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Original Article

Molecular Detection of Canine Leishmaniasis in Northern Anatolia, Turkiye

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Abstract

Background: Canin leishmaniasis (CanL), mostly caused by *Leishmania infantum*, is one of the most important vector-borne diseases in dogs in the Mediterranean region. In this study, we aimed to determine the disease profile in this region by firstly making microscopic and then molecular analyzes in the samples taken from the dogs.

Methods: Overall, 112 whole blood samples taken from dogs for clinical applications by a veterinarian in Cankırı between December 2021 and November 2022 were used. After blood collection, both thin and thick drop blood smear preparations were prepared and evaluated for Giemsa staining. *L. infantum* was investigated by Real time-PCR (RT-PCR) method from all blood samples. Sequence analysis and phylogenetic tree study were performed on positive samples.

Results: Both microscopic and RT-PCR analyzes were performed. In both studies, 3 of the 112 samples were positive. Because of the sequence analysis, they were *L. infantum*. Sequence analysis was performed from the samples found 3 positive. The phylogenetic tree was drawn by making NCBI (National Center for Biotechnology) data entries of the positive samples (Accession numbers: OQ184728, OQ184729, OQ184730).

Conclusion: Dogs are important, as they are reservoir of this disease. In this study, 3 (2.7%) positive Leishmaniasis was detected in dogs in Cankırı. Ultimately, this should prompt discussion about new strategies going forward to combat infection caused by *Leishmania*.

Introduction

The existence of *Leishmania*-like species has been documented in fossils since prehistoric times. The first fossil was found in the proboscis and digestive tract of the female of the 100-million-year-old extinct sand fly *Palaeomyia burmitis* (1). Although the study of *Leishmania* began in the late 19th century, a definitive diagnosis of the parasite was only possible before the turn of the century. In 1885, Scottish physician David Douglas Cunningham (1843–1914) saw but could not identify the *Leishmania* parasite in a Delhi boil (2). At the beginning of the twentieth century, the disease was named "leishmaniosis" by Dr William Leishman, who was working in the British Army in India. In the same period, these amastigotes, which were stained with Donovan (1903) Leishman stain, were called *Leishman-Donovan* bodies, while the causative agent was named *L. donovani* (3). Leishmaniasis is an important vector-borne (*Phlebotomine* sandflies= Diptera: Psychodidae) disease caused by infection of protozoa classified in the family Trypanosomatidae. There are about 30 species in the *Leishmania* lineage (4).

It causes visceral (VL), cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL) and post kala-azar dermal (PKDL) forms of leishmaniasis, which can be transmitted to humans and some animals. VL is produced by *L. donovani* in Asia and Africa, and by *L. infantum* in the Mediterranean basin, the Middle East, Central Asia, South America and Central America (5).

VL, caused by *L. infantum,* is a life-threatening disease that affects ≈200,000–400,000 people and causes estimated ≈20,000–40,000 deaths per year (6). Mammals can be infected asymptomatically for prolonged periods and often remain chronically infected after clinical treatment. Subclincally infected animals can transmit *Leishmania* by sandflies, blood transfusion and transplacental routes.

Leishmaniasis as one of the zoonotic diseases has spread to Europe, Asia, Africa and Latin America. Visceral leishmaniasis causes diseases in humans and dogs caused by *L. infantum*. Canine leishmaniosis (CanL) cutaneous, renal, ocular, skeletal muscle and hemolymphatic organs can be affected as target tissue. Cats get diseases similar to those in dogs with *L. infantum* (7). Dogs are important in terms of being the reservoir of this disease, as well as being symptomatic and asymptomatic. The disease creates serious problems in dogs and occasionally in humans, which can lead to death. CanL has also been declared an important disease by the World Organization for Animal Health (OIE) (8, 9). Due to its zoonotic potential, prevention of this disease in non-endemic areas is of great importance. CanL is an endemic disease in more than 70 countries and is common in the Mediterranean region (9).

Today, there is a threat of CanL spreading to higher latitudes and altitudes in Europe due to climate change (10). CanL, which is mostly caused by *L. infantum*, is one of the most important vector-borne diseases in dogs in the Mediterranean region. It should be considered as it raises human health concerns (11). New expanding molecular methods and immunological studies show that much progress has been made in CanL diagnosis, treatment and vaccine studies (12). VL is endemic in parts of the northeast, northwest, and southern regions of Iran and *L. infantum* has been reported in humans, domestic dogs, and phlebotomy vectors (13). However, wild dogs appear to be involved in this transmission cycle, similar to the other Mediterranean region country (14, 15). In addition to dogs, domestic cats and some desert rodent species (*Cricetulus migratorius, Mesocricetus auratus And Meriones persicus*) also play a role in the endemic transmission cycle of the disease (13).

In our country, in order to control CanL and prevent its spread, dogs with reservoirs should be tested, treated and precautions should be taken. With this study, we aimed to determine the disease profile in this region by firstly making microscopic and then molecular analyzes in the samples taken from the dogs living in and around Cankırı and coming to the Cankırı Municipality Animal Care and Rehabilitation Center (CMACRC).

Materials and Methods

Sample collection

Overall, 112 whole blood taken by the Veterinarian was used for clinical applications from dogs brought to CMACRC for diagnosis and treatment between December 2021 and November 2022.

This study was carried out with blood taken from dogs collected from Cankırı and its region, brought to the animal care and rehabilitation center, and evaluated clinically and regardless of breed. These samples were taken as soon as they arrived at the center and before any treatment was started. Animals that were treated and not sampled from Cankırı and its region were excluded from the study. Demographic information of the dogs was also collected by the veterinarian.

Ethical approval

The samples used in this study are not subject to 'About Working Procedures and Principles of Animal Experiments Ethics Committees' permission, since they are within the scope of "Clinical applications for diagnosis and treatment", paragraph 1 of Article 8 of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees published in the Official Gazette dated February 15, 2014 and numbered 28914. For this reason, Ethical Committee approval is not required for the study, as the samples taken for clinical purposes will be evaluated.

With the decision of the Etlık Veterinary Central Research Institute Local Ethics Committee dated 07.01.2022 and numbered 2022/04, it was decided that Ethics Committee approval is not required.

Microscopic analysis

To obtain a good blood smear preparation, blood samples should be taken in an EDTA tube and smear should be done within 1-2 hours after the sample is taken. When taking blood, it was necessary to comply strictly with the rules of asepsis and antisepsis. After blood collection, both thin and thick drop blood smear preparations should be prepared for Giemsa staining. The prepared preparations should be evaluated after staining with Giemsa dye (16). The microscopic Leica microscope was performed at 20 magnification

Molecular analysis

a- DNA extraction

One hundred and twelve blood samples taken from dogs were extracted using a spin column as suggested by the commercial kit (Gene MATRIX series, Quick blood-lot no: F/280921). The obtained DNAs were stored at -20 ºC until the PCR study.

b- Primer and probe design for detection of DNA

Primer and probe sequences designed for the target site, ITS1 (17) were synthesized (BM laboratories, Turkiye). RT-PCR mix, for a total of 25 µL for each sample; Distilled water is 5.3 µL, Master mix 12.5 µL, Primer I (10 p mol) 1µL, Primer II (10 p mol)1µL, Probe 0.2µL and DNA 5µL.

TaqMan probe: 6-Fam-TTT TCG CAG AAC GCC CCT ACC CGC-BHQ-1

Leish F: CTT TTC TGG TCC TCC GGG TAG G

Leish R: CCA CCC GGC CCT ATT TTA CAC CAA (17).

The positive control was used as the clinical isolate by diluting 1/1000 of the positive control obtained from the University of Health

Sciences. The cycle threshold value of the positive control is 27.95. Molecular great water (MGV) was used as negative control.

c- Detection of Leishmania infantum DNA using a RT-PCR assay

For the detection of *Leishmania* spp. a RT-PCR TaqMan probe was used. Before starting the PCR study, the lyophilized probes and primers were diluted with MGW at the rates recommended by the company and separated into aligots as stocks. Each was used by diluting 1/10 before the study. The prepared PCR mixture was distributed into 20 µL PCR tubes, and 5 µL of sample DNA, positive and negative controls were placed on it sequentially. Operating temperatures were performed at 95ºC for 5 minutes, at 95ºC for 20 seconds, at 60ºC for 30 seconds using a 45 cycle protocol as RT-PCR.

d- Sequencing and phylogenetic analyses

All RT-PCR positive samples were sequenced in one direction at a commercial sequencing service provider (BM laboratories, Turkiye). Nucleotide sequences were analyzed using nucleotide Blast (www.blast.ncbi.nlm.nih.gov/Blast). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (18). The tree with the highest log likelihood (-707.23) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 3 (26,52,100) nucleotide sequences. Evolutionary analyses were conducted in MEGA X (19).

Statistical Analysis phase

These analyzes with samples collected from dogs were statistically performed using SPSS version 26 software (IBM Corp., Armonk, NY, USA). Descriptive features were given using frequencies and percentages. The difference between the groups in terms of these frequencies was compared using the Chi-square test. Cases with $P<0.05$ were considered as statistically significant results.

Results

Demographic data obtained from dogs in this study and positivity rates according to RT-PCR results were given in Table 1.

Dogs brought to the clinic were at most one-year-old (57.1%) and mostly male dogs (51.8%). The most common symptom (21.5%) among the dogs in this study was skin rash and itching (17.9%)). As a result of statistical analyzes, *L. infantum* positivity rates did not yield significant results according to age, gender and symptoms (Fig. 1).

In the microscopic analysis, results were obtained in parallel with the RT-PCR test and 3 out of 112 samples were found positive. *L. infantum* was positive by RT-PCR in 2.7% of the 112 samples tested in dogs.

Table 1: Distribution of demographic data according to *Leishmania infantum* positivity (n=112)

Variable		*RT-PCR (Leishmania infantum)* Negative	Positive	Total (%)
Gender	Female	52	2	54 (48.2)
	Male	57	1	58 (51.8)
Symptoms	Alopecia	7	0	7 (6.3)
	Skin rash	24	0	24 (21.5)
	Diarrhea	1	0	1 (0.9)
	Cachexia	11	0	11 (2.6)
	Itching	17	3	20 (17.9)
	Vomitting	2	0	2(1.8)
	Vomitting +diarrea	2	0	2 (1.8)
	Breakage of nails	1	0	1 (0.9)
	None	44	0	44 (39.3)
Age	1	63	1	64 (57.1)
	2	31	2	33 (29.5)
	3	12	0	12 (10.7)
	4	3	0	3 (2.7)
Total		109 (97.3)	3 (2.7)	112 (100)

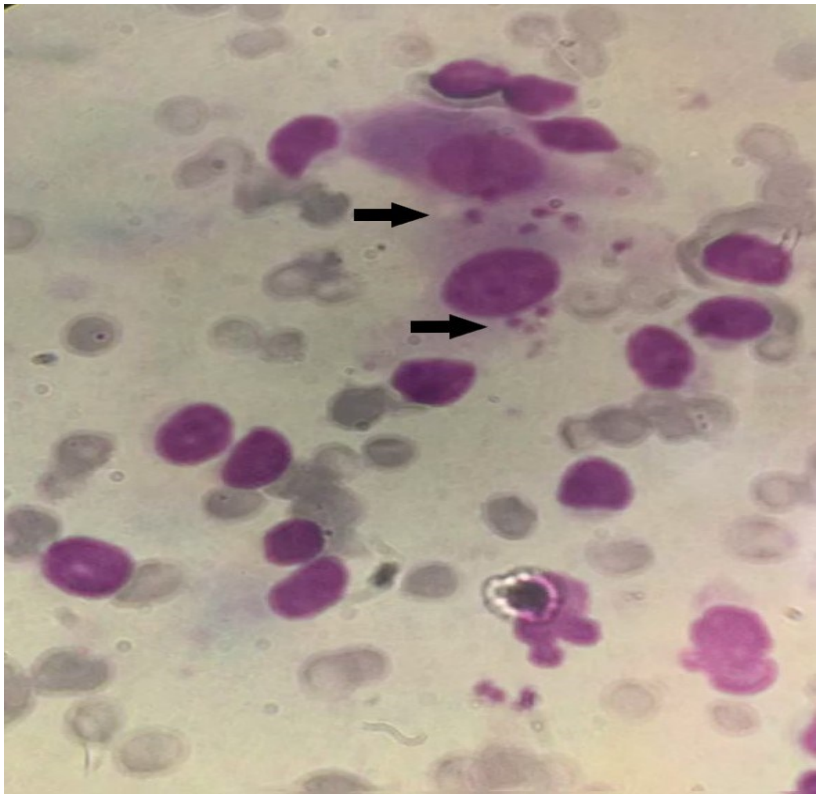

Fig. 1: Microscopic analysis of *Leishmania infantum* (Original)

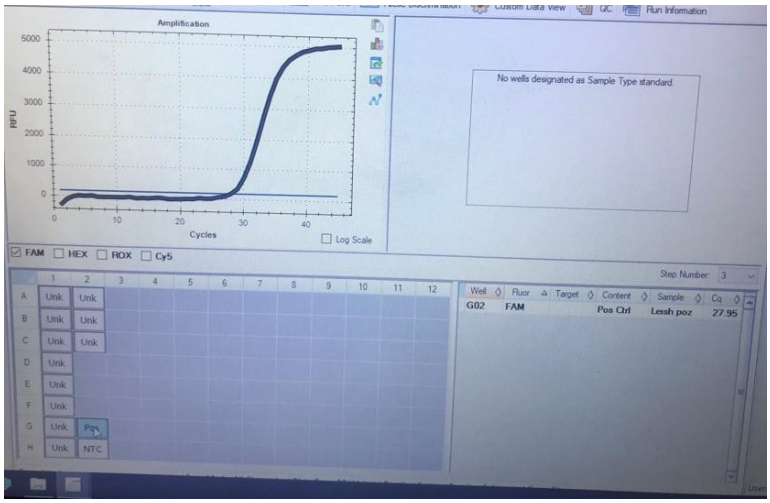

Fig. 2: *Leishmania infantum* control study

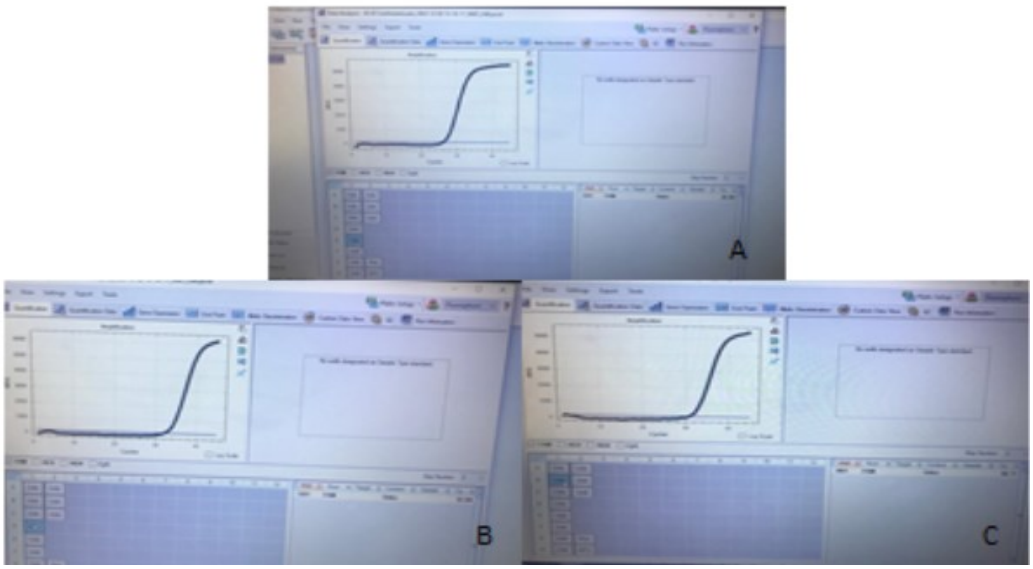

Fig. 3: *Leishmania infantum* positive study (A-B-C)

Figure 2 shows the positive control studied (obtained from the University of Health Sciences), and Fig. 3 shows the results of the study.

All positive samples were entered into the NCBI database and accession numbers OQ184728, OQ184729, OQ184730 were obtained (National Centre for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) (Fig. 4). *L. infantum* were detected in three (26, 52, 100) of the RT-PCR positive samples. A phylogenetic tree was drawn with reference strains (Fig. 4). Because of the sequence study, all samples were *L. infantum*. All of these were ancestrally in the same clade and were close to each other.

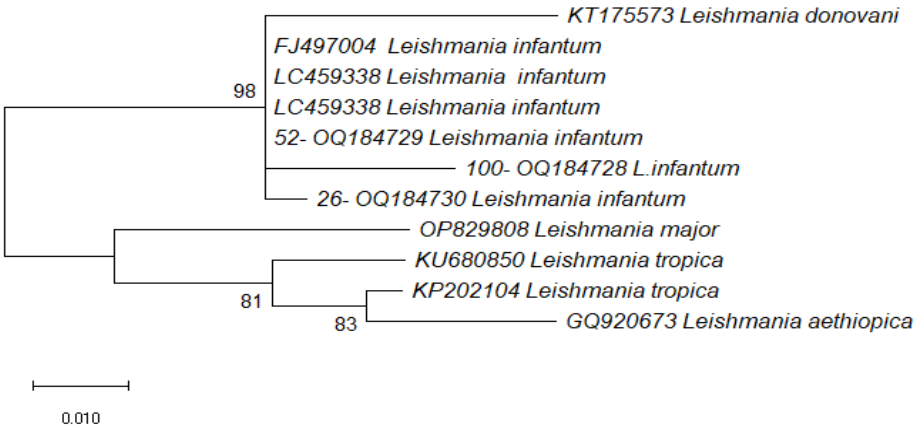

Fig. 4: Phylogenetic tree of *Leishmania infantum* (OQ184728, OQ184729, OQ184730) specimens

Discussion

Microscopic analyzes are used for the traditional diagnosis of *Leishmania*. Microscopic analyzes are difficult as they require experienced personnel. In addition to this, molecular analyzes are also challenging us as analyzes that are costly and difficult to do. Both techniques were used together in this study. The ITS 1 region of *Leishmania* were the most frequently used region in molecular studies and the taxonomic tree has been renewed (20). In this study *L. infantum* were detected in 3 of 112 dogs (2.7%). Equal positivity was found in all microscopic and PCR studies.

Exposure to phlebotomas in CanL should also be considered. Infection can sometimes show disease characterized by laboratory findings as well as absence of clinical findings. For this reason, it is necessary to consider the potential of carrying the disease in dogs with or without clinical findings (21). Unfortunately, even if dogs recover clinically after treatment, they can remain infected with *Leishmania* spp. and relapse. All *L. infantum* isolates obtained from symptomatic and asymptomatic dogs were similar in a study (22). Those with *L. infantum* in the blood and skin of symptomatic and asymptomatic infected dogs are highly contagious (22). CanL has emerged in the Balkans, with an increase in the number of infected dogs in formerly endemic areas and the spread of the disease northward. This disease, of which dogs are the main reservoir, can also be carried by wild animals and cats and is of great importance for the public health and veterinary. Researches carried out from 1934 to January 2021, it was seen that the CanL data were low in 10 countries in the Balkans (23).

There were many studies on CanL in Turkiye. These were in Aegean Region (9%), Mugla (37,4%), Antalya (7.95%), Hatay (0.8%) and Northern Cyprus (1.9%), Middle Black Sea Region (0,41%), Western Part of Turkiye, Bursa (5.5%), Edirne (100%), Istanbul (0%), Diyarbakır (0%), Western Black Sea-Karabuk (8%), Sakarya (1.45%), Kocaeli (3.07%), Kusadası (4.1%), Izmir (3.2%) (24-38). Some of these studies were studied serologically and some of them were investigated by molecular methods.

In this study, RT-PCR was 2.7% positivity. Cankırı, a localization where dog samples were collected, and its surroundings were at a transition point between North Anatolia and Central Anatolia. This is important in terms of showing the common characteristics of both regions. The region where this study was con-

ducted is in a middle area in terms of showing the Black Sea climate due to its closeness to the Black Sea Region and the continental climate due to its proximity to the Central Anatolia Region. This may be the reason why VL is less common in northern regions in our country. With the development of global warming, this climatic change has become important. This is one reason why VL tends to increase northward from the Mediterranean Region. Despite the increasing profile of VL in Europe (10), the low rates in this study were positive. However, these low rates do not eliminate the necessity of taking precautions regarding the globalizing world where human and animal mobility has increased. This study is an important study in terms of looking into the problem and showed that there are certain deficiencies in the management, surveillance and control of CanL research in North Anatolia.

Paltrinieri et al found dogs older than 2 years of age to be at high risk in their studies (21). In our study, positive samples were also found the most in 2-year-old dogs (7.2%).

VL is an infection that is becoming increasingly difficult to control, coupled with public health inadequacy and poverty. Difficulties in detecting infected and subclinical dogs in the control of the disease, not deterring seropositive dogs, vaccination problems of dogs, inadequacy of medicated dog collars and high cost of medical treatment can be counted (39). The same difficulties were identified in this study. The presence of positives and the inability to treat dogs as necessary increases the spread of the disease.

Since this disease is a vector disease, vector control is also mandatory (40, 41). Investigation of the Kızılırmak delta, located in this region where the study was carried out, in terms of vector screening is mandatory for the prevention of the disease.

Since there is a potential for transmission to humans, owners of dogs infected with *L. infantum* should be careful and warned about the issue (11). Millions of dogs have been killed for years to prevent the spread of human VL caused *by L. infantum*, which can be zoonotic, and dog culling continues today in some countries for this reason (42). It is also possible to prevent canine culling for VL by treating CanL within a one health (6). Due to its zoonotic potential, *Leishmania* may play a larger than anticipated role in human Leishmaniasis. Adult dogs may still pose a risk to human health due to their susceptibility to *Leishmania i*nfection, their role as reservoirs and their potential to be carriers. Since the detected *L. infantum* is the causative agent of the disease, also known as Kala-Azar, which can cause fatal reactions in humans, carrier dogs also negatively affect public health in our country, which is a Mediterranean country.

Because CanL is of global importance, a proactive approach is required to control the disease (6). Traditional and current developments should be used together in the diagnosis and treatment of leishmaniasis. In this study, both microscopic and RT-PCR analyzes were performed. In addition to clinical applications, specificity and sensitivity issues were also considered. It is desired to prevent this disease, which is a zoonotic disease, by evaluating current treatment options and diagnostic approaches. Fighting the infection caused by *Leishmania* in dogs will stimulate discussion about alternative new strategies to be adopted as a way forward.

Conclusion

The fact that there is still no downward trend for CanL, for which the dog breed plays a role as a reservoir, shows that leishmaniosis is of great importance in human and animal life. Today, when molecular studies are rapidly increasing, future research has also accelerated. It is essential to detect reservoirs that do not have clinical findings with PCR analysis. In addition, identifying new *L. infantum* strains isolated from dogs and humans using new sequencing techniques such as NGS and reduc-

ing the potential of vector flies and reservoir animals are important steps in eradicating the disease.

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Conflict of interest

Authors declare no conflict of interest.

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