Ayşegül BOSTANCI MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY MASTER 2016

T.R.N.C.

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DETERMINATION OF HUMAN LEISHMANIASIS SEROPREVALENCE AND DISEASE CAUSING *LEISHMANIA* SPECIES IN NORTHERN CYPRUS

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The Directorate of Health Sciences Institute

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To my beloved uncle . . .

ABSTRACT

Bostancı, A. Determination of Human Leishmaniasis Seroprevalence and Disease Causing *Leishmania* Species in Northern Cyprus. Near East University Institute of Health Sciences, M.Sc. Thesis in Medical Microbiology and Clinical Microbiology Programme, Nicosia, 2016.

Leishmaniasis is a tropical disease which is caused by *Leishmania* parasites. Cases are reported in many countries and the Mediterranean basin is an important region for the disease. Vectors of *Leishmania* spp. are *Phlebotomus* sand flies, and dogs are the main reservoir of these parasites. In Cyprus, which is located in the Mediterranean sea, human leishmaniasis and canine leishmaniasis (CanL) cases were reported previously. For this reason, this study was conducted in order to investigate human leishmaniasis seroprevalence and presence of *Leishmania* spp. in Northern Cyprus. In this study, Girne (Kyrenia) and surrounding regions were chosen as the pilot areas due to the high amount and diversity of *Phlebotomus* spp. in these regions. A total of 250 participants (242 individuals were randomly selected, and eight patients had cutaneous leishmaniasis (CL) history) were included in this research on voluntary basis. During the collection of blood and serum samples, the participants who owned dogs also provided information related with CanL history in their dogs. *Leishmania* spp. were investigated in the whole blood samples by polymerase chain reaction (PCR) while direct agglutination test (DAT) and rK39 test were used for the serologic assays. According to the test results, all of the 242 participants who were CL (-) were found to be negative. In this study, the only positive test results were obtained in the samples of four CL (+) patients (1.6% of 250 participants). Two (0.8%) of the CL (+) patients were positive for DAT with serum titers of 1:1600. One of these patients had three dogs which did not have any finding of CanL at the time of infection. Another patient (0.4%) with a history of CL was detected positive by the rK39 test. *Leishmania* spp. was detected in the blood sample of one (0.4%) patient by PCR. According to the DNA sequencing results, the agent was reported to be *L. donovani* complex. This patient was diagnosed as CL short before providing the samples for the study and one of his dogs had a sign of CanL. In the other four CL (+) patients, the serologic and molecular test results were negative. The results of this study indicate that the presence of leishmaniasis in Northern Cyprus should not be ignored. Therefore, the vector and reservoir control programmes should be implemented for prevention of the disease.

Key words: Leishmaniasis, *Leishmania*, *Phlebotomus*, Northern Cyprus

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ÖZET

Bostancı, A. Kuzey Kıbrıs'daki İnsan Leishmaniasis Seroprevelansının ve Hastalık Etkeni Olan *Leishmania* Türlerinin Belirlenmesi. Yakın Doğu Üniversitesi Sağlık Bilimleri Enstitüsü, Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Programı, Yüksek Lisans Tezi, Lefkoşa, 2016.

Leishmaniasis, *Leishmania* parazitlerine bağlı gelişen tropikal bir hastalıktır. Birçok ülkede olgular bildirilmektedir ve Akdeniz havzası bu hastalık için önemli bir bölgedir. *Leishmania* spp.'nin vektörleri *Phlebotomus* türü kum sinekleridir, ve köpekler *Leishmania* parazitlerinin esas rezervuarıdır. Akdeniz'de bulunan Kıbrıs'da, insan leishmaniasis ve kanin leishmaniasis (KanL) olguları daha önce bildirilmiştir. Bu nedenle, bu çalışma Kuzey Kıbrıs'daki insan leishmaniasis seroprevelansının ve *Leishmania* spp.'nin araştırılması amacıyla yapılmıştır. Bu çalışmada, Girne ve civar bölgeler *Phlebotomus* spp.'nin sayısı ve çeşitliliğinin fazla olması nedeniyle pilot bölge olarak seçilmiştir. İki yüz elli katılımcı (242 kişi rastgele seçilmiş, ve sekiz hastada kütanöz leishmaniasis (KL) öyküsü bulunmaktadır) bu araştırmaya gönüllülük esasına göre dahil edilmiştir. Kan ve serum örneklerinin toplanması sırasında, köpeği olan katılımcılar ayrıca köpeklerindeki KanL öyküsü ile ilgili bilgi vermişlerdir. *Leishmania* spp. tam kan örneklerine polimeraz zincir reaksiyonu (PCR) ile araştırılmış olup, direkt aglütinasyon testi (DAT) ve rK39 testi serolojik deneyler için kullanılmıştır. Test sonuçlarına göre, KL (-) olan katılımcıların hepsi de negatif bulunmuştur. Bu çalışmada, pozitif test sonuçları sadece dört KL (+) hastanın (250 katılımcının %1,6'sı) örneklerinde elde edilmiştir. KL (+) hastaların ikisinde (%0,8) DAT sonuçları pozitif, ve serum titreleri 1:1600 olarak bulunmuştur. Bu hastalardan birinin üç köpeğinin bulunduğu, ve enfeksiyon sırasında bu köpeklerde KanL bulgusunun olmadığı bildirilmiştir. KL öyküsü olan bir diğer hasta (%0,4) rK39 testi ile pozitif olarak saptanmıştır. *Leishmania* spp. bir (%0,4) hastanın kan örneğinde PCR ile saptanmıştır. DNA dizi analizi bulgularına göre, etken *L. donovani* kompleksi olarak belirlenmiştir. Çalışma için örnek verilmesinden kısa bir süre önce KL tanısı alan bu hastanın köpeklerinden birinde KanL belirtisi olduğu bildirilmiştir. KL (+) olan diğer dört hastada, serolojik ve moleküler test sonuçları negatif olarak bulunmuştur. Bu çalışmanın sonuçları Kuzey Kıbrıs'daki leishmaniasis varlığının göz ardı edilmemesi gerektiğine işaret etmektedir. Bu nedenle, hastalığın önlenmesi için vektör ve rezervuar kontrol programlarının uygulanması gerekmektedir.

Anahtar kelimeler: Leishmaniasis, *Leishmania*, *Phlebotomus*, Kuzey Kıbrıs

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SYMBOLS AND ABBREVIATIONS

CanL	Canine Leishmaniasis
CL	Cutaneous Leishmaniasis
DAT	Direct Agglutination Test
DCL	Diffuse Cutaneous Leishmaniasis
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAST	Fast Agglutination Screen Test
IFAT	Immunofluorescence antibody test
ITS-1	Internal transcribed spacer 1
kDNA	kinetoplast DNA
KATEX	LATEX Agglutination Test
L. aethiopica	*Leishmania aethiopica*
L. amazonensis	*Leishmania amazonensis*
L. braziliensis	*Leishmania braziliensis*
L. braziliensis braziliensis	*Leishmania braziliensis braziliensis*
L. braziliensis guyanensis	*Leishmania braziliensis guyanensis*
L. braziliensis panamensis	*Leishmania braziliensis panamensis*
L. chagasi	*Leishmania chagasi*
L. donovani	*Leishmania donovani*
L. donovani complex	*Leishmania donovani* complex
L. donovani donovani	*Leishmania donovani donovani*
L. donovani infantum	*Leishmania donovani infantum*
L. infantum	*Leishmania infantum*
L. major	*Leishmania major*
L. mexicana	*Leishmania mexicana*
L. peruviana	*Leishmania peruviana*
L. tropica	*Leishmania tropica*
MCL	Mucocutaneous leishmanisis

MLEE	Multi-locus Enzyme Electrophoresis
NEU	Near East University
NNN	Novy-MacNeal Nicolle
PCR	Polymerase Chain Reaction
P. alexandri	*Phlebotomus alexandri*
P. economidesi	*Phlebotomus economidesi*
P. galilaeus	*Phlebotomus galilaeus*
P. neglectus	*Phlebotomus neglectus*
P. papatasi	*Phlebotomus papatasi*
P. sergenti	*Phlebotomus sergenti*
P. tobbi	*Phlebotomus tobbi*
PKDL	Post Kala-Azar Dermal Leishmaniasis
RES	Reticuloendothelial System
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
WB	Western Blotting
WHO	World Health Organization
VL	Visceral Leishmaniasis

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1. INTRODUCTION

Leishmaniasis is a vector borne disease, caused by obligate Leishmania parasites. According to the World Health Organization (WHO) data, leishmaniasis is one of the most neglected diseases and ranked as the second most common disease following malaria. *Leishmania* species lead to 300,000 Visceral Leishmaniasis (VL) cases and one million Cutaneous Leishmaniasis (CL) cases and further 350 million people are at risk. The disease is seen endemically in 98 countries, including 72 developing countries. Clinical manifestations depend on the species of *Leishmania* genus (Gouzelou et. al., 2012; Canim Ates et. al., 2013).

Leishmaniasis is transmitted by the bite of *Phlebotomus* sand fly. In addition, blood transfusion, laboratory accidents, sexual transmission, congenital transmission are unusual transmission types of leishmaniasis (Canim Ates et. al., 2013; Elmahallawy et. al., 2014). Vectors of leishmaniasis are *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Leishmaniasis has three main clinical forms that are VL, CL and mucocutaneous leishmaniasis (MCL). Moreover, post kala-azar dermal leishmaniasis (PKDL), canine leishmaniasis (CanL) and diffuse cutaneous leishmaniasis (DCL) can also manifest clinically. *Leishmania* affects reticuloendothelial system (RES) cells and causes disease. CL is the most common form and transmitted by *Leishmania tropica* complex, *Leishmania aethiopica (L. aethiopica), Leishmania major (L. major)* and *Leishmania mexicana* complex. VL is the most dangerous form and it can be fatal if left untreated (Sundar and Rai, 2002; Elmahallawy et. al., 2014; Solano-Gallego et. al., 2014). It affects internal organs, multiplies in the RES cells and transmitted by *Leishmania donovani* complex (*L. donovani* complex). More than 90% of CL cases have been reported in Iran, Afghanistan, Syria, Saudi Arabia, Brasilia, India and Sudan, while more than 90% of VL cases have been detected in Bangladesh, Brasilia, India and Sudan (Sundar and Rai, 2002; Sharma and Singh, 2008; Canim Ates et. al., 2013).

Poverty, in fact, is the primary factor for transmission of leishmaniasis. Lack of medical treatment, resistance development by the disease vector against insecticides, drugs used against pathogens, lack of effective vaccine, migration into *Leishmania* endemic areas, increase in AIDS/HIV and other immune deficiencies, travels and ecological changes have roles in spread of the disease (Canim Ates et. al., 2013; Dutari et. al., 2014; Maia et. al., 2015; Zeyrek and et. al., 2015).

Cyprus, located in the Mediterranean Sea, with its typical climate and geographical structure provides a favorable environment for the survival of *Phelebotomus* spp. (Demir et. al., 2010). Dogs are part of daily lives in Cyprus in which considerable number of people own dogs. Due to that, dogs are mostly allowed to stay in close proximity with the citizens, even within the same house of the individuals (Canakci, 2008). Therefore, it is significantly important to investigate *Leishmania* in details. In Cyprus, CL and CanL are commonly seen types of Leishmaniasis. *Leishmania infantum (L. infantum)* zymodome MON-1 and *Leishmania donovani (L. donovani)* MON-37 are agents of CanL and CL, respectively. *Phlebotomus tobbi (P. tobbi)* is a potential vector detected in Cyprus even though *Phlebotomus neglectus (P. neglectus)* is the most abundant species in Northern Cyprus. No more VL cases were reported since 2006 in the island, however, CL and CanL cases are reported sporadically (Antoniou et. al., 2008; Ozensoy Toz et. al., 2013b).

In the light of these, this research was conducted in order to investigate human leishmaniasis seroprevalence and presence of *Leishmania* spp. in Northern Cyprus. In this study, Girne (Kyrenia) and surrounding regions were chosen as the pilot areas due to the high amount and diversity of *Phlebotomus* spp. A total of 250 individuals were included in this study on the voluntary basis. Two hundred and forty-two participants were randomly selected, while eight patients had a history of CL. Blood and serum samples were collected from each participant. The presence of *Leishmania* parasites was investigated by polymerase chain reaction (PCR) in the whole blood samples. The antibody response was examined by direct

agglutination test (DAT) and immunochromatographic rK39 test in the serum samples. The results of this study provided information on the presence of leishmaniasis and pointed out the importance of the implementation of control programmes in aiming to prevent the formation of Leishmaniasis in Northern Cyprus.

2. GENERAL INFORMATION

2.1. History of *Leishmania*

Leishmania was clarified by Cunningham, Leishman, Donovan, Borovsky, Wright, Linderberg and Vianna distinctively in the 19th century but named by Ronald Ross in 1903 (Ozbel and Ozensoy Toz, 2007).

Initially, the Old World forms were encountered in the history of *Leishmania*. There were statements about CL found on tablets located in the library of King Ashurbanipal dated back to 7th century BC (Cox, 2002). Leishmaniasis was defined by Avicenna in 10th century AD and known as since 18th century AD in the Middle East, Africa, Asia and Aleppo and Baghdad city. The disease is known as kala-azar whereas the words “kala” and “azar” mean *black* and *disease* in the native language, respectively (WHO Expert Committee, 1982; Stark, 2014).

VL had been realized after the failure of quinine application on the sick people who was thought to have malaria in 1824. A first VL epidemic was occurred in Jessore city in Bangladesh (Cox, 2002; Ozbel and Ozensoy Toz, 2007). Parasites were shown in biopsy materials that were taken from tissue lesions by English commander D.D Cuningham. However, James Homer Wright was the first doctor to define this disease clinically after he treated an Armenian patient (Crum et. al., 2005). In 1900, William B Leishman stated the agent of VL from an infected soldier’s spleen smear preparation and named these tiny oval shaped structures as corrupt trypanosomas. Sir Charles Donovan observed the same parasites from the patient’s spleen smear preparation in the same year and published these in his studies later in 1903 (Lainson, 2010). After that, Dr. Donald Ross, who described the *Leishmania* genus, named this parasite as *L. donovani* in 1903. Previously, this parasite was thought to belong to sporozoa, however in 1904, Leonard Rogers stated this parasite as a hemoflagellate. Besides, S.R. Cristophers described the pathology of VL in the same year (Chakarova et. al., 2005; Ozbel and Ozensoy Toz, 2007; Kilic et. al., 2008; Lainson, 2010).

G.C. Chattergee and Roggers Kalkuta defined promastigote structures in culture. In 1908, Nicolle and Compte named the parasitic agents of infection in dogs as *L. infantum*. Ch Nikolle produced this parasite in culture and injected them into the monkeys and dogs (Ozbel and Ozensoy Toz, 2007). By doing this, he had come up with a hypothesis stating that reservoirs of leishmaniasis in the Mediterranean basin might be dogs (Unat et. al., 1995; Chakarova et al., 2005; Ozbel and Ozensoy Toz, 2007; Kilic et. al., 2008). Russian scientists Yakimoff and Schokhor showed amastigote forms of parasites in smear preparations and named these parasites *Leishmania tropica* (*L. tropica)* and *Leishmania tropica major* (Jacobson, 2003). The vector of leishmaniasis, the genus of *Phlebotomus* sand fly, was identified in 1941 (Unat et. al., 1995).

2.2. Classification of *Leishmania*

Different species and subspecies are known to exist in the *Leishmania* genus. All *Leishmania* species have the same morphologic appearance under the light microscope. As a result of this, their classification is based on geographical distribution, epidemiological, serologic, immunological, biological and biochemical properties and diseases depending on the parasite species and subspecies. The taxonomic classification and the list of species approved by WHO are stated in Table 2.1. (Lewis, 1982; WHO Expert Committee, 1982; Unat et. al., 1995; Ozbel and Ozensoy, 2007; Paniker, 2013).

Table 2.1. Taxonomic classification of the *Leishmania* genus.

Kingdom: *Protista*

Subkingdom: *Protozoa*

Phylum: *Sarcomastigophora*

Subphylum: *Mastigophora*

Class: *Zoomastigophora*

Order: *Kinetoplastida*

Family: *Trypanosomatidae*

Genus: *Leishmania*

Subgenus: *Leishmania*

Species: *Leishmania donovani, Leishmania infantum, Leishmania chagasi, Leishmania tropica, Leishmania major, Leishmania aethiopica, Leishmania mexicana, Leishmania amazonensis*

Subgenus: *Viannia*

Species: *Leishmania braziliensis complex (Leishmania braziliensis, Leishmania peruviana), Leishmania guyanensis complex (Leishmania guyanensis, Leishmania panamensis, Leishmania shawi), Leishmania naiffi, Leishmania lainsoni*

L. donovani, L. infantum and *Leishmania chagasi (L. chagasi)* are known agents of VL (Altintas, 2002). According to the recent research studies, *L. tropica* and *Leishmania amazonensis (L. amazonensis)* could be included to this group. In addition, *L. chagasi* is the etiological agent of VL in

the New World (Schnur et. al., 2004). In the Old World, *L. tropica, L. major, L. aethiopica* and *L. infantum* are the agents of CL whilst in the New World, *Leishmania mexicana (L. mexicana), Leishmania braziliensis braziliensis (L. braziliensis braziliensis), Leishmania braziliensis guyanensis (L. braziliensis guyanensis), Leishmania braziliensis panamensis (L. braziliensis panamensis)* and *Leishmania peruviana (L. peruviana)* are the agents of MCL (Altintas, 2002; Kayser, 2002).

2.3. Morphology of *Leishmania*

Leishmania has two forms, the amastigote and the promastigote. Amastigote forms are found in the vertebrate host whereas promastigote forms are found in the vector (Unat et. al., 1995; Altintas, 2002; Ozbel and Ozensoy, 2007). The life cycle of *Leishmania* is shown in Figure 2.1.

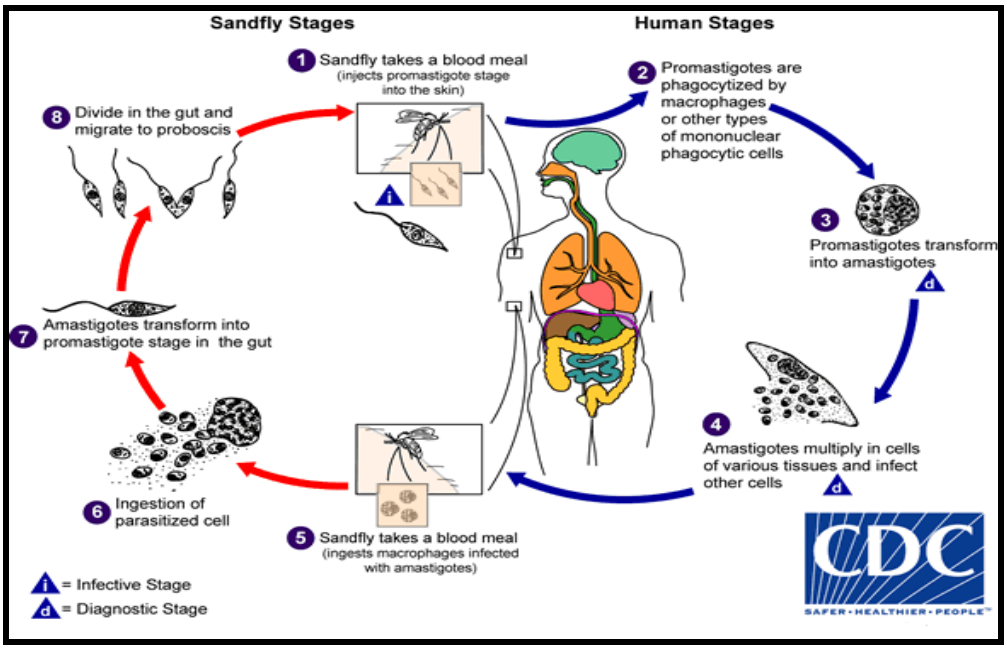

Figure 2.1. Life cycle of *Leishmania* parasites (Center for Disease Control and Prevention, 2013).

Amastigotes, shown in Figure 2.2, are 2-4 μm in length, are ovoid or round in shape. In addition, they are usually found in monocytes, polymorphonuclear leukocytes and endothelial cells. When stained with Giemsa, the cytoplasm appears in blue and nucleus in pink or dark red, respectively (Unat et. al., 1995; Ozbel and Ozensoy Toz, 2007; Paniker, 2013). Kinetoplast is rod shaped and stained in dark red, shiny red or purple. Amastigotes are nonmotile, feed on via osmosis and get nutrient from tissues. They are aerobes and proliferate longitudinal by binary fusion in macrophages. Firstly, kinetoplast and blepharoplast and then nucleus and cytoplasm are divided. There is a large nucleus close to the cytoplasm and the kinetoplast adjacent to the nucleus (Unat et. al., 1995; Ozbel and Ozensoy Toz, 2007). Additionally, there are vacuoles, blepharoplast and axonem in the cytoplasm. Flagellum does not come out of the cell freely. In all *Leishmania* species, there is only one mitochondrion, the Golgi apparatus and lysosome that helps to feed on parasites by various enzyme activities (Unat et. al., 1995; Ozbel and Ozensoy, 2007).

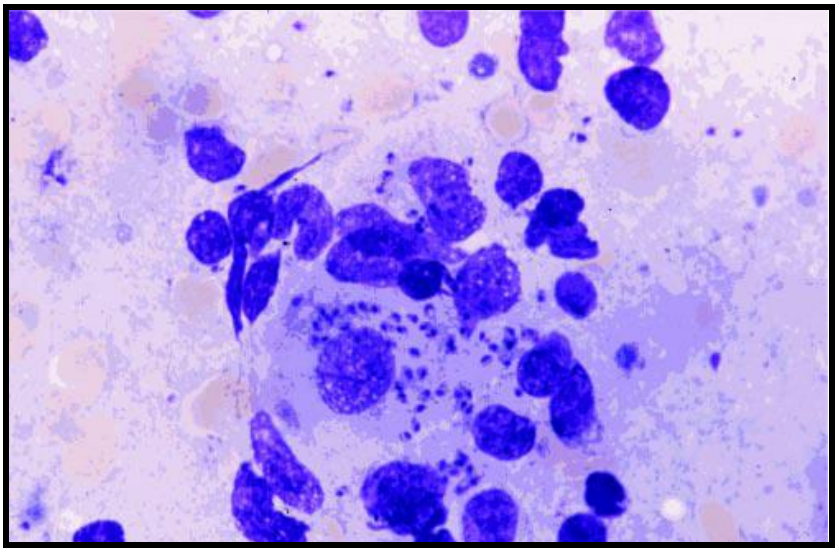

Figure 2.2. *L. donovani* amastigotes (Giemsa stained bone marrow aspirate) (Singh, 2006).

Promastigotes, shown in Figure 2.3, are 15-28 μm in length and 1.5-3.5 μm in width. One end is sharper and the other end is ovoid. Flagella come up from the front edge. As a result of dying with Giemsa, cytoplasm is stained in blue, inside the cytoplasm nucleus is stained in pink or red (Ozbel and Ozensoy Toz, 2007; Paniker, 2013). Kinetoplast is stained in lilac or shiny red in front of the nucleus. Blepharoplast is present before kinetoplast. Promastigotes are found in the midgut of the vector and the culture medium when amastigotes develop into promastigotes. There are free flagella and axonem which is located near to blepharoplast (Ozbel and Ozensoy Toz, 2007). Additionally, kinetoplast, nucleus, nucleolus and pores located in nucleus membrane are present. Moreover, the Golgi apparatus and the endoplasmic reticulum are found in the cytoplasm (Unat et. al., 1995; Ozbel and Ozensoy, 2007).

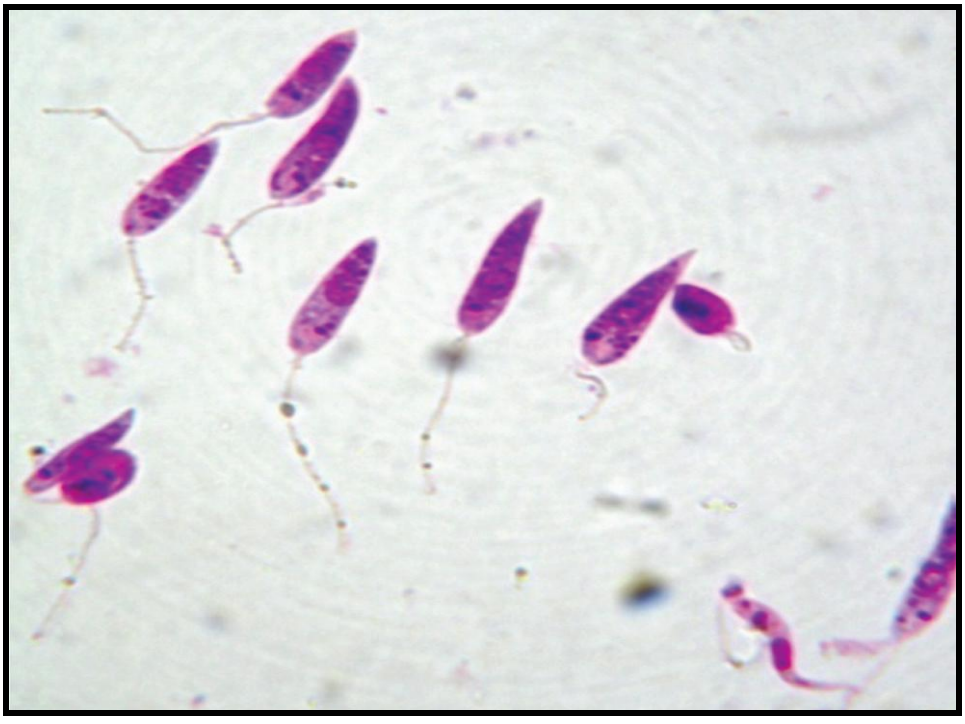

Figure 2.3. Promastigotes, from *in vitro,* culture were prepared with Giemsa stain (Gupta and Nishi, 2011).

2.4. Vectors of Leishmaniasis

More than 70 phlebotomine sand flies are the proven vectors of *Leishmania* parasites. There are more than 600 species and subspecies identified for phlebotomine subfamily (Alten and et. al, 2015). Vectors of leishmaniasis are *Phlebotomus* sand flies in the Old World and *Lutzomyia* sand flies in the New World. These sand flies live in the warm environment and are found in subtropical and tropical regions such as Africa, Asia, Australia, Central and South America and Southern Europe. Distribution in the north expands below the latitude 50°N in the northern France and Mongolia and above the latitude 50°N in Southwest Canada. The southern distribution includes latitude 40°S. However, they are absent in a region covering from Pacific Islands to New Zealand. The taxonomic classification of the vector of leishmaniasis is stated in Table 2.2. (WHO Expert Committee, 1982; Killick-Kendrick, 1999; CVBD, 2001; Dostálová and Volf, 2012; Alten and et. al, 2015).

Table 2.2. Taxonomic classification of the vector of leishmaniasis.

Phylum: *Arthopoda*
Subphylum: *Tracheata (Antennata)*
Class: *Insectea (Insecta, Hexapoda)*
Subclass: *Pterygotia (Flying insects)*
Order: *Dipterida*
Suborder: *Nematocera*
Family: *Psychodidae/ Phlebotomidae*
Subfamily: *Phlebotominae*
Genera: *Phlebotomus, Sergentomyia, Chinius* (Old World)
Lutzomyia, Bruptomyia, Warileya (New World)

Considering phlebotomine has six genera, the Old World includes *Shinuius*, *Phlebotomus* and *Sergentomyia* genera. In contrast, the New World includes *Brumptomyia, Warileya* and *Lutzomyia* genera. In New World, *Lutzomyia* is the largest genus of phlebotomine and are responsible for the transmission of CL, MCL and VL (Dujardin et. al., 1999; Azpurua et. al., 2010). *Phlebotomus* and *Lutzomyia* species are shown in Figure 2.4.

Figure 2.4. Examples of *Phlebotomus argentipes* (left) and *Lutzomyia longipalpis* (right) (Sharma and Singh, 2008).

Phlebotomine sand flies are very tiny (1.5-3.0 mm in length) and their colour is changeable from white to black. Some properties make sand flies distinct compared to other species. Firstly, they are hairy. In addition to that, they are hopping around the host before settle down for biting. A detailed picture of the sand fly is given in Figure 2.5. Also, they hold their wings at an angle above the abdomen during rest time (Dostálová and Volf, 2012). Attacks of sand flies are silent in contrast to mosquitoes. They, not all species, do not spread far from the breeding site. Except few species, rest of them bite during the night and have nocturnal or crepuscular activities. Sand flies spend time during daylight in cool and humid places such as caves, stables, latrines, fissures in the wall, cellars, bird nests, tree holes, dense vegetation, rodent burrows, rocks and soil. Insecticides cannot be applied to exophilic and exophagic species. However, they can be used for control of endophilic species (Killick-Kendrick, 1999; Dostálová and Volf, 2012). House spraying, synthetic pyrethroid impregnated bed nets are used in sand fly

control. Deltamethrin-impregnated dog collar was observed to protect dogs from sand fly biting in laboratory experiments (Killick-Kendrick, 1999; Sharma and Singh, 2008; Dostálová and Volf, 2012).

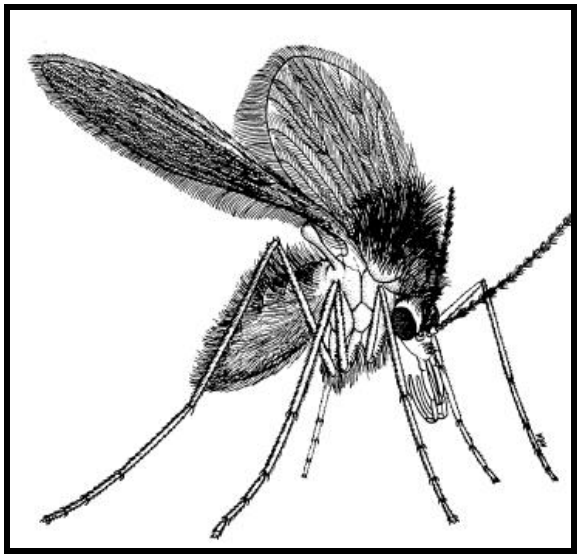

Figure 2.5. Detailed picture of female phleobotomine sand fly (Killick-Kendrick, 1999).

Natural sources of sugars are the choice of food for sand flies. However, female species also need blood as a nutrient for the production of eggs. After the bite of a female sand fly, injection of saliva may lead to the establishment of *Leishmania* parasites into the skin. According to the type of species, ambient temperature and digestion speed, maturation time of the eggs may vary. Temperature affects the period of the life cycle (Killick-Kendrick, 1999; Dostálová and Volf, 2012). Sand flies have three different stages that consist of egg, four larval stages and the pupa. Organic food, cool temperature and moisture are essential factors of these stages. Development time may vary in accordance with the temperature. After oviposition, between 50 and 100 eggs are laid by female sand fly. Hatching of eggs usually takes 7 to 10 days. Till pupation, development of eggs occurs approximately 35 to 60 days depending on the temperature and nutrients (Killick-Kendrick, 1999).

Breeding of sand flies takes place in the soil where moist and rich in humus (Killick-Kendrick, 1999; Sharma and Singh, 2008). Moreover, parasite development starts after a blood meal. Parasites transform into the promastigote stage from the amastigote phase after dividing. Division continues in the hindgut or midgut of the sand fly. After that, parasites migrate to the pharynx. Then, motile forms move to the mouth and accumulate there (Ozbel and Ozensoy Toz, 2007; Sharma and Singh, 2008).

Attachment to the inner surface of the gut is required for promastigote survival. Lipophosphoglycan, major cell surface glycoconjugate of the promastigotes, plays an important role in the attachment to the abdominal midgut of the fly. Increase in lectin production in the female sand fly's midgut after the blood sucking plays a significant role possibly in the attachment (Killick-Kendrick, 1999).

2.5. Epidemiology of Leishmaniasis in the World

Leishmaniasis is one of the neglected tropical protozoan diseases. There are more than 20 *Leishmania* species and subspecies and more than 30 sand fly species that cause disease in the New World and the Old World. Leishmaniasis is seen in 88 countries endemically, 22 tropic and subtropic regions in the New World and 66 regions in the Old World. However it has not been reported in Antarctica and Australia continents (Sundar and Rai, 2002; Ozbel and Ozensoy Toz, 2007; Sharma and Singh, 2008; Elmahallawy et. al., 2014). Most of the diseases are reported in the Mediterranean region in Europe. More than 90% of CL cases are seen in Iran, Afghanistan, Syria, Saudi Arabia, Brasilia, India and Sudan. In contrast, more than 90% of VL cases are seen in Bangladesh, Brasilia, India and Sudan. According to the WHO data, each year 300,000 new VL cases and 1 million new CL cases are reported annually. In addition, 350 million people are at risk and approximately 20,000-30,000 deaths occur annually (WHO Expert Committee, 1982; Singh, 2006; Ozbel and Ozensoy, 2007; Sharma and Singh, 2008; Center for Disease Control and Prevention, 2013; Elmahallawy

et. al., 2014). Endemicity of CL is given in Figure 2.6 and endemicity of VL is given in Figure 2.7, respectively.

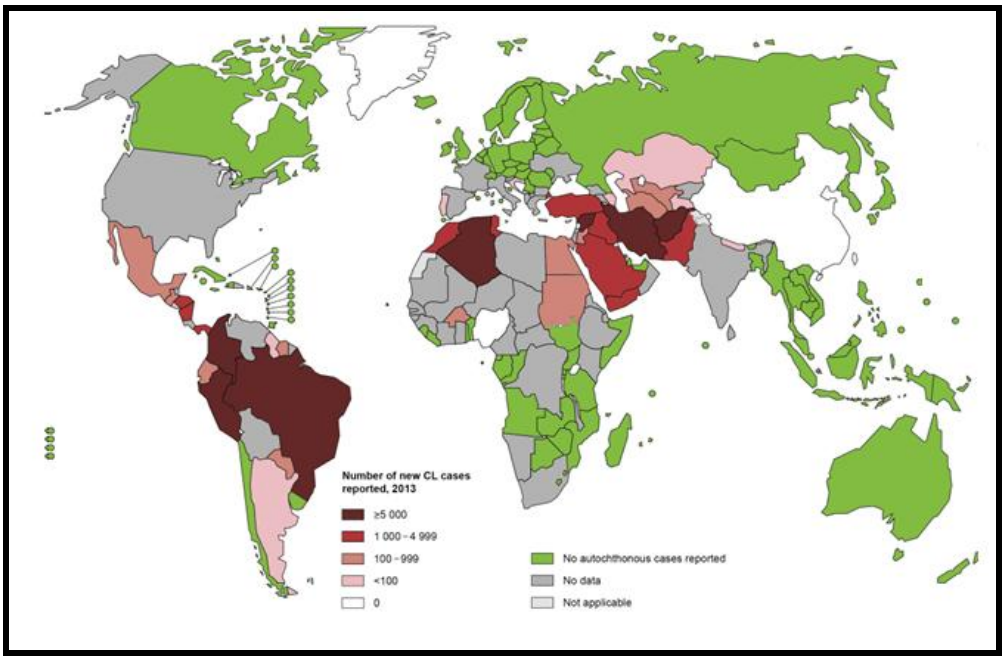

Figure 2.6. Status of endemicity of CL, worldwide, 2013. Burgundy areas indicate more than 5000 CL cases while red areas indicate 1000-4999 CL cases and pink areas specify 100-1000 CL cases. Pale pink areas show less than 100 cases reported in CL. No CL cases were reported in white areas. Additionally, green areas show no autochthonous cases while grey indicate no data and light grey indicates not applicable areas, respectively (WHO, 2015b).

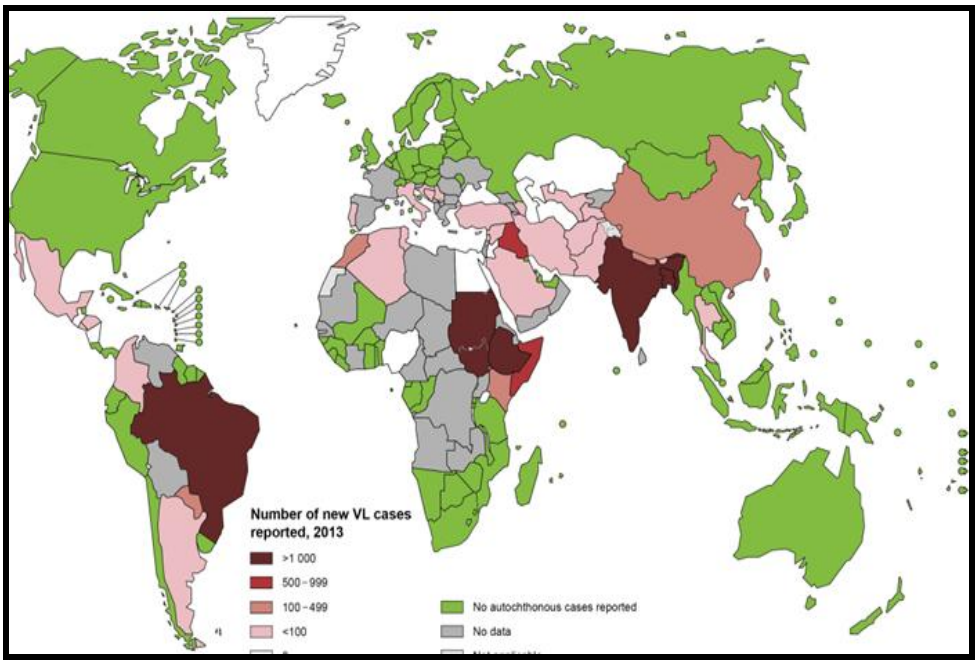

Figure 2.7. Endemicity status of VL, worldwide 2013. Burgundy areas indicate more than 5000 CL cases, while red areas indicate 1000-4999 VL cases and pink areas specify 100-1000 VL cases. Pale pink areas show less than 100 cases reported in VL. No VL cases were reported in while areas. Additionally, green areas show no autochthonous cases while grey indicate no data and light grey indicates not applicable areas, respectively (WHO, 2015c).

Anthroponotic leishmaniasis is primarily seen in urban areas of Islamic Republic of Iran, Morocco, Afghanistan and Syrian Arab Republic, whereas zoonotic leishmaniasis is mainly observed in rural areas. Zoonotic VL and anthroponotic VL are endemic and anthroponotic VL is seen in Sudan and South Sudan (12% of world cases) (WHO, 2015a). Zoonotic VL is mainly seen in the Mediterranean basin. Moreover, India, Nepal, and Bangladesh are the most affected VL regions. PKDL cases are mostly seen in East Africa and the Indian subcontinent (50% and 10% of patients, respectively) (WHO Expert Committee, 1982; Chappuis et. al., 2007; Center for Disease Control and Prevention 2013). According to the researches, *L. infantum* is the etiological agent of zoonotic VL and *L. tropica* is the etiological agent of

anthroponotic CL in Turkey. In addition, *L. major was* reported in the South Anatolia in Turkey. *L. infantum* MON-1 and *L. infantum* MON-98 were isolated from dogs and L. *infantum* MON-1 was isolated from humans in the Mediterranean basin (Ozensoy Toz et. al., 2013a; Zeyrek and et. al., 2015). More than 50,000 *Leishmania* cases have been reported since 2012 in Syria. WHO report of 2010 revealed dramatic change in CL cases in Syria. However, due to the chaotic situation in Syria, most of the *Leishmania* cases were left undeclared. As a result of that, total numbers of *Leishmania* cases are unclear in Syria. *Leishmania*-HIV coinfection is reported at high rates from southern Europe, Ethiopia, Brazil and Bihar in India (WHO Expert Committee, 1982; Sundar and Rai, 2002; Ozbel and Ozensoy, 2007; Center for Disease Control and Prevention 2013; Hayani et. al, 2015).

Epidemiology of leishmaniasis is attributed to environmental, climatic and social factors. The primary factor can be regarded as the poverty. Deforestation, the building of dams, urbanization, irrigation schemes, population mobility, malnutrition and socioeconomic conditions are the main factors that lead to disease progression (WHO, 2015a). Transmission of the disease occurs in rural areas, periurban areas and villages in mountain regions. It occurs in places where rich in humidity and has heavy annual rainfall, like vegetation areas, subsoil and alluvial soil. Moreover, leishmaniasis is also seen in agricultural villages in South East Asia where houses are built with mud walls and earthen floors and where animals like cattle live next to residential homes. CL occurs in East Africa especially in villages that are settled in rock hills, riverbank or natural hyraxe’s habitats (WHO Expert Committee, 1982; Ozbel and Ozensoy Toz, 2007; Center for Disease Control and Prevention, 2013; Hacaloglu, 2014).

2.6. Epidemiology of Leishmaniasis in Cyprus

Cyprus is the third largest island in the Mediterranean Sea (9,251 km^2), with coordinates of 32°16 - 34°36 eastern longitudes and 34°33 - 35°42 northern latitudes (Figure 2.8). Cyprus is located at 105 km west of Syria, 380 km north of Egypt and 75 km south of Turkey (Deplazes et. al., 1998; Demir et. al., 2010; Embassy Of Cyprus, 2015). The weather is mild in winter and hot in summer, which is appropriate for the of the sand flies. Mountains of Cyprus are Beşparmak (Pentadactylos) and Trodos (Troodos) that run along the north coastline and the south coastline, respectively. In addition, Mesarya (Mesaoria) Plain, located between Trodos (Troodos) and Beşparmak (Pentadactylos) Mountains, is the area where production of dry crops such as wheat, oats and barleys takes place (Antoniou et. al., 2008; Demir et. al., 2010).

The first study, about the sand flies, had carried out in 1944 by Adler in Girne (Kyrenia) and Trodos (Troodos) mountains. According to the survey results, three *Sergentomyia* and seven *Phlebotomus* fauna were elicited in Cyprus (Antoniou et. al., 2008; Demir et. al., 2010). The vector of *L. infantum* in the Eastern Mediterranean area is *P. neglectus* and the presence of *P. neglectus* in the island was stated by Demir et al (Demir et. al., 2010). *P. neglectus* was found in the northern part while *Phlebotomus galilaeus (P. galilaeus),* P*. tobbi, Phlebotomus sergenti (P. sergenti), Phlebotomus papatasi (P. papatasi)* were found in the island. These species are responsible from *L. donovani* transmission in Cyprus. Based on the WHO data, *L. infantum* is stated as the etiological agent and *P. tobbi* as the potential vector in Cyprus, respectively. Additionally, *Phlebotomus economidesi (P. economidesi) and Phlebotomus alexandri (P. alexandri)* may cause *L. donovani* MON-37 transmission in Southern Cyprus (Antoniou et. al., 2009; Demir et. al., 2010; Mazeris et. al., 2010).

In Southern Cyprus, Mintr and Eitrem carried out a sand fly survey in 1985. Another study conducted by Depaquit in Southern Cyprus revealed 3 *Sergentomyia* and 8 *Phlebotomus* species (Demir et. al., 2010).

Figure 2.8. Map of Cyprus. Black arrow shows the location of Girne (Kyrenia) (Cyprus Map, Google).

VL cases and CL cases were stated sporadically in research articles since 1935 in Cyprus. In addition, infantile VL cases were reported in 1990 and two CL cases were reported in 1987 from the Northern Cyprus, respectively. CL cases had reported sporadically from foothill villages in Girne (Kyrenia) by Desjeux et al (Deplazes et. al, 1998; Ozensoy Toz et. al., 2013b). According to the data from Eresh and colleagues in 1990, a number of cases increased year by year. *L. infantum* was identified from a skin biopsy by deoxyribonucleic acid (DNA) hybridisation in this study. *Leishmania* skin test positivity was found 35% of young adults in Lapta (Lapithos) and 10% in Girne (Kyrenia), respectively, in a study conducted by Eresh (Deplazes et. al., 1998; Mazeris et. al., 2010; Ozensoy Toz et. al., 2013b). Moreover, two VL and three CL cases were reported in Southern Cyprus in 2006. VL cases reported were 9 months and 73 years old patients and CL cases reported were 44, 50 and 55 years old, respectively. In fact, the origins of VL cases were Cyprus and United Kingdom. All CL cases were from

Cyprus and *L. donovani* MON-37 was isolated from all cases (Antoniou et. al., 2008; Demir et. al., 2010; Mazeris et. al., 2010).

L. infantum MON-1 and *L. infantum* MON-98 were isolated from dogs as a result of CanL research studies in Southern Cyprus (Deplazes et. al., 1998; Mazeris et. al., 2010). Isolation of *L. infantum* MON-1 and *L. infantum* MON-98 from canine isolates from CL areas suggested a coinfection in dogs. Serology and PCR were performed for diagnosis (Antoniou et. al., 2008; Mazeris et. al., 2010; Ozensoy Toz et. al., 2013b). Malaria eradication campaigns lead to the reduction in sand fly fauna in 1940's (Ozensoy Toz et. al., 2013b). Likewise, dog population reduced due to anti-echinococcosis campaign held on between 1970-1975 in Southern Cyprus (Deplazes et. al., 1998). Thus, CanL was virtually eradicated in 1970's. However, CanL reemerged in Southern Cyprus due to increase in sand fly and dog population. The results indicated that CanL in Northern Cyprus, held on in 2008, revealed that CanL was also present in Northern Cyprus but no detailed data exist (Deplazes et. al., 1998; Canakci, 2008; Ozensoy Toz et. al., 2013b).

2.7. Clinical Forms of Leishmaniasis

2.7.1. Visceral Leishmaniasis

VL is a systemic disease that affects the RES. *L. donovani complex* leads to VL. Especially, VL affects immunocompromised people and infants and leads to many clinical manifestations. It might be fatal if left untreated (Gunay et. al., 2005; Pagliano et. al., 2005; Santarém et. al., 2010; Balci et. al., 2011; De Souza et. al., 2012; Lakhal et. al., 2012; Hasker et. al., 2013; Gul et. al., 2014).

VL is endemic in the Mediterranean basin, Latin America, Middle East, East Africa, Indian subcontinent, Northeastern Brazil, tropical and subtropical areas of the world (Sundar and Rai, 2002; Ferroglio et. al., 2007; De Souza,

et. al., 2012; Hasker et. al., 2013). As it is seen endemically, 90% of VL cases occur in Nepal, Bangladesh, East Africa, Brazil, Sudan and Indian subcontinent (Chappuis et. al., 2007; Palatnik-de-Sousa and Day, 2011; Bhattarai et. al., 2012; Abbasi et. al., 2013; Ferroglio et. al., 2013; Clemente et. al., 2014). Co-infection with other diseases and misdiagnose with leprosy, tuberculosis, malaria, brucellosis, amoebiasis, are commonly observed. These issues lead to the delay in clinical diagnosis of leishmaniasis in the endemic areas (Iqbal et. al., 2002; Costa et. al., 2012; Akhoundi et. al., 2013; Kaur and Kaur, 2013). Prompt therapy is necessary for VL and decreases mortality rate (Meredith et. al., 1995; Akhoundi et. al., 2013). In the Mediterranean basin, *L. infantum* leads to infant VL whereas, in Latin America, infant VL is caused by *L. chagasi*. In addition, *L. amozonensis* also leads to VL while *L. donovani* leads to VL cases in India (Sundar and Rai, 2002). As *L. donovani* and *L. infantum* cause VL, *L. infantum* leads to VL in children and immunosuppressed people. However, *L. donovani* leads to disease in all ages (Chappuis et. al., 2007). Apart from these, VL is sporadically seen in Italy, Old Yugoslavia, Spain, Malta, Portugal, Bulgaria and Greece (Chakarova et. al., 2005).

Clinical manifestations of VL include prolonged fever, fatigue, cachexia, hepatosplenomegaly (shown in Figure 2.9), icterus, proteinuria, splenomegaly, pancytopenia, leucopenia, anemia, rapid weight loss, malaise, hypergammaglobulinemia, lymphadenopathy, discomfort in left hypochondrium and suppression of the cellular immune response (Iqbal et al., 2002; Sundar and Rai, 2002; Bodur et. al., 2003; Chappuis et. al., 2005; Romero and Boelaert, 2010; Palatnik-de-Sousa and Day, 2011; De Souza et. al., 2012; Lakhal et. al., 2012; Gul et. al., 2014). In addition to these, diarrhea and cough might be seen in some cases. Furthermore, wasting, malnutrition, paleness in mucosal membranes is the common manifestations observed in VL patients. The incubation period varies from 10 days to 10 years. Generally, it is between two and six months (Gunay et. al., 2005; Chappuis et. al., 2007; Balci et. al., 2011). Different symptoms can be seen depending on the species. For example, darkening of the skin, abdomen, hands, feet

and face are commonly seen in India. Mucosal lesions, cutaneous ulcer and nodules are seen in Sudan (frequently) and East Africa (rarely). Fever in VL is abrupt, and after exposure, it begins in 2 weeks to 2 years. After the first two weeks, fever rises and drops as two daily peaks happen in the morning and in the evening with plenty of sweating. Chills, profound malaise and drenching sweats are the other symptoms that are seen in VL. Moreover, acute renal damage, several mucosal hemorrhage and severe hemolytic anemia are developed by VL patients (WHO Expert Committee, 1982, Unat et. al., 1995; Sundar and Rai, 2002; Ozbel and Ozensoy Toz, 2007). After bitten by sand flies, promastigotes are phagocytosed by macrophages under the skin and these forms turn into amastigotes. They multiply continuously by binary fusion. Small granuloma occurs at the site of the bite and then amastigotes migrate to the RES. Daughter cells produced by amastigotes lead to distend and rupture of macrophages (Paniker, 2013). As a consequence of macrophage destruction, released amastigotes continue to infect macrophages and spread to spleen, liver, bone marrow and lymph nodes (Gunay et. al., 2005; Balci et. al., 2011).

Figure 2.9. VL patient, suffering from hepatosplenomegaly (WHO, 2014).

In VL, the liver, the spleen, the bone marrow, the lymph nodes, the lymphoid tissues and the small intestine mucosa are affected due to the reticuloendothelial hyperplasia (WHO Expert Committee, 1982; Gunay et. al., 2005). Anemia and granulocytopenia occur due to reduced life spans of lymphocytes and erythrocytes. The increase in the number of mononuclear phagocytes leads to progressive hypertrophy in the spleen and liver. As a result, spleen expands extremely and parasitic mononuclear cells replace splenic lymphoid tissue. In Kupffer cells, parasitic mononuclear cells lead to hepatomegaly (Altintas, 2002; Gunay et. al., 2005). After the parasitic invasion of hepatocytes, prothrombin production decreases in the liver. Correspondingly, the prothrombin depletion, together with thrombocytopenia, leads to mucosal hemorrhage. Malnutrition and edema cause hypoalbuminemia. Ulceration and parasitemia in intestine lead to diarrhea. In addition, production of numerous defenseless antibodies leads to hypergammaglobulinemia, commonly seen in VL. Anemia is associated with complement activation (WHO Expert Committee, 1982; Gunay et. al., 2005).

According to the transmission types, there are two types of VL which are anthroponotic and zoonotic VL. In zoonotic VL, transmission occurs from the animal reservoir to humans via vector and it is found in *L. infantum* transmission areas. In zoonotic VL, dogs are the main reservoir and they are the most important risk factors for predisposition of diseases. In anthroponotic VL, transmission occurs from human to human *via* vector and it is found in the areas where *L. donovani* transmission occurs. The reservoir of this disease is dogs and humans are considered as accidental host (Chappuis et. al., 2007; Petersen and Barr, 2009).

Diagnosis of VL can be done by direct parasitological examination, serologic and molecular tests. However, each test has its advantages and disadvantages. Demonstration of amastigotes from biopsy material, tissue smears or bone marrow is used for definitive diagnosis of VL. However, these procedures have low sensitivity. Since these procedures are invasive, they need precision and highly experienced staff (Santarém et. al., 2010; Hasker et. al., 2013).

After recovery of VL, some patients develop PKDL that has a significant role in VL transmission (Sundar and Rai, 2002). PKDL is the complication of VL and is seen in Sudan, Indian subcontinent and East Africa occasionally after treatment (WHO Expert Committee, 1982; Chappuis et. al., 2007). In addition, it can be seen in immunocompromised people living in *L. infantum* endemic areas. Nodular, macular or maculopapular rash are characteristic properties of PKDL. Interval period between treated VL and PKDL is 0 to 6 months in Sudan. However, it is from 6 months to 3 years in India. Depigmented macules, erythematous patches and nodules are the lesion types seen in PKDL. PKDL is not zoonotic and human is the only host and reservoir. As the nodular lesions consist of many parasites, PKDL patients are very infectious (Chappuis et. al., 2007; Alam et. al., 2009; Paniker, 2013).

2.7.2. Cutaneous Leishmaniasis

CL is the most prevalent form of leishmaniasis and characterized by ulcers in the skin, in exposed parts of the body. CL is also known as Delhi boil, Bagdad boil, oriental sore or Aleppo button (Paniker, 2013; Elmahallawy et. al., 2014). However, CL parasite was first found in tissues of Delhi boil in Calcutta. CL leads to deformation of the skin and causes physiological as well as social problems. According to the WHO data, 1 million CL cases develop each year (Monge-Maillo and Lopez Velez, 2013; Paniker, 2013; Mouttaki et. al., 2014; Bsrat et. al., 2015; Hayani et. al, 2015).

Different types of *Leishmania* parasites lead to different types of CL. *L. mexicana* causes localized skin lesions; *Leishmania venezuelensis, L. amazonensis* and *Leishmania pifanoi* causes DCL and *Leishmania braziliensis complex* causes MCL (Guan et. al., 2013; Monroy-Ostria et. al., 2014). *L. tropica, L. major* and *L. aethiopica* leads to the Old World CL. *L. major* and *L. tropica* are found in Afghanistan, India, Middle East, North Africa, Eastern Mediterranean countries. However, *L. aethiopica* occurs in Kenya and Ethiopia. *L. aethiopica* leads to three types of lesions which are

CL, MCL and DCL. In this type, ulceration may be late or absent, lesions progress slowly and healing occurs in 1-3 years or longer (WHO Expert Committee, 1982). *L. amozenensis, Leishmania braziliensis (L. braziliensis)* and *L. mexicana* cause CL in the New World. New World CL shares the similar clinical presentation with Old World CL, however, lesions are more severe and chronic (De Oliveira et. al., 2003). Depending on the *L. braziliensis braziliensis,* single or multiple lesions occur. These lesions seldom heal spontaneously, in primary stages. The size of lesions is variable. Lymphatic involvement might be seen in early stages. If left untreated, it turns into MCL. Then, *Leishmania mexicana mexicana* leads to 'bay sore' or 'chiclero's ulcer'. Lesions are painless and heal in few months. Moreover, most of the lesions are single and ears are commonly involved. Sometimes, lesions tend to be chronic and destruction of the ear is commonly seen (WHO Expert Committee, 1982; Altintas, 2002; De Oliveira et. al., 2003). Single, indolent nodular lesions can be seen in the infection caused by *Leishmania mexicana venezulensis* (WHO Expert Committee, 1982).

L. braziliensis guyanensis, agent of pian bois, leads to dry, single and persistent lesions. Ulceration all over the body and metastases along the lymphatic system are commonly seen (WHO Expert Committee, 1982). 'Uta' is caused by *L. peruviana* and it commonly affects children. Lesions are single and heal spontaneously in four months. *Leishmania mexicana amozonensis (L. mexicana amozonensis)* leads to single or multiple skin lesions that rarely heal spontaneously. Infection is seen in forest rodents, however, people who're affected by this parasite have DCL (WHO Expert Committee, 1982; Akcali et. al., 2007; Es-Sette et. al., 2014; Mouttaki et. al., 2014). Species that lead to CL are stated in Table 2.3 (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007).

Table 2.3. Species that lead to cutaneous leishmaniasis.

Old World Cutaneous Leishmaniasis	*Leishmania aethiopica* *Leishmania infantum* *Leishmania major* *Leishmania tropica*
New World Cutaneous Leishmaniasis	*Leishmania amazonensis* *Leishmania brasiliensis* *Leishmania garnhami* *Leishmania guyanensis* *Leishmania mexicana* *Leishmania panamensis* *Leishmania peruviana* *Leishmania pifanoi* *Leishmania venezuelensis*

CL begins with a lesion at the inoculation site. After that, a crust is formed in the center and may deteriorate to gradually healing ulcers. As a result, depressed scars with altered pigment are developed (WHO Expert Committee, 1982). CL has different types of lesions which are important for clinical manifestations. Skin lesions could be seen as the papule, plaque, ulcer and nodular purigo. Of these, ulcers (shown in figure 2.10) and papules are the most commonly observed lesions. Numerous parasites are present in the infected macrophages in the case of papule and plaque. In addition, reduction or disappearances are seen in collagenous fibres. Lesions can heal spontaneously, disseminate to the nasopharyngeal mucosa in MCL, cause secondary infections or spread through the entire body in DCL. Multiple *Leishmania* species might be coexisting in the endemic areas (WHO Expert Committee, 1982; Paniker, 2013; Monroy-Ostria et. al., 2014).

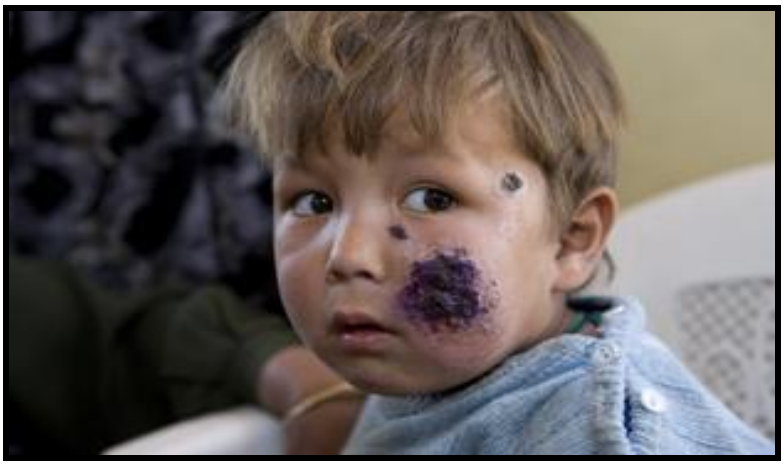

Figure 2.10. A CL lesion affecting the skin (WHO campaigns, 2014).

CL occurs in the RES cells of the skin and lymphoid tissues and leads to dermal lesions. In CL, parasites do not exceed to internal organs. Inflammatory granulomatous reaction with infiltration of lymphocyte and plasma cells are present. Papulation in early lesion then turns into ulceration necrosis. Papules and ulcers heal over months to years and leave scars (Paniker, 2013).

CL has two types that are anthroponotic CL and zoonotic CL (Akcali et. al., 2007; Guan et. al., 2013**;** Alam et. al., 2014**;** Mouttaki et. al., 2014).

Anthroponotic type of CL is caused by *L. tropica* and leads to dry painless lesions with ulceration and results in disfiguring scars. It is commonly seen in the Middle East and North Western India. *P. sergenti* is the most important vector. Anthroponotic CL is known as oriental sore or Delhi boil and seen in children in the endemic areas (Ozbel and Ozensoy Toz, 2007). Anthroponotic CL starts as an elevated papule then turns into nodule and eventually into ulcer in a few weeks. Single or multiple lesions, varying from 0.5 to 3 cm in size, can be seen. Lymph glands are involved and lymphatic spread is a distinctive feature (Altintas, 2002). Ulcers have indurated and raised margins. Ulcers are painless if there is no secondary infection. In the case of a secondary infection, lesions are painful. In *L. tropica* and *L. major* infection, satellite lesions are present (WHO Expert Committee, 1982; Paniker, 2013).

Zoonotic CL leads to inflamed ulcers that are usually multiple, and it is caused by *L. major*. In contrast to anthroponotic type, the incubation period is shorter that is less than four months (Altintas, 2002; Paniker, 2013). Lesions caused by the infection with *L. major* are painless, ulcerated and often inflamed and they heal more rapidly when compared to *L. tropica* ulcers. Multiple lesions are confluent and infected with secondary infections. Zoonotic CL is seen in Africa, Middle East and lowland zones of Asia. The most important vector is *P. papatasi*, while rodents, rats where gerbils are the main reservoirs. Ulcers heal slowly and leave disabling and disfiguring scars (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007; Paniker, 2013; Samy et. al., 2014).

L. tropica is the etiological agent of dry leishmaniasis which is endemic in Asia. It is also commonly seen in North and West Africa and the Mediterranean countries (Ozbel and Ozensoy Toz, 2007; Paniker, 2013). The incubation period of dry leishmaniasis is between 2-12 months. *P. sergenti* and *P. papatasi* are the vectors of dry leishmaniasis. CL starts with an infiltration at the biting site and then papulation is seen. Papules, which evolve slowly, are itchy. After that, nodule becomes hypertrophic. Overtime, epidermis becomes thinner and ulceration occurs. Ulcers reach 1-3 cm wideness in 3-4 months period with a crust on the lesion. In dry leishmaniasis, thorn like structures (typical for dry leishmaniasis) can be seen under the crust (Behcet's sign). Lesions might be single or multiple. Ulcers are painless if secondary infection is absent. Lesions start healing when granulation tissue is formed after a year. Cicatrix is formed in the involved area (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007; Paniker, 2013).

L. major leads to moist CL. It is seen in Turkmenistan, Russia, Kazakhstan, Uzbekistan, Middle East, North and Central Africa, particularly in the rural areas (Altintas, 2002). The incubation period, which is 2 weeks to 3 months, is very short in contrast to dry leishmaniasis. On the other hand, the progress of the disease is very fast, and lymph glands are affected (Altintas, 2002). Ganglion becomes large and multiple lesions can be seen.

Infiltration edema and papule are seen on the site of *Phlebotomus* bite and the papule turns into ulcers. Secondary lesions may form around the ulcer. Ulcers with crust heal in 3-6 months and leave profundus scars that mostly occur in the arms and legs (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007; Paniker, 2013).

Recidivan CL is characterized by a scar formation with a peripheral activity on the face. Lesions are disfiguring and destructive. It is described as relapsing. They progress slowly and do not usually respond to treatment. Amastigotes are rarely found in the lesion, so this manifestation can be misdiagnosed as lupus vulgaris (WHO Expert Committee, 1982; Ozbel and Ozensoy Toz, 2007; Crowe et. al., 2014).

2.7.3. Mucocutaneous Leishmaniasis

L. braziliensis braziliensis, L. braziliensis panamensis and *L. braziliensis guyanensis* are responsible for MCL called ‘espundia’ (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007). It is seen in Peru, Equator, Brazil, Colombia and Venezuela. The primary lesions are similar with CL,. However, metastatic spread to the oronasal/pharyngeal mucosa can be seen in the presence of the primary lesion or up to 30 years (WHO Expert Committee, 1982). Soft tissue and cartilage of oronasal/pharyngeal cavity are destroyed by ulceration and erosion. Secondary granulomatous lesions have formed in nose, mouth, pharynx mucosa due to the blood and lymph expansion. These lesions have numerous macrophages and plasma cells. Incubation time is between 10 days to 2 months in Brazil. Ulcers have formed in uraniscus, lips, ears, pharynx, larynx and trachea via invasive agents from the first lesion. Tapir nose is developed due to the swelling of nose and lips. Conditions may be painless or painful. Secondary infections are commonly seen. Dissemination may occur in the eyes or genital area in years (WHO Expert Committee, 1982; Altintas, 2002; Ozbel and Ozensoy Toz, 2007). On the contrary, lesions do not heal spontaneously as in the case of CL. Mutilation and

suffering are severe, and malnutrition, bronchopneumonia, septicemia or gasping leads to death. MCL in the Old World is reported from Sudan. Agent of MCL in Sudan is *L. donovani* and ulceration of buccal mucosa is slowly evolved. In Ethiopia, infection of the lesions with *L. aethiopica* leads to MCL (WHO Expert Committee, 1982; Altintas, 2002).

2.7.4. Diffuse Cutaneous Leishmaniasis

Many species and subspecies of *Leishmania* cause DCL. Disseminated thickening of the skin in papules, plaques or multiple nodules is seen in DCL (WHO Expert Committee, 1982). It affects the face and exterior surfaces of the limbs and seems like the lepromatous leprosy (Ozbel and Ozensoy Toz, 2007). However, mucosal involvement and ulceration are not seen. Unlike the other types of CL, DCL does not heal spontaneously and is prone to relapses after treatment. It is difficult to treat the disease and lesions remain for years or entire life (WHO Expert Committee, 1982). It is seen in people with low humoral or cellular immunity. In the Old World, DCL is caused by *L. aethiopica*. On the other hand, DCL is caused by *L. mexicana amazonensis* in the New World. *Leishmania mexicana pifanoi* is the only species that leads to DCL in Venezuela (WHO Expert Committee, 1982; Paniker, 2013).

2.7.5. Canine Leishmaniasis

CanL, which is a zoonotic disease, is caused by *L. infantum* and is fatal for dogs. It is characterized by dull of haircut, depression, decrease in muscle mass, loss of condition, splenomegaly, lymphadenopathy, serosanguinous nasal discharge, diarrhea, vomiting, melena, epistaxis, long brittle nails, dry brittle hair coat. Fever is seen in some cases (Petersen and Barr, 2009). There are many clinical signs of the CanL. The most common manifestation is the skin lesions in dogs. From mild proteinuria to nephrotic syndrome or an end stage renal disease may be the manifestations of CanL

(Solano-Gallego et. al., 2011). The main cause of mortality is the chronic renal failure. Age, breed and genetic properties are predisposing factors that affect the CanL development. It is stated that Cocker Spaniel, Rottweiler, Boxer and German Shepher dog breeds are more susceptible to develop CanL. In contrast, some dog breeds like the Ibizian hound rarely develop the disease. CanL is often seen in dogs younger than 3 years and older than 8 years (Petersen and Barr, 2009; Solano-Gallego et.al., 2011)

CanL is seen in more than 70 countries endemically in Asia, Africa, South and Central America, USA and Southern Europe. Especially, CanL is endemic in Spain. Importation from endemic areas to non-endemic areas generates public health problems (Solano-Gallego et. al., 2011). Distribution of CanL in Europe has changed due to the climatic and socioeconomic factors. Movement of infected dogs, changes in vector distribution and increase in travel lead to the change in the epidemiology. CanL is seen in northern Spain, the foothills of the Alps in Italy and the Pyrenees in France. CanL is reported from Italy in the late 1990's. Infections may be seen in different types that can be self-limiting, severe, subclinical or fatal (Aisa et. al., 1998; Solano-Gallego et. al., 2011; Ferroglio et. al., 2013; Mattin et. al., 2014). Distribution of CanL in Europe is given in Figure 2.11.

Figure 2.11. Canine *L.infantum* distributions map in Europe (2011). Burgundy area shows endemic areas and white question marks in these areas indicate missing information while green area shows non-endemic areas. Yellow question marks in green area indicate autochthonous cases while yellow spots on green area indicate autochtonous foci (Solano-Gallego et. al., 2011).

Diagnostic tests of the CanL are not 100% specific and sensitive. Indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) can be used for diagnosis. In addition, PCR can also be used (the preffered method of diagnosis). Detection of the kinetoplast DNA (kDNA) by PCR is the most sensitive method for diagnosis. PCR, which is conducted by using the biopsy material, has a high sensitivity in dogs (Solano-Gallego et. al., 2011). Even though, renal insufficiency have lower recovery rate in comparison to other mild syndromes. Using a protective collar, which containing synthetic pyrethroids, permethrin or deltamethrin, supplies protection against sand flies and decreases the infection risk. In addition, reducing habitats of sand flies, keeping dogs inside and using indoor

insecticides might also be protective for disease (Solano-Gallego et. al., 2011; Allahverdiyev et. al., 2012).

Symptomatic and asymptomatic dogs are the reservoirs of the CanL. Thus, detection of the infected dogs is crucial. Internal and cutaneous lesions are seen in the CanL. Skin lesions may cause skin pallor and inflammation, lameness, anemia, anorexia, epistaxis, manginess, somnolence, weight loss, local or general lymphadenopathy, diarrhea, ocular lesions, eye inflammation, keratoconjunctivitis, uveitis, thirst and frequent urination, fever, renal failure, sinking in the nails and neural and vascular diseases. Cutaneous lesions with alopecia, epidermal thickening, depigmentation and chapping of footpads are seen (El-Harith et. al., 1989; Ica et. al., 2008; Petersen and Barr, 2009; Solano-Gallego et. al., 2011). Splenomegaly, hepatomegaly, enlarged lymph nodes, are observed in dogs due to the parasitic invasion of the RES cells. However, clinical symptoms are not shown all the time. Kidney disease may be the only clinical manifestation and is the cause of nephrotic syndrome that begins with mild proteinuria. Chronic renal failure usually leads to an increase in the mortality rate of CanL. Clinical signs observed in dogs may confuse with malignant tumours and disease like lupus, endocrine disease and lymphoproliferative disease. Serologic and molecular methods are in use for CanL detection (Ica et. al., 2008; Solano-Gallego et. al., 2011). Some of the clinical manifestations of CanL are given in Figure 2.12. Drugs which are used in the treatment of human VL cannot be effective in the management of CanL and cause resistance and relapses. Dogs which have CanL more than two years cannot be treated. Therefore, using of collars and insecticides and early diagnosis of CanL are very important (El-Harith et. al., 1989; Petersen and Barr, 2009; Solano-Gallego et. al., 2011; Paulan et. al., 2013; Quinnell et. al., 2013; Hosseininasab et. al., 2014).

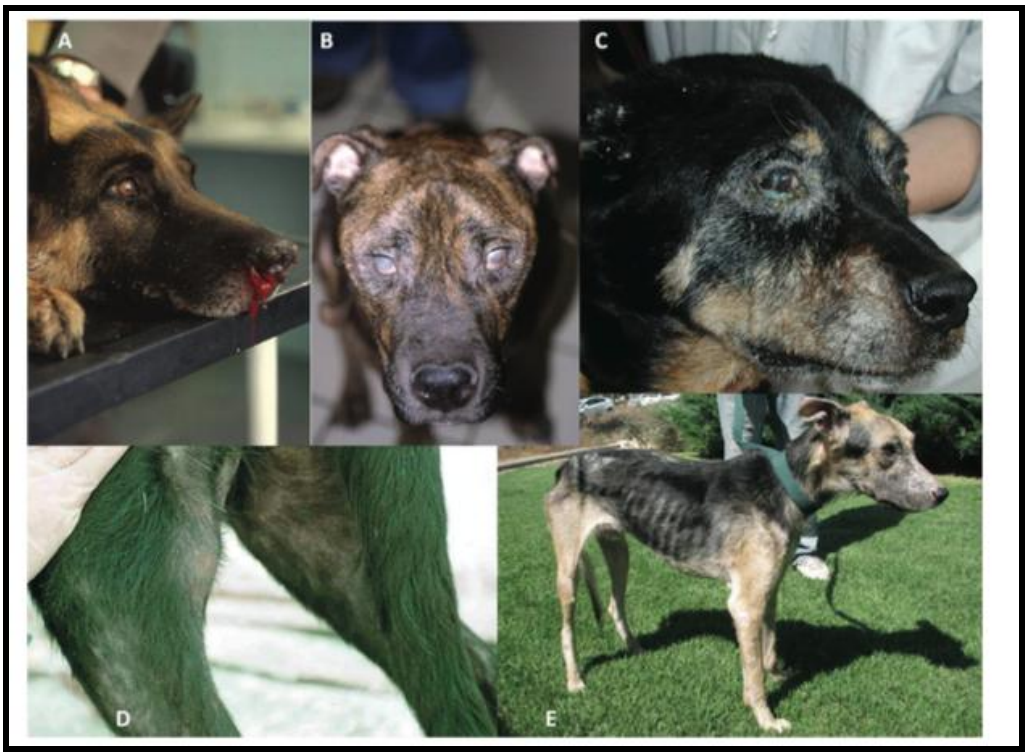

Figure 2.12. Clinical sings of CanL. (A: Epistaxis, B: Bilateral uveitis and corneal opacity, C: Purulent conjunctivitis and blepharitis, D: Exfoliative alopecia in the rea leg and popliteal lymphadenomegaly, E: Marked cachexia and generalized exfoliative alopecia) (Solano-Gallego et. al., 2011).

2.8 Diagnosis of Leishmaniasis

Diagnosis of leishmaniasis relies on microscopic examination of bone marrow, lymph node, splenic aspirate material or investigation of nucleated cells from peripheral blood analysis. However, these methods are invasive and cumbersome (Ozbel and Ozensoy Toz, 2011). Moreover, they have low sensitivity and give cross-reaction with leprosy, Chagas' disease and malaria (Lakhal et. al., 2012). Aspiration material can be used directly in PCR that is the most sensitive method for VL diagnosis and high sensitivity in peripheral blood in contrast to blood culture and microscopy (Allahverdiyev et. al., 2012). rK39 strip test has an advantage over ELISA, IFAT and DAT. Parasite

detection by microscopic examination has a high specificity but its sensitivity depends on the type of aspirates; this is 53-65% of the lymph node, 53-86% for the bone marrow, and 93-99% for the spleen aspirate. However, aspiration is an invasive and life treating procedure (Allahverdiyev et. al., 2005; Chappuis et. al., 2007; Ferroglio et. al., 2007; Ozbel and Ozensoy Toz, 2007; Romero and Boelaert, 2010; Ozbel and Ozensoy Toz, 2011; Allahverdiyev et. al., 2012; Lakhal et. al., 2012).

Serologic tests depend on detection of the anti-leishmanial antibody, have high sensitivity and are widely used in the diagnosis of VL. However, due to the low antibody levels in the blood of asymptomatic patients, the sensitivity of serologic tests decreases. These tests cannot be used by itself as a diagnostic test in CL diagnosis (Ozbel and Ozensoy Toz, 2011). In contrast to CL, IFAT, DAT, fast agglutination screening test (FAST) and ELISA are serologic tests in use for diagnosis of VL. However, crude antigen preparations limit specificity of these tests and give cross reactivity to other endemic diseases such as trypanosomiasis and malaria (Lakhal et. al., 2012). Antigen type is very important for serologic tests. Preparations with amastigotes are more sensitive than promastigotes. IFAT, Western Blotting (WB), ELISA has high specificity but cannot be used in field studies. However, DAT and rK39 strip test are used in field studies but serology does not discriminate active and past infections (Chappuis et. al., 2007; Ferroglio et. al., 2007; Ica et. al., 2008; Alam et. al., 2009; Santarém et. al., 2010; Solano-Gallego et. al., 2011; Lakhal et. al., 2012; Solano-Gallego et. al., 2014).

Serologic and parasitologic tests such as direct examination are used in the diagnosis of CanL. Serologic tests include DAT, IFAT, ELISA and WB. Microscopy is used in the diagnosis, however, it has low sensitivity and requires an invasive procedure. PCR is the preferred method because of its high sensitivity and specificity. Asymptomatic dogs can be detected by PCR (Aisa et. al., 1998; Ferroglio et. al., 2007; Kilic et. al., 2008; De Souza et. al., 2012).

2.8.1. Microscopy

Microscopic examination of bone marrow, splenic aspirates and biopsy materials from the liver, lymph nodes, blood and buffy coats are used in VL diagnosis. Microscopic examination of aspirates is still considered as gold standard in leishmaniasis diagnosis. However, it is a cumbersome method and needs experienced staff (Reithinger and Dujardin, 2007; Ica et. al., 2008; Elmahallawy et. al., 2014). The sensitivity of microscopy depends on sample type used in diagnosis. Staining with Giemsa helps to see amastigotes under the light microscope. After microscopic examination, these materials can culture into Novy-MacNeal-Nicolle (NNN) agar and motile promastigotes can be seen in two weeks. Sampling should be done with expertise staff in order to prevent fatal hemorrhage. Splenic aspiration has the highest sensitivity. Bone marrow aspiration sensitivity is changeable (Reithinger and Dujardin, 2007; Ica et. al., 2008; Elmahallawy et. al., 2014). Lymph node aspiration is performed in patients who develop lymphadenopathy. However, parasites cannot be always detected. Bone marrow aspiration is performed under local anesthesia. Spleen aspiration is a very dangerous procedure in the acute phase. Caution risk of the lethal hemorrhage is very high. Thus, it should not be preferred in the first place (Ozbel and Ozensoy Toz, 2007; Reithinger and Dujardin, 2007; Ica et. al., 2008; Ozbel and Ozensoy Toz, 2011; Elmahallawy et. al., 2014).

In contrast to VL, in CL and MCL patients, scrapings, lesion smears of biopsies and smears are used as the sample. Ulcer based materials give the highest yield (Elmahallawy et. al., 2014). Samples should be taken from the edge of the ulcer, not in the center. The first aspirated sample should be used for microscopy and the last sample should be used for culture. By this way, contamination risk is minimized. Microscopy and culture together increase the sensitivity (Ozbel and Ozensoy Toz, 2007; Reithinger and Dujardin, 2007; Ozbel and Ozensoy Toz, 2011; Elmahallawy et. al., 2014).

2.8.2. Culture

Diagnosis of VL is done by the samples taken from the RES organs such as the spleen, bone marrow, liver or lymph nodes. These preparations are stained with the Wright or Giemsa stains and these materials are cultivated in the NNN medium (consist of defibrinated rabbit blood, sodium chloride, distilled water and peptone) under sterile conditions. This procedure has a long incubation time and is time consuming. Thus, it cannot be used for the prompt diagnosis. Since culture is prone to contamination., caution must be taken toavoidthis risk (Unat et. al., 1995; Ozbel and Ozensoy Toz, 2011; De Vries, 2015). Promastigotes start to proliferate in the culture after approximately 10 days of inoculation. Culture is accepted as negative after one month control check. Promastigotes proliferate more quickly in the liquid media such as RPMI-1640, M199, and fetal calf serum added Schneider's Drosophila medium. Cultures are incubated at 24-27°C. Cultures are the samples of active parasites obtained from patients' aspirate materials (Ozbel and Ozensoy Toz, 2011; Elmahallawy et. al., 2014). The sensitivity of culture is very high in the symptomatic patients, however, it is very low in the asymptomatic patients (Allahverdiyev et. al., 2012).

In CL patients, samples are taken from the border of lesion or epidermis-dermis component. During sample aspiration, saline solution is injected into the area and aspirated back. By this way, amastigotes are seen more properly (De Vries, 2015). Recently developed microculture method is more sensitive and easy to perform. Fewer medium is required in contrast to the classical culture method. However, differentiation of species could not be performed by this method. When compared to the other methods, microculture is more sensitive in the diagnosis of asymptomatic infections (Allahverdiyev et. al., 2005; Allahverdiyev et. al., 2012; Elmahallawy et. al., 2014; De Vries, 2015).

2.8.3 Serologic Tests

Direct Agglutination Test

DAT, developed by Harrith and colleagues, is one of the serologic tests that are used since 1986 in the diagnosis of VL and CanL. It is a very reliable, specific, sensitive, simple and cost-effective method (Silva et. al., 2005; Hamzavi et. al., 2012; Akhoundi et. al., 2013; Bamorovat et. al., 2014). DAT uses whole promastigotes for the screening of antibody formation. By this way, DAT can easily detect high titers. It is approved for the diagnosis of VL in the endemic areas and is very easy to perform, does not require electricity and cold storage of reagents (Mohebali et. al., 2006; Maia et. al., 2012). On the contrary, it has some drawbacks such as long incubation time, cumbersome procedure and antigen type (Silva et. al., 2005; Bamorovat et. al., 2014). Due to low levels of anti-*Leishmania* antibodies in HIV patients, DAT does not detect leishmaniasis. Therefore, results should be confirmed by other methods (Meredith et. al., 1995).

The type of antigen is important in DAT studies. Freeze-dried trypsin-fixed and stained *L. donovani* promastigotes are preferred over liquid antigens. Antigens should be stored between 2-8°C after they are liquidified. In DAT, microtitre plates are used for diluting the sample which is mixed with the killed *L. donovani* promastigotes. Agglutination can be seen after 18-hour incubation (Mohebali et. al., 2006; Chappuis et. al., 2007; Adams et. al., 2012; Elmahallawy et. al., 2014).

Promastigotes are formalin fixed, coomassie blue stained and ready to use. A serum sample is added to the dilution fluid and then antigens are gently mixed and incubated. Agglutination is observed after the incubation time. Adding 2-mercaptoethanol to the dilution fluid prevents cross reactivity of DAT with other diseases. DAT does not differentiate between the past, subclinical or current leishmaniasis (Meredith et. al., 1995; Sundar and Rai, 2002; Chappuis et. al., 2007; Adams et. al., 2012).

FAST is the improved form of DAT. One serum dilution is used in FAST test and the incubation time is 3 hours in comparison to DAT (Silva et. al., 2005). However, further validation with DAT is needed in positive results (Chappuis et. al., 2007; Ozbel and Ozensoy Toz, 2007; Elmahallawy et. al., 2014).

rK39 Immunochromatographic Dipstick Test

The recombinant protein rK39 is composed of 39 amino acid repetitive immunodominant B cell epitope antigen conserved in the kinesin region of *L. donovani* complex. The immunochromatographic rapid rK39 dipstick test has high sensitivity (Chappuis et. al., 2005; Chappuis et. al., 2007; Santarém et. al., 2010; Kumar et. al., 2011; Maia et. al., 2012; Hosseininasab et. al., 2014). This test does not discriminate between active, past and asymptomatic infections (Meredith et. al., 1995; Sundar and Rai, 2002). However, it cannot be used in the diagnosis of relapses since the anti-leishmania antibodies remain positive for years (Mohebali et. al., 2006). rK39 is found in *Leishmania* amastigotes however, it cannot be used for the diagnosis of CL and MCL due to the low antibody levels (Ozbel and Ozensoy Toz, 2011). The test has 100% specificity and sensitivity in human VL and CanL however it has a limited sensitivity if the antibody level is low (Chappuis et. al., 2006; Ozbel and Ozensoy Toz, 2007; Costa et. al., 2012).

Enzyme Linked Immuno-Sorbent Assay

ELISA uses enzymes for screening antigen-antibody reactions and has been utilized for the diagnosis of VL for a long time. It has a high sensitivity and long storage time of reagents. It is easy to perform and allows high numbers of sample screening. However, sensitivity is limited by antigen type used in the test. ELISA is based on the addition of enzyme-labeled conjugate within the solid material or on the generated antigen-antibody

complex, following with addition of the substrate and then observing the colour formation in the positive samples. Total soluble antigens are used with 80-100% sensitivity and 85-95% specificity. However, using total soluble antigen causes problems (Santarém et. al., 2010; Maia et. al., 2012). Different types of antigens used in ELISA are the whole or soluble extract of amastigotes, recombinant proteins, purified antigens and whole or soluble extract of promastigotes. Antigen types determine specificity and sensitivity. Use of amastigote antigen was found to be more sensitive in dogs. In the diagnosis of CL, use of recombinant Heat Shock Protein 83 was shown to have a high performance (Kaur and Kaur, 2013; Solano-Gallego et. al., 2014; De Vries, 2015).

K39 antigen is also used in ELISA as a recombinant antigen, and this is known as rK39 ELISA. It is very important in the diagnosis of acute VL and has a high sensitivity in HIV-positive patients. However, CL and MCL patients do not respond to rK39 antigen (Ozbel and Ozensoy Toz, 2011; Elmahallawy et. al., 2014).

Immunofluoresence Antibody Test (IFAT)

IFAT is widely used in the diagnosis of VL. It remains positive for 9-12 months after treatment. It gives positive results in the early phases of the disease. Thus, it is important for diagnosis and relapses (Ozbel and Ozensoy Toz, 2011).

IFAT is widely used in the diagnosis of CanL, however, it has some drawbacks. Each laboratory has different cut off values for IFAT and results cannot be repeated (Ferroglio et. al., 2007; Ferroglio et. al., 2013). In addition, IFAT requires specialized equipment for the microscopic examination. Another limitation is the preparation of the antigens which is very sensitive. Promastigotes, which are produced in culture, are commonly used for minimizing the cross-reactivity with the trypanosomal infection for

IFAT. In addition, protecting the shape of amastigotes or promastigotes in the antigen preparation is very important (Ozbel and Ozensoy Toz, 2007; Elmahallawy et. al., 2014). In CL, IFAT alone, cannot be used as a diagnostic test. Further confirmation with other tests is needed. It gives high titers in mucosal lesions or multiple lesions but other types of lesions give low antibody titers. Antigen types are important (Ozbel and Ozensoy Toz, 2011).

LATEX Agglutination Test (KATEX)

LATEX Agglutination test (KATEX) is one of the serologic tests detecting heat stable, low molecular weight carbohydrate antigen in urine samples. This test is in use in the Indian subcontinent and East Africa. It has high specificity but low sensitivity. It is used in VL diagnosis especially in immunocompromised people. KATEX, boiling of urine sample prior to testing allows reduction of false-positive results (Ozbel and Ozensoy Toz, 2011; Akhoundi et. al., 2013; Elmahallawy et. al., 2014). Additionally, these tests do not distinguish negative and weakly positive results (Chappuis et. al., 2007; Ozbel and Ozensoy Toz, 2007; Kaur and Kaur, 2013; Elmahallawy et. al., 2014).

Western Blotting

Western Blotting (WB), which uses whole promastigotes and detects low levels of antibody, is regarded to be more sensitive than serologic techniques. However, in the diagnosis of CL, WB is not preferred due to the low titers of antileishmania antibodies (Aisa et. al., 1998; Kaur and Kaur, 2013). WB is found to be more sensitive than IFAT and ELISA. However, it requires expert laboratory staff and expensive equipment and thus, its use is limited. It is used for validation of the results obtained from other techniques. It is stated that WB is more sensitive than IFAT in CanL diagnosis (Aisa et

al., 1998; Ferroglio et. al., 2007; Ferroglio et. al., 2013; Elmahallawy et. al., 2014; Solano-Gallego et. al., 2014).

Other Tests

Leishmania Skin test and Formol gel test were preferred for leishmaniasis diagnosis. Since their results were not specific to the disease, nowadays they are currently replaced with the other tests such as IFAT, DAT and ELISA.

Leishmania Skin test is also known as Montenegro Skin Test and is usually used in leishmaniasis epidemiological studies. In CL, this test turns into positive after 2-3 months of lesion formation and stays positive the rest of the life. In CL diagnosis, this test is used as the marker for cellular response. However in VL, it remains negative in the acute phase and turns into positive after two years of treatment (Ozbel and Ozensoy Toz, 2007). Montenegro skin test cannot be used by itself in VL diagnosis due to the development of strong *Leishmania*-specific cell-mediated immunity after treatment. This test is positive in MCL and very important diagnostic test for detection of low parasite titers. Results in PKDL are changeable. It might be positive or negative in Africa. However, it is always negative in India (Ozbel and Ozensoy Toz, 2007). This test does not differentiate past and present infections. In addition, it requires culture facilities to produce antigens (Ozbel and Ozensoy Toz, 2007; Reithinger and Dujardin, 2007; De Vries, 2015).

Formol gel test is performed easily and is cheap. In addition, it detects polyclonal immunoglobulin (hypergammaglobulinemia) (Chappuis et. al., 2005). The production of high titers of nonspecific antibodies is the most commonly seen in VL patients. This test is used in the Indian subcontinent and East Africa, however, sensitivity is very poor. Positive results do not approve VL diagnosis, however, it raises clinical controversies (Chappuis et. al., 2005; Ozbel and Ozensoy Toz, 2007). The test starts to become positive after one month and stays positive after 3-4 months of treatment. Two drops

of formol solution are added to one ml serum sample. If the results are positive, gelling and whitening are observed. Moreover, VL relapses cannot be detected by Serologic test due to anti-*leishmania* antibodies that remain positive for a long time after treatment (Unat et. al., 1995; Chappuis et. al., 2007; Ozbel and Ozensoy Toz, 2007; Paniker, 2013).

2.8.4. Molecular Methods

Polymerase Chain Reaction (PCR) is highly specific and sensitive than serologic and microscopic methods in the diagnosis of leishmaniasis. It also detects asymptomatic infection (Chappuis et. al., 2007; Ferroglio et. al., 2007; Ferroglio et al., 2013; Clemente et. al., 2014). In addition, PCR can detect relapses and reinfection (Sundar and Rai, 2002).

PCR, uses whole blood samples, gives 98.5% sensitivity and specificity (Ica et. al., 2008; Maia et. al., 2012; Abbasi et. al., 2013). Buffy coat samples were shown to be ten times more sensitive than the whole blood in PCR detection for leishmaniasis. However, PCR needs experienced staff, expensive machines and has a high cost (Chappuis et. al., 2007; Ica et. al., 2008; Maia et. al., 2012; Salam et. al., 2012; Abbasi et. al., 2013). Besides, samples need to be frozen. Samples stored at room temperatures are stable for 6 months but the use of filter paper samples allows detection after years (Alam et. al., 2009; Eslami et. al., 2014).

Moreover, *Leishmania* genus can be identified by PCR methods but species cannot be determined by genus-specific methods. Different targets, like kDNA minicircles, telomeric sequences, gp63, miniexons, β-tubulin or ribosomal RNA encoding genes, have been applied for the identification of species in *Leishmania* parasites (Ozensoy Toz et. al., 2013a). kDNA is a unique organelle found in *Leishmania* and thousands of copies of DNA is possed by kDNA (Sundar and Rai, 2002). Using PCR with the kDNA primers is promising as it has a high sensitivity (Lakhal et. al., 2012). ITS-1 PCR is

able to distinguish nearly all *Leishmania* species with the restriction enzymes (Alam et. al., 2009; Eslami et. al., 2014).

Specific discrimination of *Leishmania* species can be accomplished by DNA sequencing, restriction fragment length polymorphism (RFLP) and multi-locus enzyme electrophoresis (MLEE) (Gouzelou et. al., 2012; Ozensoy Toz et. al., 2013a). RFLP technique allows direct species identification. Results can be obtained from blood, serum or tissue samples. RFLP gives accurate results, thus, there is no need for culturing or analysis by microscopy or other techniques. MLEE is reference method for typing *Leishmania* strains. However, prior isolation and mass culturing of parasites are required (Gouzelou et. al., 2012; Eslami et. al., 2014).

2.9. Treatment

Treatment of leishmaniasis is based on *Leishmania* species, clinical presentations and geographic regions (Monge-Maillo and Lopez Velez, 2013).

2.9.1. Treatment of VL

The first line treatments of VL, pentavalent antimonials are the drugs of choice. Sodium stibogluconate, sodium antimony gluconate, pentamidine isethionate, meglumine antimoniate are the drugs that are used for the treatment of VL (Altintas, 2002; Chappuis et. al., 2006). However, antimonials have very toxic side effects and sometimes they can be life threatening besides, resistance to antimony compounds was demonstrated (Chappuis et. al., 2007). Due to the resistance, amphotericin B deoxycholate (intravenously or intramuscularly) or miltefosine (oral) are used instead as first line drugs. Furthermore, these drugs are used in the endemic regions in the case of a treatment failure with the pentavalent. In addition, they have side effects which include fever or chills. In the United States and Europe,

liposomal amphotericin B, and miltefosine (the first oral drug) are prescribed for VL (Chappuis et. al., 2005; Chappuis et. al., 2006; Chappuis et. al., 2007; Georgiadou and et. al., 2015).

Liposomal amphotericin B is recommended for the protection against secondary bacterial infections and is applied without toxicity in VL therapy. Paromomycin that is given intramuscularly is also preferred. Drugs used in VL therapy are also used for the treatment of PKDL (Ozbel and Ozensoy Toz, 2007; Chappuis et. al., 2007; Paniker, 2013). Relapses are common in VL, especially in HIV-infected patients (Kayser et. al., 2002; Georgiadou and et. al., 2015).

2.9.2. Treatment of CL

CL treatment might be topical or systemic. Topical treatment includes paromomycin ointment, thermotherapy, cryotherapy, and local application of local antimonials. Antimonial preparations, can be applied intralesional, are used in *L. major* and *L. tropica* infections (Kayser et. al., 2002, Monge-Maillo and Lopez Velez, 2013).

Old World CL heals spontaneously. However, it is necessary to consider the existence of secondary infections, therefore, the attention has to be paid for the cleaning of the wound. Pentavalent antimonials are the drugs of choice in CL therapy. Sodium stibogluconate, meglumine antimoniate and ketoconazole are used in the therapy (Altintas, 2002). Treatment of CL is the same as VL. However, pentamidine is used when resistance occurs. Amphotericin B, antifungal agents, miltefosine and paromomycine might be alternative to pentavalent antimonials. A paste of 10% charcoal in sulphuric acid or liquid nitrogen is used in the topical treatment (Paniker, 2013; De Vries, 2015).

2.9.3. Treatment of MCL

Treatment of MCL includes antimonials, amphotericin B and pentamidines (Kayser et. al., 2002). Pentavalent antimonial compounds, pyrimethamine, metronidazole and amphotericin B are used in MCL therapy (Altintas, 2002). Pentavalent antimony compounds are used for the treatment of MCL but have a moderate effect. Systemic antimonials are preferred in the treatment of *L. braziliensis* infections (Paniker, 2013; De Vries, 2015).

2.10. Prevention

Eliminating the sand fly fauna completely is difficult due to the different resting, feeding and reproduction areas of sand flies. Sand flies proliferate easily in the humid areas and they easily hide in the dark and windless places. Indoor protection with insecticides is effective (Sharma and Singh, 2008). Using repellent lotions and permethrin-deltamethrin bed nets provides protection. Insecticides could be applied in both indoors and outdoors, barns, tree holes and nests and could protect against vector. Using air conditioning systems and fans are also protective (Killick-Kendrick, 1999; Sharma and Singh, 2008; Solano-Gallego et. al., 2011).

Dogs should be checked for the infection twice a year by the serologic tests. Dogs with the positive test results should be removed from the area or treated with drugs. Using deltamethrin-treated collar is also protective. Early diagnosis is very important for the achievement of treatment (Solano-Gallego et. al., 2011).

3. MATERIALS AND METHODS

3.1. Selection of the Volunteers and the Ethical Approval

In this study, Girne (Kyrenia) and surrounding regions were chosen as the pilot areas due to the high amount and diversity of *Phlebotomus* spp. in these regions. This study was conducted from September 2014 until September 2015 and a total of 250 participants were included in this study. Volunteers who were living in these areas at least for one year were chosen and people who owned dogs were given priority in the study. The leftover serum and blood samples in the routine tests were used from a total of 242 volunteers who admitted to Girne (Kyrenia) Akçiçek Hospital (n: 166), and those who were redirected to the Girne (Kyrenia) Akçiçek Hospital from Lapta (Lapithos) Health Center (n: 71) and Esentepe (Agios Amvrosios) Health Center (n: 5). Apart from the randomly selected volunteers, patients with a clinical history of CL were informed about the research and kindly requested to participate in this study. A total of eight individuals were included in the study on the voluntary basis. Among seven individuals who were previously diagnosed as CL and received the treatment, three participants attended the study at Girne (Kyrenia) Akçiçek Hospital while the samples of four patients were redirected from Lapta (Lapithos) Health Center. In addition to these participants, a patient from Mağusa (Famagusta) who was recently diagnosed as CL and began to receive the treatment attended the study. The residential areas and CL history of the participants were summarized in Table 3.1.

Ethical approval for the study was obtained from the Near East University Scientific Research Assessment Ethics Committee (Project no: YDU/2014/22-128).

Table 3.1. The residential areas and CL history of the participants, leishmaniasis survey, Northern Cyprus, 2015.

Residential areas of the participants	Participants without a history of CL	Participants with a history of CL	Total number of the participants
Girne (Kyrenia) Akçiçek Hospital	166	3	169
Lapta (Lapithos) Health Center	71	4	75
Esentepe (Agios Amvrosios)	5	-	5
Mağusa (Famagusta)	-	1	1
Total number of the participants	242	8	250

3.2 Sample Collection

In this study, whole blood and serum samples were collected from 250 participants. Whole blood samples were used in order to search for *Leishmania* spp. by molecular methods. These samples containing ethylenediaminetetraacetic acid (EDTA) were kept at -20°C until DNA isolations were carried out. The serum samples, collected from the participants, were used for the serologic tests (DAT and rK39). Sera were centrifuged at 4000 rpm for 10 minutes and then these samples were separated into 1.5 ml Eppendorf tubes and stored at -80°C until used.

3.3. Questionnaire

During the sample collection, the participants were kindly requested to fill the personal information form and the questionnaire. The volunteers' name, surname, age and gender were documented. In the next stage,

questions related with leishmaniasis were answered. The participants were questioned whether they had dogs, what gender and how old their dogs were, and whether their dogs had protective collar against leishmaniasis. The dog owners were kindly requested to provide information about whether their dogs had the previous history of CanL, or whether they noticed one or more signs or symptoms of leishmaniasis in their dogs. In the questionnaire, all of the volunteers also answered whether they had any information about leishmaniasis (Appendix 1-4).

3.4. Serologic Tests

In this study, serologic tests (DAT and rK39 dipstick test) were conducted by using the serum samples collected from 250 participants. The serologic tests were performed at the Near East University (NEU) Hospital Clinical Microbiology Laboratory, Northern Cyprus.

3.4.1. Direct Agglutination Test

DAT was conducted according to the manufacturer's protocol (El Harith et. al., 1988; Adams et. al., 2012). Frozen serum samples were taken out from the freezer and incubated at 56°C for 30 minutes. Heat-inactivated serum samples were then left at the room temperature until thawing completely. Five ml physiological saline (0.9% NaCl) was added to the bottle containing the freeze-dried antigen. This preparation was mixed gently and left at room temperature for a minimum of 5 minutes prior to use. In the meantime, serum dilution fluid consisting of 0.9% (wt/vol) NaCl + 0.78% (vol/vol) β-mercaptoethanol was prepared. Microplates were labeled with the numbers of participants and filled with serum dilution fluid. First well of rows were filled with 100 µl dilution fluids and the rest three wells were filled with 50 µl. One µl of the serum sample was added to the first well of the row. After adding the serum sample, dilution was made by removing 50 µl from the first well and adding to the second well of the row. After well mixing, 50 µl of the

dilution were taken from the second well and added to the third well of the row. This process was repeated until the last well of the row. In this study, four wells were used for each patient and suspicious positive results were repeated by using full wells of each row. One row was left blank for the negative control. Eventually, 50 µl of the antigen were added to each well and plates were tapped gently. Plates were then left for incubation at the room temperature for 18 hours. Microplates were read on a white background after the 18-hour incubation (Figure 3.1). Enlarged blue button with traces of agglutination was considered as positive. Cut-off titer was ≥1:1600.

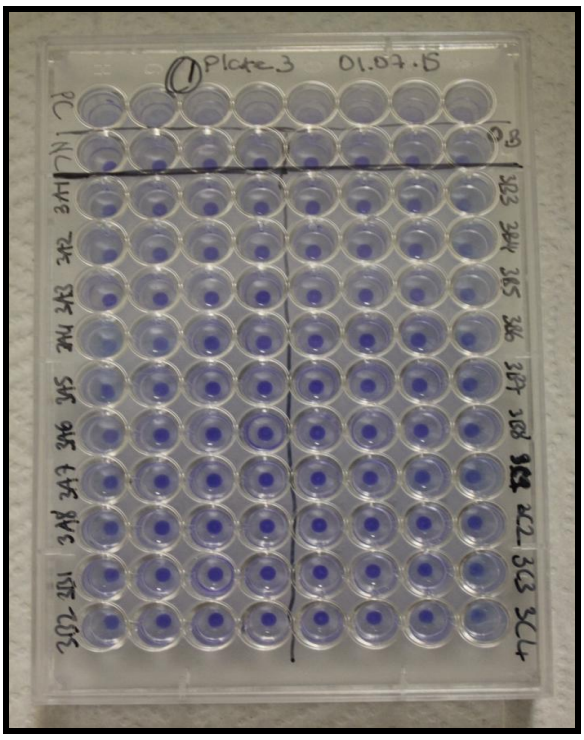

Figure 3.1. Example of DAT in a microplate at the end of the 18-hour incubation period, the NEU Hospital, Northern Cyprus, 2015.

3.4.2. rK39 Dipstick Test

The rK39 dipstick test was performed according to the manufacturer's protocol (Standard Diagnostic Bioline Leishmania Ab). Serum samples from patients were taken into collection tubes (without anticoagulants) and centrifuged at 4500 rpm for 10 minutes. After that, the serum specimens in the supernatant were collected into 1.5 ml Eppendorf tubes. Twenty µl of the serum samples were transferred into new Eppendorf tubes and left at room temperature for 30 minutes. Meanwhile, contents of the kits were left at room temperature prior to testing. Three drops of the assay diluents (approximately 100 µl) were added to the test tube (supplied by the kit). After that, 20 µl of the serum sample were added to the test tube using a capillary pipette (supplied by the kit) and stirred gently. The test strip was removed from the foil punch and then inserted vertically into the test tube that contained diluted sample (Figure 3.2). Results were read within 15 minutes. The results were considered positive when two distinct red or pink lines, one in the test region and another in the control region, appeared.

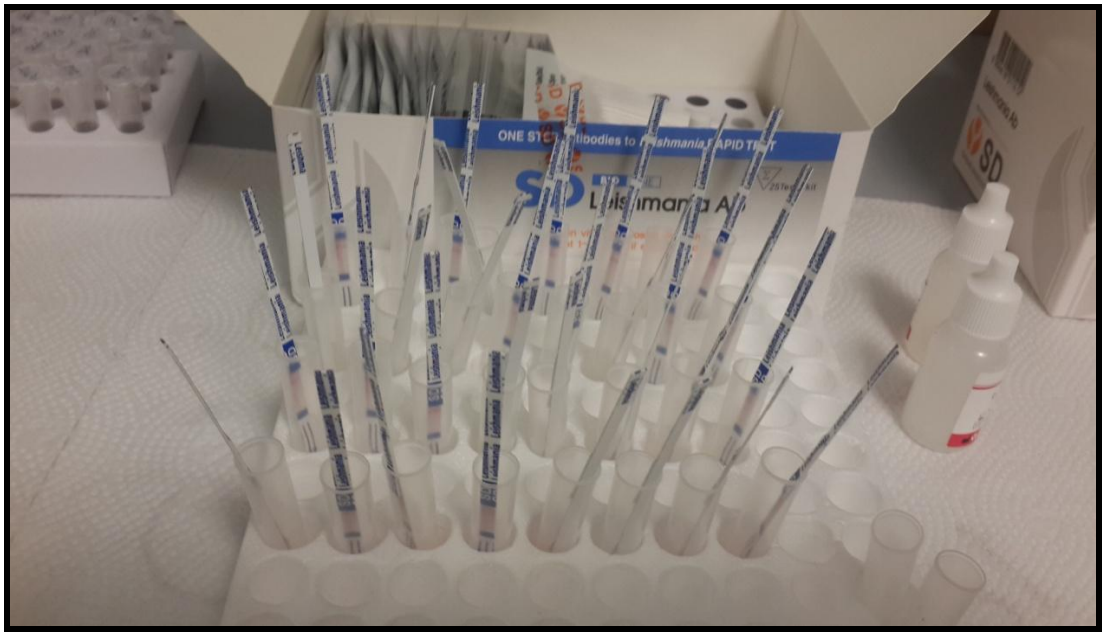

Figure 3.2. Example of the rK39 dipstick test, the NEU Hospital, Northern Cyprus, 2015.

3.5. Molecular Methods

3.5.1. DNA Extraction

Blood samples were previously collected in the tubes containing EDTA for DNA isolation. After collection, blood samples were stored at -20°C until used. Molecular tests were carried out in “BM Labosis” company in Ankara, Turkey. DNA isolation was conducted according to the manufacturer’s protocol (GeneMATRIX Quick Blood DNA Purification kit):

1- 40 µl of Buffer QB were added to the spin column.

2- 200 µl of the blood sample were added to 1.5 ml Eppendorf tube. Ten µl Proteinase K and 200-µl SOL-QB buffer were added to the blood sample respectively.

3- Mixture was vortexed thoroughly and centrifuged at 12000 rpm for 1 minute.

4- After centrifugation, the lysate was transferred to the spin-column which was placed in the collection tube. Spin column was centrifuged at 12000 rpm for 2 minutes.

5- The spin column was taken out from the collection tube and the flow was discarded. Spin column was placed back into the collection tube. 500 µl from the QBX1 buffer were added to the spin column and centrifuged at 12000 rpm for 1 minute.

6- The spin column was taken out and flow was discarded from the collection tube. Spin column was placed back into the collection tube and 500 µl from the QBX2 buffer were added to the spin column. Spin column was centrifuged at 12000 rpm for 2 minutes.

7- After centrifugation, spin column was placed to a new collection tube and 100 µl of elution buffer, heated at 70°C, were added to the mixture to elute DNA. The mixture was incubated at room temperature for 3 minutes.

8- After incubation, the mixture was centrifuged at 12000 rpm for 1 minute. After centrifugation, spin column was discarded and DNA was stored at 4°C for further analysis.

3.5.2. Investigation of *Leishmania* spp. by PCR

PCR was performed in order to search for *Leishmania* spp. in 250 blood samples collected from the participants. Amplification reactions were carried out by using a thermal cycler (Kyratec).

The list of PCR materials used for the molecular detection of *Leishmania* spp. was given in Table 3.2.

Table 3.2. The list of PCR materials used for the molecular detection of *Leishmania* spp., Northern Cyprus, 2015.

The list of PCR materials
PCR Buffer (Solis Biodyne)
$MgCl_2$, (Solis Biodyne)
dNTP mix (Solis Biodyne)
LSP805bp BM-F (BM Labosis)
Lsp805bp BM-R (BM Labosis)
Taq polymerase (Solis Biodyne)
DNA, Negative control (Distilled water)
1X TBE (BM Labosis), Agarose (Bioshop)

Leishmania specific primers (Table 3.3) which were used for *Leishmania* spp. detection amplifies the internal transcription spacer 1 (ITS-1). This is a noncoding region placed at SSUrRNA, bounded by the genes 18S and 5.8S (Guimaraes et. al., 2014), and produces an 805 bp fragment of *Leishmania* spp.

Table 3.3. The primer sequences in PCR used for the molecular detection of *Leishmania* spp., Northern Cyprus, 2015.

The primers	The primer sequences
LSP805bp BM-F	(5'GGATAACGGCTCACATAACG-3')
Lsp805bp BM-R	(5'-GGTCTGTAAACAAAGGTTGTCG-3')

Amplification reactions were performed in a 35 µl volume containing 2.0 mM MgCl (Solis Biodyne), 200 µM dNTPs (Solis Biodyne), 0.5 µM of each primer, 2.0 U of *Taq* DNA polymerase (Solis Biodyne), 5 µl of the template DNA and 22.25 µl water (Table 3.4). The following cycling conditions were applied for the PCR: 95˚C for 4 minutes; 42 cycles of 95˚C for 40 seconds, 56˚C for 35 seconds, 72˚C for 38 seconds; and a final extension at 72˚C for 5 minutes (Table 3.5). In each run, molecular-grade water was used as the negative control, while DNA from cultured promastigotes served as the positive control.

Following the amplification of DNA samples, 5 µl of the PCR products were run on 1.5% agarose gel in 1X Tris-Borat-EDTA buffer and visualized by staining with ethidium bromide (Bioshop), using 1 kb DNA ladder (Solis Biodyne) as a molecular marker. The electrophoresis (Major Science) was conducted at 100V for 100 minutes. Separated products were visualized under an ultraviolet transilluminator (Biostep) and the product bands were evaluated.

Table 3.4. Calculation of the PCR mix used for the molecular detection of *Leishmania* spp., Northern Cyprus, 2015.

Reagents	Stock concentration	Reaction concentration	Microliters per reaction
PCR Buffer	10X	1X	3,5 µl
$MgCl_2$	25 mM	2,0 mM	1,75 µl
dNTPmix	20 mM	0,2 mM	0,35 µl
LSP805bp BM-F	20 µM	0,5 µM	0,875 µl
Lsp805bp BM-R	20 µM	0,5 µM	0,875 µl
Taq polymerase	5 U/µl	2 U	0,4 µl
DNA	5 µl		
Water	22,25 µl		

Table 3.5. The cycling conditions in PCR used for the molecular detection of *Leishmania* spp., Northern Cyprus, 2015.

Temperature	Duration	Cycle
95	4 min	1
95	40 sec	42
56	35 sec	
72	38 sec	
72	5 min	1
8	∞	

3.5.3. DNA Sequencing

In this study, the blood sample that was detected positive by PCR was further tested by DNA sequencing. For detection of *Leishmania* spp. by DNA sequencing, the PCR product was sent to “Macrogen” laboratory company in Amsterdam, Netherlands. The sequencing was conducted according to Sanger’s method (Sanger et. al., 1977). The results were assessed by using BLAST software.

4. RESULTS

This study was conducted from September 2014 until September 2015 and Girne (Kyrenia) and surrounding regions were chosen as the pilot areas due to the high amount and diversity of *Phlebotomus* spp. in these regions for *Leishmania* species detection in Northern Cyprus.

A total of 250 samples were collected from the volunteers who attended the study. Among 250 individuals, 149 participants were female (59,6%) and 101 were male (40,4%). The mean age of the participants was 42.84 ± 17.42, as the median age was 40.00 (7.00-86.00). The highest number (n: 113, 45.2%) of the participants was recorded in the age group 25-44 which was followed by the age group 45-64 (n: 63, 25.2%). Forty (16.0%) individuals belonged to the age group 65+, while the age groups 15-24 and 1-14 had twenty-eight (11.2%) and six (2.4%) participants, respectively (Table 4.1).

Table 4.1. Distribution of the participants according to the age groups in the leishmaniasis study, Northern Cyprus, 2015.

Age groups	Number of the participants	
	Number (n)	Percentage (%)
1-14	6	2.4
15-24	28	11.2
25-44	113	45.2
45-64	63	25.2
65+	40	16.0

The residential areas of the participants were stated in Table 4.2. According to the results, the highest number (n: 82, 32.8%) of participants was living in Lapta (Lapithos). This was followed by Girne (Kyrenia) and Alsancak (Motides) which had 61 (24.4%) and 43 (17.2%) participants, respectively.

Table 4.2. Distribution of the participants according to the residential areas in the leishmaniasis study, Northern Cyprus, 2015.

Residental areas of the participants	Number of the female participants	Number of the male participants	Total number of the participants
Alsancak (Motides)	29	14	43
Arapköy (Kepini)	1	0	1
Balabayıs (Bellapais)	3	0	3
Beşparmak (Trapeza)	0	2	2
Boğazköy (Mpogadzi)	1	1	2
Çatalköy (Agios Epiktitos)	4	3	7
Dikmen (Dikomo)	1	0	1
Erdemit (Trimithi)	1	0	1
Esentepe (Agios Amvrosios)	3	2	5
Girne (Kyrenia)	33	28	61
Karakum (Karakoumi)	1	0	1
Karaoğlanoğlu (Agios Georgios)	8	1	9
Karşıyaka (Vasilia)	5	4	9
Kayalar (Oga)	0	1	1
Kozanköy (Larnakatis Lapithos)	0	1	1
Lapta (Lapithos)	46	36	82
Lefkoşa (Nicosia)	4	2	6
Mağusa (Famagusta)	0	1	1
Ozanköy (Kazafani)	6	1	7
Tepebaşı (Diorios)	0	1	1
Yeşiltepe (Elia)	0	1	1
Zeytinlik (Templos)	3	2	5
Total number of the participants	149	101	250

During the sample collection, a questionnaire was also conducted where information related with leishmaniasis were obtained. According to the questionnaire, 133 (%53.2) participants had one or more dogs. Ninety-two (69.2%) of 133 participants had one dog, while 26 (19.5%) of them had two dogs. Twelve (9.0%) of those participants had three dogs, and three (2.3%) individuals had four dogs. Thirty-six (27.1%) of 133 participants were using the protective collar in their dogs for prevention against *Phlebotomus* sand flies, while 97 (72.9%) of them were not using the protective collar.

The information obtained from the questionnaire revealed that 70 of 250 participants (28.0%) had information about leishmaniasis disease, while 180 of them (72.0%) did not have information about the disease. Among 70 participants who had information about the disease, 50 individuals (71.4%) were noted to have dogs, while 20 of them (28.6%) did not have any dog. Twenty of 50 participants (40.0%) who had both information about the disease and had dogs were using the protective collar, while 30 of them (60.0%) were not using the protective collar.

In the questionnaire, 21 of 133 participants (15.8%) who owned dogs declared that they observed one or more findings of CanL in one or more of their dogs. Five of 21 participants (23.8%) stated that one or more of their dogs were euthanized.

Information obtained from the questionnaire of eight patients with CL history was also evaluated. Two of these patients had dogs at the time of infection. One patient (Patient no: 1, male, 68 years old) who was previously diagnosed with CL declared that he owned three dogs when he had the disease. The patient used the protective collar for his dogs and did not observe any finding of CanL in his dogs at the time of infection. The other patient (Patient no: 4, male, 40 years old) who owned two dogs observed epistaxis, a sign for CanL, in one of his dogs. This patient who was recently diagnosed as CL before attending the study stated that he did not use the protective collar for his dogs (Figure 4.1).

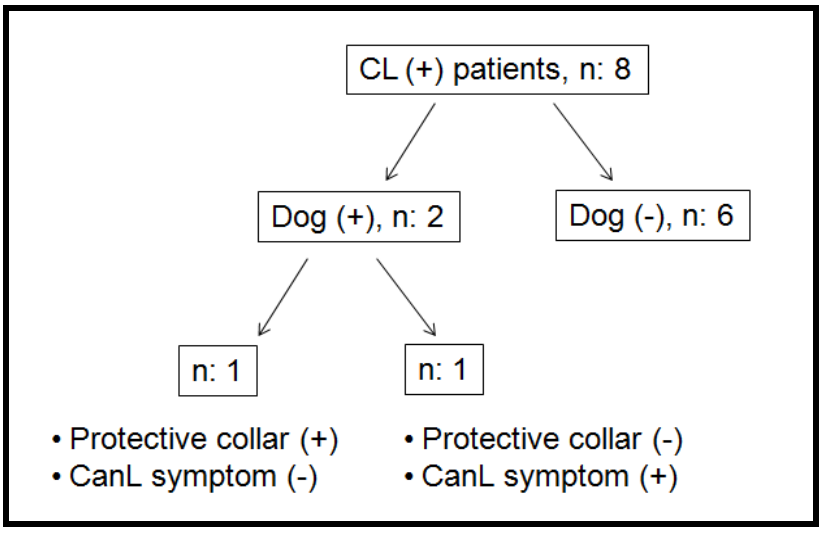

Figure 4.1. Schematic explanation of eight CL (+) patients, leishmaniasis survey, Northern Cyprus, 2015.

Serologic tests were performed at the Near East University Hospital, Clinical Microbiology Laboratory. Firstly, DAT was performed, and two (0,8%) of 250 participants were found to be positive for the anti-*Leishmania* antibodies. The serum titers of both patients were detected to be 1:1600. These patients were previously diagnosed as CL and received treatment before. One of these patients (Patient no: 1, male, 68 years old) was living in Çatalköy (Agios epiktitos). This patient owned three dogs, he used the protective collar for his dogs and did not observe any finding of CanL in his dogs at the time of infection. The other patient (Patient no: 2, male, 54 years old) from Karşıyaka (Vasilia) stated that he did not have any dog. PCR and rK39 results of these patients were negative. DAT plate, having the positive results after an 18-hour incubation period, was given in Figure 4.2.

In the second part of the serologic tests, rK39 strip test was performed. According to the rK39 test results, one (0.4%) patient (Patient no:3, male, 18 years old) was detected to be positive (Figure 4.3). This patient attended the study from Lapta (Lapithos) and had been previously diagnosed as CL and treated. The patient did not have any dog. PCR and DAT results of this patient were negative.

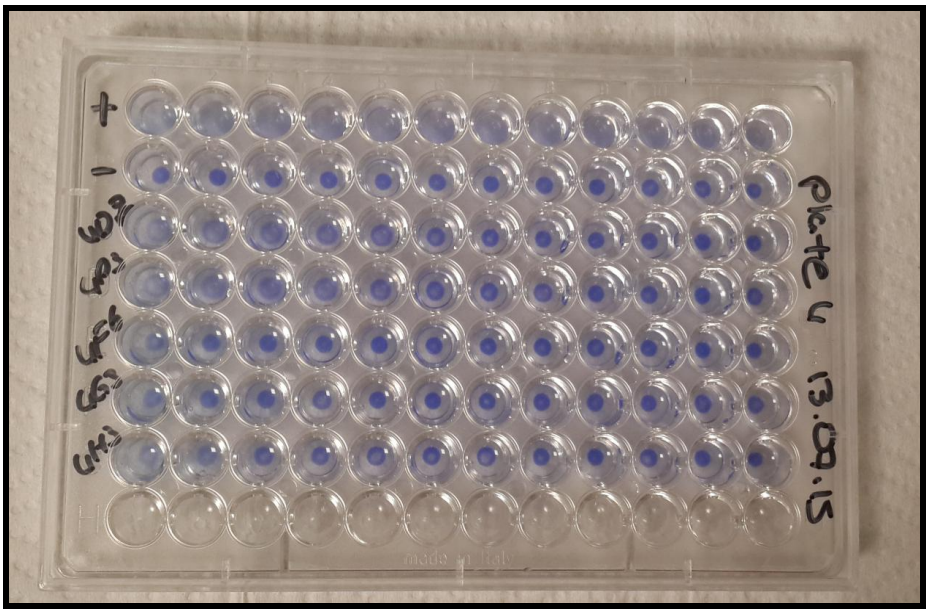

Figure 4.2. DAT plate having the positive results (Patient no: 1, 4D2 and patient no: 2, 4D3) after 18-hour incubation time, performed at the NEU Hospital, Northern Cyprus, 2015 (The first row: positive control; the second row: negative control; patients 4F6, 4G3 and 4H3: negative results.).

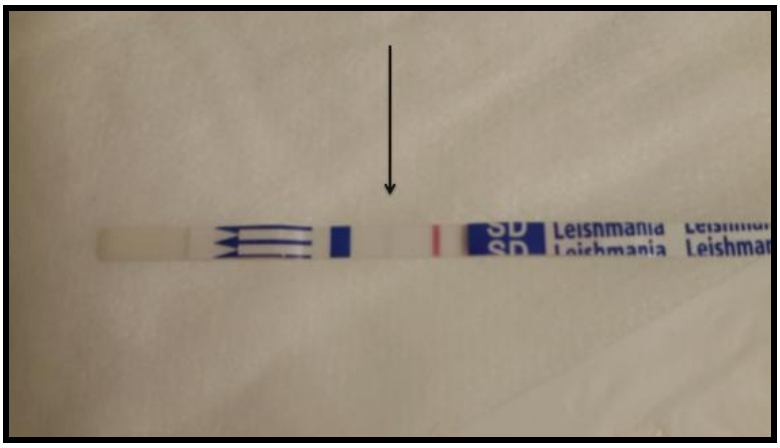

Figure 4.3. The rK39 strip test having positive result, performed at the NEU Hospital, Northern Cyprus 2015 (Black arrow shows the positive reaction.).

In this study, the presence of *Leishmania* parasites were evaluated by PCR in the whole blood samples collected from 250 participants. The PCR results revealed that *Leishmania spp*. was detected in one (0.4%) patient (Patient no: 4, male, 40 years old) (Figure 4.4). This patient was living in Mağusa (Famagusta) and had two dogs. The patient stated that he did not use the protective collar for his dogs, and one of his dogs had epistaxis. This patient was diagnosed as CL and began to receive the treatment short before providing the blood and serum samples for the study. Unlike the PCR results, DAT and rK39 test results were negative for this patient.

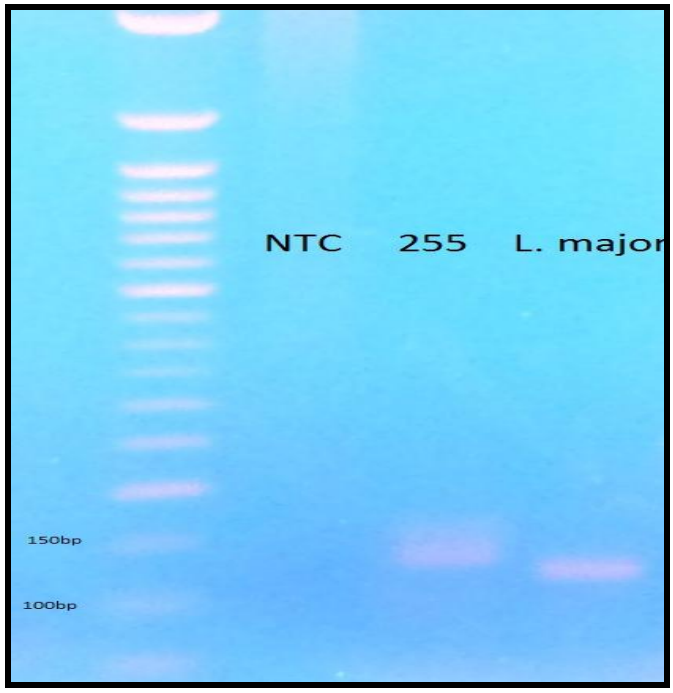

Figure 4.4. Positive PCR result according to the gel electrophoresis, performed at “BM Labosis” company, Ankara, 2015.

The DNA sample that was positive for *Leishmania* spp. was further searched by DNA sequencing. According to the DNA sequencing results, the agent was reported to be *L. donovani* complex. Differentiation between *Leishmania donovani donovani* (*L. donovani donovani)* and *Leishmania donovani infantum* (*L. donovani infantum)* could not be conducted due to the

similar gene sequences of these subspecies. Phylogenetic analysis based on the partial sequence of the ITS-5.8S rDNA using the BLAST software showed that the sequences KF815214.1 (Figure 4.5) and KF815215.1 (Figure 4.6) were 94% similar to the *L. donovani* sequences deposited in the GenBank. Besides, the BLAST analysis indicated that the sequence KJ573795.1 was also 94% similar to the *L. infantum* sequence deposited in the GenBank (Figure 4.7).

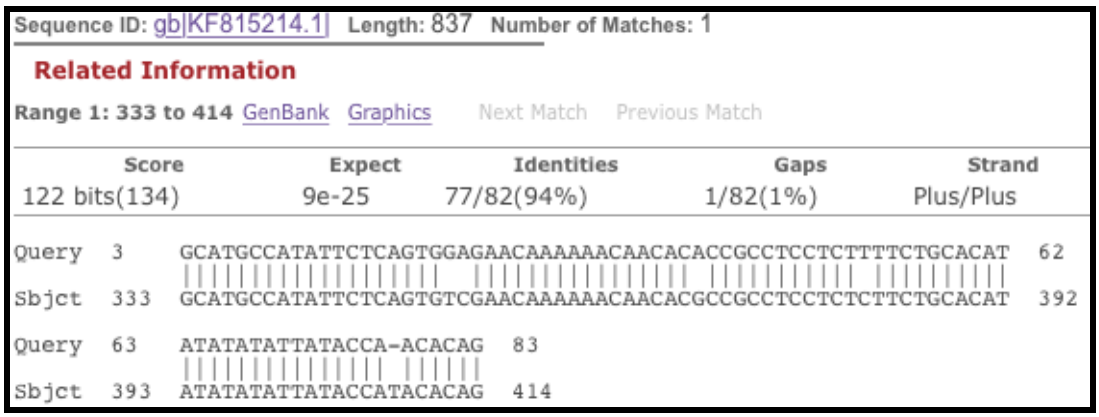

Figure 4.5. The BLAST analysis showing 94% similarity of the sequence KF815214.1 with *L. donovani.*

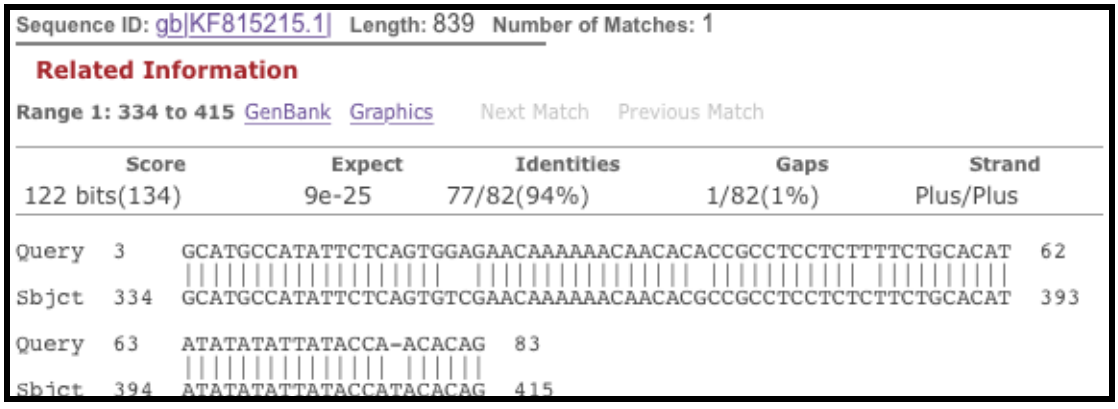

Figure 4.6. The BLAST analysis suggesting 94% similarity of the sequence KF815215.1 with *L. donovani.*

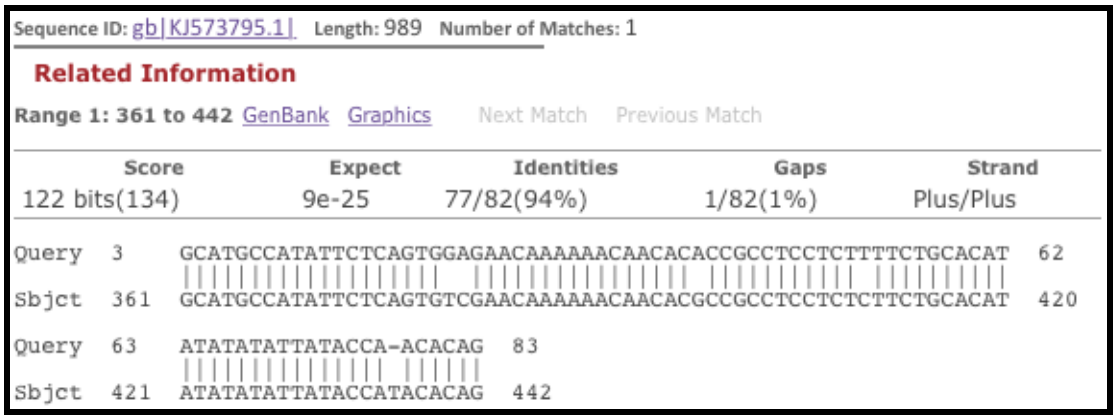

Figure 4.7. The BLAST analysis indicating 94% similarity of the sequence KJ573795.1 with *L. infantum.*

In this study, a total of 250 participants (242 individuals who were randomly selected and eight patients with a history of CL) were included for investigation of *Leishmania* spp. or the antibody response against leishmaniasis. All of the 242 participants who were CL (-) were detected negative with the serologic and molecular tests used in the study.

The only positive test results were obtained in the samples of four CL (+) patients (1.6% of 250 participants). Two (0.8%) of the CL (+) patients (Patient no: 1 and no: 2) were positive for DAT with serum titers of 1:1600. One of these patients (Patient no: 1) had three dogs, however he used the protective collar in his dogs and did not observe any finding of CanL in his dogs at the time of infection. The second patient (Patient no: 2) did not have any dog. In the rK39 strip test, one (0.4%) patient with a history of CL (Patient no: 3) was detected positive. This patient did not have any dog. *Leishmania* spp. was detected in the blood sample of one (0.4%) patient (Patient no: 4) by PCR. The BLAST analysis indicated 94% similarity with *L. donovani* and *L. infantum.* This patient was recently diagnosed as CL before the time of sampling and one of his dogs had epistaxis. In the other four CL (+) patients, the serologic and molecular test results were negative.

Detailed demographic information and the test results of eight CL (+) patients were given in Table 4.3.

Table 4.3. Detailed demographic information and the test results of eight CL (+) patients, leishmaniasis survey, Northern Cyprus, 2015.

Patient number	Age	Gender	Number of dogs at the time of infection	Use of the protective collar at the time of the infection	Signs of CanL at the time of the infection	Residential area	Test results		
							DAT	rK39	PCR
Patient 1	68	M	3	Yes	No	Çatalköy (Agios epiktitos)	+	-	-
Patient 2	54	M	0	-	-	Karşıyaka (Vasilia)	+	-	-
Patient 3	18	M	0	-	-	Lapta (Lapithos)	-	+	-
Patient 4	40	M	2	No	Yes, epistaxis	Mağusa (Famagusta)	-	-	+
Patient 5	60	M	0	-	-	Lapta (Lapithos)	-	-	-
Patient 6	36	F	0	-	-	Lapta (Lapithos)	-	-	-
Patient 7	7	M	0	-	-	Lapta (Lapithos)	-	-	-
Patient 8	18	F	0	-	-	Lapta (Lapithos)	-	-	-

5. DISCUSSION

Leishmaniasis is considered as one of the important neglected tropical diseases by the WHO. According to the WHO, it is endemic in 98 countries (Ozensoy Toz et. al., 2013a). Leishmaniasis has three main clinical forms which are VL, CL and MCL. Three hundred thousand VL cases and 1 million CL cases occur annually. More than 90% of the VL cases are seen in Ethiopia, Bangladesh, India, Brazil, Sudan, and South Sudan. In addition, one third of the CL cases occur in the Mediterranean basin, Americas, Middle East, and East Africa (Mugasa et. al., 2010; Ozensoy Toz et. al., 2013a; Elmahallawy et. al., 2014; WHO, 2015a).

Leishmaniasis is endemic in the Mediterranean basin, in all countries of southern Europe and the Middle East (Deplazes et. al., 1998; Mazeris et. al., 2010; Ntais et. al., 2013; Ozensoy Toz et. al., 2013a). Four *Leishmania* species cause leishmaniasis in the Mediterranean basin, which are *L. infantum, L. donovani, L. major* and *L. tropica. L. infantum* is the most common species in the Mediterranean basin and responsible for CL, VL and CanL transmission. *L. donovani* causes anthroponotic CL and VL in Cyprus, *L. tropica* causes anthroponotic CL in Europe, and *L. major* causes zoonotic CL in North Africa and the Middle East. *L. infantum*, *L. tropica* and *L. major* are also reported to exist in Turkey, which has a geographical importance as a neighbour of Cyprus (Deplazes et. al., 1998; Mazeris et. al., 2010; Ntais et. al., 2013; Ozensoy Toz et. al., 2013a; Hosseininasab et. al., 2014).

Cyprus is located in the eastern Mediterranean basin where leishmaniasis is seen endemically (Deplazes et. al., 1998; Demir et. al., 2010; Ozensoy Toz et. al., 2013b). In Cyprus, two different transmission cycles appear to be involved. First transmission cycle is *L. infantum* MON-1 cases which cause CanL. Second transmission cycle is *L. donovani* MON-37 cases that cause anthroponotic CL and VL cases in Cyprus. Besides these cycles, Cyprus has localities that are rich in diversity of *Phlebotomus* species, vector of *Leishmania*, that help the active circulation of the parasite in the island (Deplazes et. al., 1998; Demir et. al., 2010; Mazeris et. al., 2010;

Koliou et. al., 2014). Previous studies held in Cyprus revealed that *L. infantum* MON-1 and *L. infantum* MON-98 are found in canine isolates and *L. donovani* MON-37 is found in humans in Southern Cyprus, while *L. infantum* MON-1 is found in both canine and human isolates in Northern Cyprus (Deplazes et. al., 1998; Demir et. al., 2010; Mazeris et. al., 2010; Ozensoy Toz et. al., 2013b).

A case report from the Baf (Paphos) district in Southern Cyprus revealed four CL cases caused by *L. donovani* MON-37. This study is particularly important as it involves the first CL case caused by *L. donovani* in a child and the first cluster of CL cases with adults reported in Europe (Koliou et. al., 2014). Koliou et. al. showed that *L. donovani* MON-37 bears genetic similarities to isolates from Turkey but bears genetic differences to isolates from India, Israel, Kenya and Sri Lanka (Koliou et. al., 2014).

VL cases have been reported from Cyprus since 1935 with very low incidence. Additionally, sporadic CL cases were reported in 1990 from Girne (Kyrenia) region in Northern Cyprus. Earlier studies indicated that CL cases had gradually increased from 2 to 36 between 1985-1990. Additionally, *L. infantum* was isolated by isoenzyme electrophoresis in London from a 61-year-old female from Northern Cyprus (Deplazes et. al., 1998; Demir et. al., 2010). According to the researchers, *Leishmania* skin test positivity was provided as 10% in Girne (Kyrenia) and 35% in Lapta (Lapithos), respectively in 1990. In addition, *L. infantum* managed to be identified by *in vitro* cultivation of a skin biopsy material from this area (Deplazes et. al., 1998; Ozensoy Toz et. al., 2013b).

CanL studies were also conducted in both areas and *L. infantum* MON-1 and *L. infantum* MON-98 were isolated from canine isolates in Southern Cyprus while *L. infantum* MON-1 was isolated from canine isolates in Northern Cyprus. Studies from Southern Cyprus showed that CanL seroprevalence had almost 9-fold increase in the last 10 years in some areas with 1.7%-14.2% (Mazeris et. al., 2010; Ozensoy Toz et. al., 2013b). Also, positive results in CanL have been obtained in Girne (Kyrenia), Güzelyurt

(Morphou) and Yeni İskele (Trikomo) in previous studies. However, there were no positive results in Lefkoşa (Nicosia) (Canakci, 2008). These results indicate that all parts of the Northern Cyprus, which are close to the seaside and have high humidity, provide favorable breeding conditions for sandflies and dogs and hence play an important role in transmission in such areas. Lefkoşa (Nicosia) is relatively more developed, most of the dogs are under control and insecticides are more commonly used than the other areas. Therefore, the absence of CanL in Lefkoşa (Nicosia) may be explained by these reasons (Canakci, 2008).

Many *Phlebotomus* species have been detected in both parts of the island. Species isolated from Northern Cyprus were *P. galilaeus, P. papatasi, P. tobbi, P. alexandri, P. sergenti, P. economidesi, P. neglectus, Phlebotomus kyreniae, Phlebotomus jacusieli, Sergentomyia azizi, Sergentomyia minuta*, and *Sergentomyia fallax* (Demir et. al., 2010). *P. tobbi, P. galilaeus, P. papatasi, P. sergenti, P. alexandri, Phlebotomus mascittii*, and *P. economidesi* were isolated from Southern Cyprus (Mazeris et. al., 2010). Among these species, *P. galilaeus, P. neglectus, P. papatasi* and *P. tobbi* have an importance for public health. *P. alexandri, P. galilaeus* and *P. tobbi* are putative vectors in both Northern and Southern Cyprus. Cyprus. Especially, *P. tobbi* appears to be responsible for the transmission of *L. infantum* and *L. donovani,* as well as *L. infantum* which cause CanL in Northern Cyprus (Demir et. al., 2010; Ozensoy Toz et. al., 2013b). Ergunay et. al. conducted a field study in Northern Cyprus about sandflies. According to their results, *Phlebotomus perfiliewi* sensu lato complex is the most abundant species in Northern Cyprus followed by *P. tobbi* and *P. neglectus*. As *P. tobbi* has been reported on the island as the most abundant species in Lapta (Lapithos) and the vector of CanL in Cyprus, it was also reported as a vector of CL from Çukurova, Turkey. In that study, *L. infantum* DNA was detected in *P. tobbi* collected from Gecitkoy (Panagra) and Lapta (Lapithos) (Ergunay, 2014).

The present study was conducted in order to search for human leishmaniasis seroprevalence and presence of *Leishmania* spp. in Northern Cyprus. In this research, Girne (Kyrenia) and surrounding regions were chosen as the pilot areas due to the high amount and diversity of *Phlebotomus* spp. A total of 250 participants (242 individuals were randomly selected, and eight patients had cutaneous leishmaniasis (CL) history were included in this research on the voluntary basis. During the collection of blood and serum samples, the participants who owned dogs also provided information related to CanL history in their dogs. The whole blood samples of the participants were tested for the presence of *Leishmania* spp. by PCR. The antibody response was searched by DAT and rK39 strip test in the serum samples of the participants.

The results obtained from the molecular and serologic assays revealed that all of the 242 participants who were diganosed as CL (-) previously were also detected negative in the present study study. The only positive test results were obtained in the samples of four CL (+) patients (1.6% of 250 participants).

In DAT assay, two (0.8%) of the CL (+) patients (Patient no: 1 and no: 2) were found to be positive with serum titers of 1:1600. One of these patients (Patient no: 1, male, 68 years old) was living in Çatalköy (Agios epiktitos). According to the information obtained from the questionnaire, this patient owned three dogs, he used the protective collar for his dogs and did not observe any finding of CanL in his dogs at the time of infection. The other patient (Patient no: 2, male, 54 years old) from Karşıyaka (Vasilia) stated that he did not have any dog. PCR and rK39 results of these patients were negative. According to the test results of 250 participants, the result of DAT revealed a higher rate of infection than those of PCR and the rK39 strip test. The results of DAT assay could not be compared to those of other studies held in Cyprus, because in our records, DAT was used for the first time in leishmaniasis studies in Cyprus. In this study, positive DAT results indicate that DAT is able to detect even low levels of antibodies. However, in CL, the poor humoral response might lead to negative results. In line with this, DAT

was demonstrated to be more sensitive and more specific in contrast to the rK39 strip test (Maia et. al., 2012; De Vries, 2015).

In this study, one (0.4%) patient (Patient no: 3, male, 18 years old) was detected to be positive by the rK39 strip test. This patient was living in Lapta (Lapithos) and had been previously diagnosed as CL and treated. In the questionnaire, this patient stated that he did not have any dog. PCR and DAT results of this patient were negative. The result obtained from the rK39 strip test could not be compared to other studies carried out in Cyprus, because in our records, this is the first study that used the rK39 test in leishmaniasis studies in Cyprus. The patient with the positive rK39 test result was diagnosed as CL four years ago and received treatment. Thus, the positivity can be explained in two ways. First, the serum antibody levels decrease after successful treatment but they remain detectable up to several years. Second, people who live in an endemic area may have an asymptomatic infection and produce positive results (Sundar and Rai, 2002; Chappuis et. al., 2007; Elmahallawy et. al., 2014). For this study, the first scenario is much more likely for this patient's condition. Anti-rK39 antibodies persist for a long time and do not differentiate between active, past or subclinical infections. Moreover, it might be negative in the early stages of the disease or it remains positive well beyond the time of cure. Due to that, drawbacks are limiting their use for the diagnosis of relapses or reinfection (Sundar and Rai, 2002; Elmahallawy et. al., 2014).

According to the PCR results, *Leishmania spp*. was detected in the whole blood sample of one (0.4%) patient (Patient no: 4, male, 40 years old) who was living in Mağusa (Famagusta). This patient was diagnosed as CL and began to receive the treatment short before providing the blood and serum samples for the study. According to the DNA sequencing results, the agent was reported to be *L. donovani* complex. The BLAST analysis indicated 94% similarity with *L. donovani* and *L. infantum.* This patient who had two dogs stated that he did not use the protective collar for his dogs, and one of his dogs had epistaxis. DAT and rK39 test results were found to be negative for this patient. Due to the recent CL infection short before the time

of sampling, positive PCR results were obtained. Negative serologic test results suggested that the parasite species was not *L. infantum* or antibodies did not appear or the level of antibodies was too low for detection. Since the serologic tests are based on screening of *L. infantum* infections, positive PCR results might be linked to *L. donovani donovani.* Furthermore, this patient was living in a region other than Girne (Kyrenia). Thus, another question might bring to mind that the infection might not be linked to *L. donovani infantum* but possibly to *L. donovani donovani*. This patient was living in Mağusa (Famagusta). Mağusa (Famagusta) is close to Larnaka (Larnaca) which has a high amount of *P. papatasi* (vector of *L. donovani*). For this reason, the area should be investigated in terms of *Phlebotomus* and *Leishmania* species, respectively (Mazeris et. al., 2010).

Results of this study can be combined with other studies held on both sides of the island and findings indicate that *L. donovani* complex is actively circulating in both parts of the island (Poeppl et. al., 2011). Two different transmission cycles, *L. donovani* MON-37 and *L. infatum* MON-1, occur in Southern Cyprus (Mazeris et. al., 2010). In Northern Cyprus, *L. donovani* MON-37 transmission is absent and *L. infantum* transmission was reported. Studies confirmed human to human transmission in Southern Cyprus (Ozensoy Toz et. al., 2013b; Koliou et. al., 2014).

In this study, during the collection of blood and serum samples, the knowledge of the participants on leishmaniasis was evaluated by conducting a questionnaire. A total of 133 (%53.2) participants declared that they had one or more dogs. According to the information obtained, 36 (27.1%) of 133 participants were using the protective collar in their dogs, while 97 (72.9%) of them were not using the protective collar. In the questionnaire, 70 (28.0%) of 250 participants declared that they had information about leishmaniasis disease. Fifty (71.4%) of 70 individuals were noted to have dogs. Although these participants had information about the disease, only 20 (40.0%) of them were using protective collar in their dogs. The results of the questionnaire suggest that measures should be taken to increase the public awareness of leishmaniasis.

Investigating asymptomatic and infected dogs should be performed as a precaution for the disease. Therefore, dog owners should be investigated and recorded, and dogs should be checked up regularly for control of leishmaniasis. This step may provide a major contribution to reducing the incidence of the disease.

Vegetation, climatic conditions and animal shelters in Cyprus provide a suitable breeding ground for the vector of this disease. The presence of *Phlebotomus* spp. in Northern Cyprus constitutes a danger against CanL. This implies that precaution should be encouraged for vector eradication. Using the protective collars in the dogs that includes anti-parasitic drugs is important for preventing infection in the reservoir.

The results of this study indicate the presence of leishmaniasis in Northern Cyprus and suggest that the vector and reservoir control programmes should be implemented for prevention of the disease.

This study was conducted by including a limited number of individuals from certain regions. Research studies including more participants from different locations should be performed in order to have a more comprehensive data on the epidemiology of leishmaniasis in Northern Cyprus.

6. CONCLUSION

This study was conducted for investigation of seroprevalence of human leishmaniasis and determination of *Leishmania* spp. in Northern Cyprus. A total of 250 individuals participated in this research on the voluntary basis. Two hundred and forty-two participants were randomly selected and eight patients who were diagnosed as CL were included in this study. During the collection of blood and serum samples, the participants provided information on awareness of leishmania, and the individuals who owned dogs were questioned about CanL history in their dogs. The whole blood samples of the participants were tested for the presence of *Leishmania* spp. by PCR. The antibody response was investigated by DAT and rK39 strip test in the serum samples of the participants.

According to the results obtained from the molecular and serologic assays, all of the 242 participants who were CL (-) were detected negative in the study.

Test results of the eight CL (+) patients were evaluated and four (1.6% of 250 participants) patients were found to be positive. In DAT assay, two (0.8%) of the CL (+) patients (Patient no: 1 and no: 2) were detected positive and serum titers of both patients were recorded as 1:1600. One of these patients (Patient no: 1) had three dogs, however, he used the protective collar in his dogs and did not observe any finding of CanL in his dogs at the time of infection. According to the information obtained from the questionnaire, the second patient (Patient no: 2) did not have any dog. PCR and rK39 test results of these patients were negative.

Positive rK39 test result was obtained in the serum sample of one (0.4%) patient who was previously diagnosed as CL (Patient no: 3). This patient did not have any dog. PCR and DAT results of this patient were negative.

PCR results revealed that, *Leishmania* spp. was detected in the blood sample of one (0.4%) patient who was diagnosed as CL short before the time of sampling (Patient no: 4). According to the DNA sequencing results, the

agent was reported to be *L. donovani* complex. The BLAST analysis indicated 94% similarity with *L. donovani* and *L. infantum.* In the questionnaire, this patient stated that one of his dogs had epistaxis. In the other four CL (+) patients, the serologic and molecular test results were negative.

In this study, the results of the questionnaire were also evaluated in order to determine the knowledge of the participants on leishmaniasis. According to the questionnaire, 36 (27.1%) of 133 participants who owned dogs stated that they were using the protective collar in their dogs, while 97 (72.9%) of them were not using the protective collar. According to the questionnaire, 70 (28.0%) of 250 participants declared that they had information about leishmaniasis disease. Among these participants, 50 (71.4%) individuals were noted to have dogs. Although these participants had information about the disease, only 20 (40.0%) of them were using the protective collar in their dogs. The results of the questionnaire suggest that measures should be taken to increase the public awareness of leishmaniasis.

The results of this study indicate that the presence of leishmaniasis in Northern Cyprus should not be ignored. Due to its typical climate and geographical structure, Cyprus has a favourable condition for the living of *Phlebotomus* spp. Besides, dogs are distributed in the places where people live. Therefore, the vector and reservoir control programmes should be implemented for prevention of the disease.

Since a limited number of individuals from certain regions participated in this study, research studies including more participants from different locations should be conducted. By this way, a more comprehensive data will be obtained on the prevalence of leishmaniasis in Northern Cyprus.

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APPENDIX 1

CLARIFIED CONSENT FORM FOR THE RESEARCH STUDY

TRNC Ministry of Health and the Near East University Faculty of Medicine, Department of Medical Microbiology and Clinical Microbiology are conducting a collaborative project for investigation of the human leishmaniasis cases in Northern Cyprus.

The aim of this study is to have information about the presence of human leishmaniasis seroprevalence and disease causing *Leishmania* species in Northern Cyprus. In this study, blood samples will be collected from 250 people living in Girne (Kyrenia), Lapta (Lapithos), Esentepe (Agios Amvrosios) and surrounding area that have a high amount and diversity of sand flies – the vector of the disease. The participants in this study should be living in Northern Cyprus for at least one year and have dogs (or be in contact with the dogs). Participation in the study will be on the voluntary basis, and the presonal information of the participants and the test results will be kept confidential. The participants will not be charged for conducting the tests, and in case of obtaining positive test result, the patients will be informed and given the appropriate treatment. In addition, control programmes on the reservoir dogs, and the vector sandflies will be started. By this way, this study will contribute to the elimination and eradication of leishmaniasis, which is an important tropical disease, in Cyprus.

Participant	**Physician that informed the participant**
Name and surname:	Name and surname:
Address:	Address:
Telephone:	Telephone:
Signature:	Signature:

Witness of the interview	**Coordinator of the research**
Name and surname:	Name and surname:
Address:	Address:
Telephone:	Telephone:
Signature:	Signature:

APPENDIX 2

ARAŞTIRMA AMAÇLI ÇALIŞMA İÇİN AYDINLATILMIŞ ONAM FORMU

KKTC Sağlık Bakanlığı ile Yakın Doğu Üniversitesi Tıp Fakültesi Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı işbirliğinde Kuzey Kıbrıs'daki insan leishmaniasis olgularının araştırılacağı bir proje yürütülmektedir.

Bu çalışmanın amacı Kuzey Kıbrıs'daki insan leishmaniasis seroprevelansı ve etken parazit *Leishmania* türleri hakkında bilgi sahibi olunmasıdır. Çalışmada, bu parazitlerin vektörü olan ve halk arasında küp düşen olarak bilinen kum sineklerinin sayısı ve tür çeşitliliği açısından zengin olan Girne, Lapta, Esentepe ve civar bölgelerde yerleşim gösteren 250 insandan kan örnekleri alınacaktır. Çalışmaya katılacak kişilerin en az bir yıldır Kuzey Kıbrıs'da yaşıyor olmaları ve köpek sahibi olmaları (veya köpeklerle temas halinde olmaları) gerekmektedir. Çalışmaya katılım gönüllülük esasına göre olacak ve katılımcıların kişisel bilgileri ile test sonuçları gizli tutulacaktır. Katılımcılardan herhangi bir ücret talep edilmeden yapılacak testler sonucunda pozitiflik saptanması durumunda, hastalarla iletişime geçilerek, uygun tedavi verilecektir. Ayrıca, gerek vektör olan kum sinekleri gerekse rezervuar olan köpeklerin kontrol altına alınmasına yönelik programların başlatılması için adım atılacaktır. Bu şekilde, önemli bir tropikal hastalık olan leishmaniasisin Kıbrıs'da eliminasyon ve eradikasyonuna katkıda bulunulacaktır.

Katılımcı
Adı, soyadı:
Adres:
Tel:
İmza:

Katılımcı ile görüşen hekim
Adı, soyadı, unvanı:
Adres:
Tel:
İmza:

Görüşme tanığı
Adı, soyadı:
Adres:
Tel:
İmza:

Sorumlu araştırmacı
Adı, soyadı, unvanı:
Adres:
Tel:
İmza:

APPENDIX 3

PARTICIPANT INFORMATION FORM

Participant's;

1) Barcode number:

2) Name and surname:

3) Age:

4) Gender:

5) Profession:

Questions:

6) Do you have any dog? If your answer is yes, how many dogs do you have?

7) What is your dog's age? (Please indicate for each of your dogs)

8) What is your dog's gender? (Please indicate for each of your dogs)

9) Do you have information about leishmaniasis?

10) Did you notice one or more signs or symptoms of leishmaniasis in your dogs (for example, hair loss particularly in the head, blurred eyes or discharge from the eyes, nose bleeding, ulcers in the nose or ears, etc.)? If the answer is yes, which findings did you come across with?

11) Do you use protective collar that contains antiparasitic drug for your dog?

Thank you for your interest and your participation in this study.

APPENDIX 4

KATILIMCI BİLGİ FORMU

Katılımcının;

1) Barkod numarası:
2) Adı-soyadı:
3) Yaşı:
4) Cinsiyeti:
5) Mesleği:

Sorular:

6) Köpek sahibi misiniz? Eğer cevabınız evet ise, kaç adet köpeğiniz bulunmaktadır?
   7) Köpeğinizin yaşı nedir? (Lütfen her bir köpeğiniz için cevaplayınız)
   8) Köpeğinizin cinsiyeti nedir? (Lütfen her bir köpeğiniz için cevaplayınız)
9) Leishmaniasis hastalığı ile ilgili bilginiz var mı?
10) Sahibi olduğunuz köpeklerde leishmaniasis bulgularından bir veya birkaçına (örneğin, özellikle baş bölgesinde kıl dökülmeleri, gözde bulanıklaşma veya akıntı, burun kanaması, burun veya kulakta ülserler, vs...) rastladınız mı? Eğer yanıtınız evet ise, hangi bulgulara rastladınız?
11) Köpeğinize, koruyucu antiparaziter ilaç içeren tasma takıyor musunuz?

Çalışmaya olan ilginiz ve katılımınız için teşekkür ederiz.