. infantum* in dogs in Burdur is reported for the first time using a molecular method. The importance of vector fly control has been remembered once again in order to protect from the disease both animals that can be host of parasites, especially dogs, and human society, since it is a zoonotic disease.

Keywords: Burdur, Canine leishmaniasis, *Leishmania infartum*, PCR, Prevalence.

Öz: Kanin leishmaniasis (CanL); Okyanusya haricindeki tüm kıtalarda görülen ve tedavi edilmediği durumlarda ölümcül olabilen, köpeklerin en önemli vektör kaynaklı paraziter hastalıkları arasında yer almaktadır. Bu çalışma ile Burdur ilinde kanin leishmaniasis etkeni olan *L. infantum*'un moleküler yaygınlığının araştırılması amaçlanmıştır. Bu amaçla 120 köpekten toplanan kan örnekleri önce cins düzeyinde *Leishmania* spp. sonra da tür düzeyinde *L. infantum* primerleri kullanılarak Polimeraz Zincir Reaksiyonu (PCR) yöntemi ile incelenmiştir. Sonuç olarak birinci aşama PCR ile incelenen 120 köpek kan örneğinin beş tanesinde *Leishmania* DNA'sı saptanmış, böylece *Leishmania* cins düzeyinde moleküler pozitiflik oranı %4,16 olarak bulunmuştur. Cins düzeyinde pozitiflik saptanan bu beş örneğin *L. infantum* için yapılan ikinci basamak PCR sonucunda ise dört örnek pozitif sonuç vermiş ve *L. infantum* prevalansı %3,33 şeklinde bulunmuştur. Cinsiyet, yaş ve ırk ile *L. infantum* pozitifliği arasında istatistiksel olarak anlamlı bir farklılık bulunmamıştır. Aynı zamanda kan frotileri de mikroskobik bakı ile incelenmiş ancak örneklerin hiçbirinde *Leishmania* amastigotlarına rastlanmamıştır. Elde edilen bu veriler ile Burdur'da ilk defa moleküler tabanlı bir yöntem olan PCR ile köpeklerde *Leishmania* spp. ve *L. infantum* pozitifliği bildirilmektedir. Hastalıktan hem köpekler başta olmak üzere parazite konaklık yapabilen hayvanları hem de zoonoz bir hastalık olması nedeniyle insan toplumunu koruyabilmek için vektör sinek mücadelesinin önemini bir kez daha hatırlanmıştır.

Anahtar Kelimeler: Burdur, Kanin leishmaniazis, *Leishmania infartum*, PCR, Prevalans.

*Corresponding author : Onur KÖSE
e-mail : onurköse@mehmetakif.edu.tr
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Introduction

Leishmaniasis is a vector-borne, zoonotic protozoan disease mostly seen in tropical and subtropical climates (Lamotte et al., 2017; Steverding, 2017). *Leishmania* species, the causative agents of the disease, are flagellated protozoans in the Trypanosomatidae family; use some carnivores, rodents, lizards, insectivores, especially humans and dogs as definitive, *Phlebotomus* (in

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Europe, Asia and Africa) and *Lutzomyia* sandflies (in America) as intermediate hosts and transmitted by infected female sandflies sucking blood (Ayele and Seyoum, 2016; Alemayehu and Alemayehu, 2017). *Leishmania* species are heteroxene parasites with a life cycle between sandflies where they multiply as free promastigotes in the intestinal lumen and mammalian hosts where they multiply as obligate intracellular amastigotes in mononuclear phagocytic cells (Handman, 1999; Lamotte et al., 2017). The disease may be observed in different clinical forms of varying characters and severities, based on the parasitized species and immune response against the infection. According to this, the disease may have various names such as; visceral leishmaniasis (VL or Kala Azar), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and diffuse cutaneous leishmaniasis (DCL) (Akhoundi et al., 2016; Steverding, 2017; Galluzzi et al., 2018).

Dogs are in a critical position for public health, because of they are reservoir hosts of zoonotic *Leishmania* species, in addition canine leishmaniasis (CanL) is among the most important vector-borne parasitic diseases of all ages and breeds of dogs in both cutaneus and visceral forms (Slappendel and Greene, 1990; Ribeiro et al., 2018). Leishmaniasis may occur in all continents except Oceania and can be fatal when untreated (Baneth et al., 2008; Dantas-Torres et al., 2012; Travi et al., 2018; Olias-Molero et al., 2019). The primary responsible pathogen of canine leishmaniasis is *L. infantum* in the *L. donovani* group, in almost every region of the world, especially in Mediterranean countries such as Turkey, Italy, Spain and Portugal (Ready, 2014; Ribeiro et al., 2018; Teimouri et al., 2018; Olias-Molero et al., 2019). In addition, canine leishmaniasis cases have been reported caused by *L. tropica* and *L. major* (Bamorovat et al., 2015; Baneth et al., 2014; 2017; Hakkour et al., 2019). In the Americas (especially Central and South parts), the most important agent responsible for canine leishmaniasis is *L. chagasi*, which is synonymous (identical) of *L. infantum* (Akhoundi et al., 2016; Marcondes and Day, 2019).

Various prevalence studies have been carried out for leishmaniasis in Turkey and the most commonly reported species were *L. tropica* and *L. infantum* (Özbel et al., 2000; Ertabaklar et al., 2005; İça et al., 2008; Özensoy Töz et al., 2009; Aydenizöz et al., 2010; Bakırcı et al., 2016; Ünlü et al., 2019). There are also reports for *L. major* and *L. donovani* (Koltaş et al., 2014; Zeyrek et al., 2014). According to the results of the previous studies; the prevalence of canine leishmaniasis (regardless of diagnostic method) were reported between 37.4% from different geographical regions of Turkey.

Studies on obtaining epidemiological data, especially prevalence of pathogens, have priority for the design and implementation of effective prevention and control strategies against diseases. Therefore, the aim of the present study was to investigate the prevalence of *L. infantum*, mainly causative agent of CanL, in dog blood samples in Burdur province by using Polymerase Chain Reaction (PCR).

Materials and Methods

Ethical Approval

The present study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (date: 17.02.2021 and decision number: 2021/722).

Blood samples and Deoxyribonucleic acid (DNA) extraction

The blood specimens were obtained from dogs visiting Animal Hospital of Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine and private veterinary clinics with various complaints and municipal animal shelter in Burdur city. A total of 120 dogs with different age, sex and breed were sampled and 5-6 ml of blood specimens were taken from cephalic vein (*vena cephalica antebrachii*) into EDTA-containing blood tubes. All samples were collected between June and October 2021. DNA extraction was performed from 300 µL of each blood sample via

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Promega Wizard Genomic DNA Extraction Kit (Madison, WI, USA) by manufactural instructions.

Microscopical examination

In case of detecting amastigote forms of the parasite, blood smears were prepared for each sample, fixed in methanol, stained with 5% Giemsa solution and then examined under 100x objective of light microscope.

Polymerase Chain Reaction (PCR)

Examination of the samples for *Leishmania* sp. DNA was performed using a two-stage PCR protocol. In the first step; RV1 (5'- CTTTTCTGGTCCCGCGGGTAGG - 3') and RV2 (5'- CCACCTGGCCTATTTTACAC - 3') primer pairs were used to amplify the 145 base pair (bp) region of kinetoplast DNA (kDNA) minicircles of the *Leishmania* genus, which includes *L. donovani*, *L. infantum*, *L. tropica* *L. major* and *L. braziliensis* (le Fichoux et al., 1999; Gao et al., 2015). In the second step, *Leishmania* spp. positive isolates were examined using MC1 (5' - GTTAGC CGATGGTGGTCTTG - 3') and MC2 (5' - CACCCATTTTTCCGATTTTG - 3') primer pairs amplifying a 447 bp region of *L. infantum/donovani* complex kDNA minicircles (Cortes et al., 2004). Total final volume for each PCR mix was 50 µL. PCR mixes consisted of; 1 × PCR buffer, 1.5 mM MgCl2, 200 mM of each dNTP, 2.5 U of hotstart Taq DNA polymerase, 25 pmol of forward and reverse primers and 2 µL of template DNA. Reaction conditions were as follows: an initial denaturation at 94˚C for 10 min was followed by 45 cycles of denaturation (95˚C for1 min), annealing (62˚C for 1.5 min for RV1/RV2 primers, 50˚C for 1.5 min for MC1/MC2 primers) and extension (72˚C for 30 sec), then followed by 72˚C for 10 min as a final extension. Following PCR amplification, 9 µL of each sample were electrophoresed in a 1.5% agarose gel prepared with 1 x TAE (Tris-asetik asit-EDTA) buffer at 100 Volt about 45 minutes and visualized by using ultraviolet (UV) light.

Statistical analysis

The data obtained from the present study was analysed using Minitab 16 Statistical Software. The Chi-Square test was performed to analyse the association among age, sex, breed parameters and PCR positivity. Differences for which the P value was less than 0.05 were considered statistically significant during comparisons for each parameter within itself.

Results

Due to microscopic examination, none of the samples were found positive in terms of amastigote forms of *Leishmania* spp. even positive ones in PCR, however, *Ehrlichia* spp. gamonts were detected in one sample (Figure 1).

In the first step PCR, *Leishmania* spp. DNA was detected in five of the 120 dog blood samples, therefore the molecular positivity rate of *Leishmania* spp. was was found 4.16%. As a result of the second step PCR performed for *L. infantum*, four samples were positive, therefore, the prevalence of *L. infantum* was found to be 3.33% (Table 1). The agarose gel electrophoresis images of the PCR results for both primer pairs are shown in Figure 2.

According to data analysis, no statistical significance was determined in the *L. infantum* positivity rates among age groups (Table 2) and sex (Table 3).

Considering the relationship among breeds and *L. infantum* positivity, crossbred and kangal breed dogs were found positive as 8.57% (3/35) and 12.5% (1/8), respectively, while positivity was not found in other 22 different breeds. Besides, P value could not be obtained by chi-square test, since the number of dogs found positive by breed was insufficient for statistical analysis.

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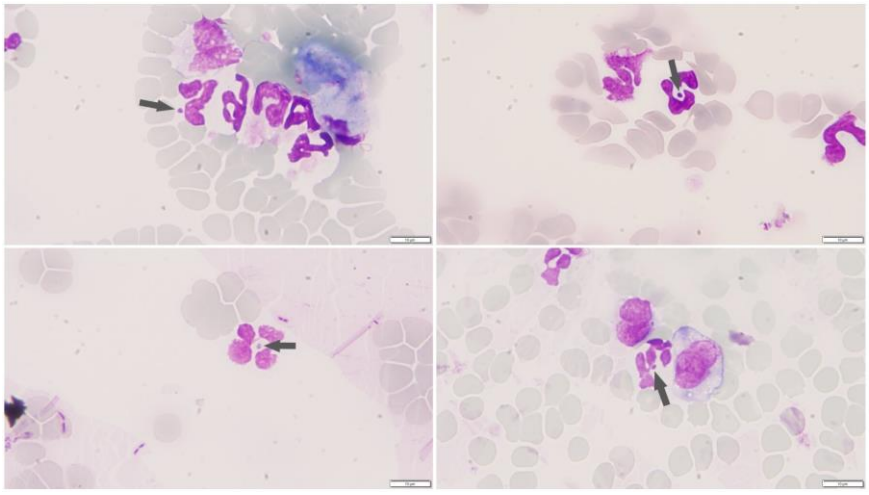

Figure 1. *Ehrlichia* spp. gamonts (arrows), 100x objective, scalebar: 10 µm

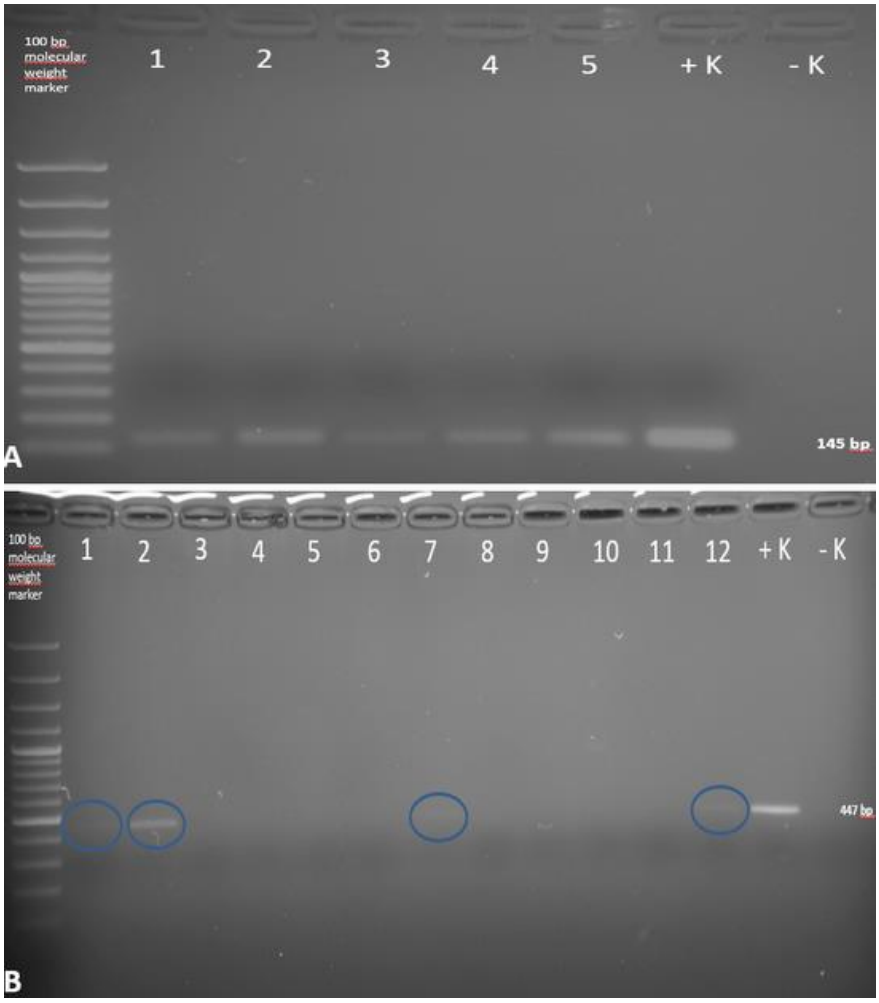

Figure 2. Agarose gel electrophoresis images of amplified DNA using; RV1-RV2 (A) and MC1-MC2 (B) primer pairs. A: 1-5 positive samples, +K positive control, -K negative control. B: 1,2,7,12 positive samples (1, 7 and 12 were very faint), +K positive control, -K negative control.

Table 1. Number and percentages of *Leishmania* spp. and *L. infantum* positive dogs

Number of examined dog	Number and percentage of *Leishmania* spp. positive dogs	Number and percentage of *L. infantum* positive dogs
120	5 (4.16%)	4 (3.33%)

Table 2. Distribution of *L. infantum* positive dogs according to age groups

Age groups (year)	Number of examined dogs	Number and percentage of *L. infantum* positive dogs
0-3	57	2 (3.5%)
3-6	29	1 (3.44%)
6-12	27	1 (3.7%)
>12	7	0 (0%)
P		> 0.05

Table 3. Distribution of *L. infantum* positive dogs according to sex

Sex	Number of examined dogs	Number and percentage of *L. infantum* positive dogs
Female	63	3 (4.76%)
Male	57	1 (1.75%)
P		0.359

Discussion

According to literature review; microscopic examination, in vitro cultivation, serological and molecular based methods are commonly used for studies on the epidemiology of canine leishmaniasis. Sensitivity of traditional methods such as microscopic examination are low especially in reservoir hosts and are insufficient in distinguishing different *Leishmania* species (İça et al., 2008; Özerdem et al., 2009; Aydenizöz et al., 2010; Töz et al., 2013). Various serological methods, such as Indirect Fluorescent Antibody Test (IFAT), Direct Antiglobulin Test (DAT), Enzyme Linked Immunosorbent Assay (ELISA), C-ELISA and Dot-ELISA have been used to determine the seroprevalence of canine leishmaniasis (Schalling and Oskam, 2002; İça et al., 2008; Özerdem et al., 2009). However, these methods may not always detect current infections, and the commonly used IFAT method can produce cross-reactivity between different species (Solano-Gallego et al., 2009; Bourdeau et al., 2014). Molecular based diagnosis methods such as PCR, multiplex and nested PCR, Restriction Fragment Length Polymorphism (RFLP) have been used for more reliable results and discrimination of *Leishmania* species in the recent decades (Schallig and Oskam, 2002; Andrade et al., 2006; Töz et al., 2013; Ayele and Seyoum, 2016). These techniques allow researchers to determine *Leishmania* species by targeting various regions of the nuclear and kinetoplast DNA (le Fichoux et al., 1999; Cortes et al., 2004). The mini-circle regions of kinetoplast DNA are particularly important in detecting *Leishmania* species (Noyes et al., 1998; Lachaud et al., 2002; Bensoussan et al., 2006). Therefore, in the present study, the PCR method

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was used to amplify the regions of kinetoplast DNA (kDNA) minicircles of the *Leishmania* genus and the *L. infantum/donovani* complex. Additionally, microscopic examinations were performed; however, no amastigotes were found in any of the samples. Similarly, Ozerdem et al. (2009) reported that microscopic examination of peripheral blood for amastigotes had a sensitivity of 76% and 34.5% compared to the rK39 dipstick test and PCR methods, respectively, whereas the PCR method showed a sensitivity of 100%. These results indicate that amastigotes cannot always be detected in peripheral blood, even when the animal is infected with parasites. Hence, it is concluded that examining different materials using multiple methods would be beneficial for obtaining the most accurate results.

In the present research, three out of the five samples that were found positive for *Leishmania* at the genus level were also *Leishmania* positive in the rapid test kits applied in the veterinary clinics where the samples were taken. However, interestingly, among the 120 samples analyzed, five other samples found positive with the rapid test kit but yielded negative results in the PCR analysis in this study. Rapid test kits are commonly used in clinics for the diagnosis of diseases due to their practical application and ability to provide quick results. Whereas, as demonstrated in this study, it is important to note that these test kits may not always yield accurate results. On the other hand, it should be noted that PCR testing may not always give accurate results for *Leishmania* species, especially in peripheral blood samples. Therefore, if possible, it is essential to examine biopsy samples from spleen, liver, skin, bone marrow or lymph node aspirates or blood serum in addition to blood for confirmation (Schallig and Oskam, 2002). Besides, using multiple diagnostic methods (molecular, serological, etc.) together ensures the reliability and accuracy of the results.

According to the results of the previous studies; the prevalence of canine leishmaniasis (regardless of diagnostic method) were reported between 0-37.4% from different geographical regions of Turkey (Aslan Çelik et al., 2019) and the most prevalent is Aegean region with a rate of 37.4% (Bakırcı and Topçuoğlu, 2021). Then followed by Mediterranean (12.96%), Central Anatolia (5.82%), Black Sea (5.38%), Eastern Anatolia (4.38%), Marmara (2.40%), and Southeastern Anatolia regions (0%) (Aslan Çelik et al., 2019). According to literature review, a serological prevalence study from Burdur province was encountered where 49 dogs were examined, and all were found to be negative (Beyhan et al., 2016). In the present research, molecular positivity rates were found to be 4.16% for *Leishmania* spp. and 3.33% for *L. infantum* in dog blood samples. With this result, molecular-based PCR method was used for the first time to report *Leishmania* spp. and *L. infantum* positivity from Burdur.

In the present study, a higher percentage of positivity was found in female dogs (4.76%) compared to males (1.75%). However, the difference was not statistically significant among genders. Similarly, there was no statistically significant difference among age groups. In addition, the number of positive dogs by breed was insufficient for statistical analysis. Therefore, inadequate sample size and the number of positive samples hinders a comprehensive evaluation of the impact of gender, age, and breed on canine leishmaniasis positivity. To better understand whether these parameters are predisposing factors for leishmaniasis, further studies with a larger number of samples are needed. However, it is crucial to remember that vector fly control remains a much more prioritized issue in preventing the spread of the disease.

Regionally, differences in climate conditions, significantly influencing vector populations and the presence of animal reservoir hosts, as well as environmental and ecological changes and migration movements are other important factors affecting the spread of leishmaniasis (Parker et al., 2021). Therefore, rehabilitating the habitats of vector flies holds great importance in disease prevention. Additionally, controlling human and animal movements, conducting regular screening

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studies to obtain epidemiological data on the prevalence of the pathogen and vectors, treating carrier humans and animals, are all extremely important for both animal and human health.

Another crucial aspect in terms of leishmaniasis epidemiology is determining the habitats and distributions of sand fly species that act as vectors for *Leishmania* species. In Burdur, where this study was conducted, the presence of species such as *Phlebotomus kandelakii* s.l., *Adlerius* spp., *P. tobbi*, *P. major* s.l., *Larroussius* spp., *P. papatasi*, *Transphlebotomus* spp., *P. sergenti* s.l., *Paraphlebotomus* spp., *P. perfiliewi*, *P. halepensis* and *S. dentata* has been reported (Kaynaş, 2019). Among them, *P. kandelakii*, *P. perfiliewi* and *P. tobbi* are proven vectors for *L. infantum*, while *P. halepensis* is a suspected vector (Cunze et al., 2019). The presence of these three proven and one suspected vector species in the region supports the presence of *L. infantum* (3.33%) detected in dog blood samples in the present study. In addition, in the same study (Kaynaş, 2019), sand fly species identified, such as *P. sergenti* and *P. papatasi* are proven vectors for *L. tropica* and *L. major* respectively (Cunze et al., 2019). Some reports indicating the presence of both *L. tropica* and *L. major* exists in Turkey (Akman et al., 2000; Töz et al., 2013; Zeyrek et al., 2014). In the present study, although the isolate showed *Leishmania* spp. positivity at the genus level, its species-specific PCR result indicated it was not positive for *L. infantum*. Due to the presence of its vectors in this region, the isolate could potentially be *L. tropica* or *L. major*. Therefore, due to the rapid changes in climate conditions caused by global warming, which lead to alterations and expansions in vector habitats, it becomes evident that further studies are needed concerning both the epidemiology of vectors and *Leishmania* species in Burdur, as well as in other regions of Turkey and the rest of the world.

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