SS. CYRIL AND METHODIUS UNIVERSITY IN SKOPJE

FACULTY OF VETERINARY MEDICINE – SKOPJE

Betim B. Xhekaj

EPIDEMIOLOGY OF CANINE LEISHMANIOSIS IN KOSOVO AND THE ROLE OF *PHLEBOTOMUS* SPP. IN ITS SPREAD

Doctoral thesis

Skopje, 2024

SS. CYRIL AND METHODIUS UNIVERSITY IN SKOPJE

FACULTY OF VETERINARY MEDICINE – SKOPJE

Department of Parasitology and Parasitic Diseases

Betim B. Xhekaj

EPIDEMIOLOGY OF CANINE LEISHMANIOSIS IN KOSOVO AND THE ROLE OF *PHLEBOTOMUS* SPP. IN ITS SPREAD

Doctoral thesis

Skopje, 2024

Doctoral student:
BETIM B. XHEKAJ

Topic:
EPIDEMIOLOGY OF CANINE LEISHMANIOSIS IN KOSOVO AND THE ROLE OF *PHLEBOTOMUS* SPP. IN ITS SPREAD

Mentor:
PROF. JOVANA STEFANOVSKA, Ph.D.
Faculty of Veterinary Medicine – Skopje

Defense Panel:
Ph.D. NAME AND SURNAME, (Chairman)
Member's Institute

Ph.D. NAME AND SURNAME,
Member's Institute

Ph.D. NAME AND SURNAME,
Member's Institute

Ph.D. NAME AND SURNAME,
Member's Institute

Ph.D. NAME AND SURNAME,
Member's Institute

Scientific area:
PARASITIC DISEASES OF ANIMALS

Date of defense:
(If the date is not specified, it is added manually)

Betim B. Xhekaj

EPIDEMIOLOGY OF CANINE LEISHMANIOSIS IN KOSOVO AND THE ROLE OF *PHLEBOTOMUS* SPP. IN ITS SPREAD

- ABSTRACT -

Canine leishmaniosis (CanL) is a neglected zoonotic disease caused by *Leishmania* spp. Etiological agents are intracellular protozoa belonging to the genus *Leishmania* (Kinetoplastida, Trypanosomatidae). The life cycle of the *Leishmania* parasite is heteroxenous and alternates between the female phlebotomine sand flies (Diptera, Psychodidae) as vectors and numerous mammalian species (including humans). *Leishmania infantum* is the species responsible for the zoonotic form of the disease where dogs are reservoir hosts. The Mediterranean sand fly fauna is diverse, and leishmaniasis, mainly caused by *Leishmania infantum*, is endemic in the Balkan countries. Despite recent entomological surveys, only some districts of Kosovo have been sampled for sand flies, with no proof/confirmation of *L. infantum*. This study aimed to determine the seroprevalence of CanL in asymptomatic dogs in Kosovo, and to gain further insights into the species composition of natural sand fly populations in previously unsampled districts and areas in Kosovo without reports of leishmaniasis and to detect *Leishmania* DNA in sand flies. Dog samples were collected from 285 dogs in all seven districts in Kosovo (35–50 samples per district) from summer 2021 to spring 2022. Whereas a sand fly survey was conducted during the sand fly season in 2022 in all seven districts of Kosovo. Dog sera were tested using enzyme-linked immunosorbent assay (ELISA), and the presence of anti-*Leishmania* IgG was confirmed by an indirect fluorescent antibody test (IFAT). The true overall seroprevalence of CanL of asymptomatic dogs in Kosovo with ELISA was 4.21% (95% CI: 2.42–7.21), while with IFAT was 3.51% (95% CI: 1.92–6.34). The highest rates were found in the Prishtina district to be 8.0% (4/50) by ELISA and 6.0% (3/50) by IFAT, and in the Mitrovica district, the prevalence was 0% (0/40). Sand fly females were screened for *Leishmania* DNA by PCR. Positive samples were sequenced and subjected to maximum likelihood analysis with reference sequences for further molecular characterization. The trapping activities at 114 different localities resulted in 3272 caught specimens, comprising seven sand fly species of two genera, namely *Phlebotomus neglectus*, *Ph. perfiliewi*, *Ph. tobbi*, *Ph. papatasi*, *Ph. simici*, *Ph. balcanicus* and *Sergentomyia minuta*. *Leishmania infantum* DNA was

detected in three individual sand flies of *Ph. neglectus* and *Ph. perfiliewi* from one rural location in the Prizren district (Semetisht village). In dog sera, there were no significant differences among the different districts, gender, age, health status, and breed. These findings highlight the presence of CanL in most districts of Kosovo and underline the veterinary relevance of clinically asymptomatic dogs infected with *Leishmania*. On the other hand, the entomological study of sand fly vectors provides the most extensive sand fly survey in Kosovo and reports the first record of *L. infantum* DNA in sand flies, indicating the autochthonous circulation of *L. infantum* and the crucial role of vectors in its spread.

Keywords: *Leishmania infantum*, Leishmaniosis, Dogs, *Phlebotomus* spp., Kosovo, ELISA, IFAT, PCR

Бетим Б. Џекај

ЕПИДЕМИОЛОГИЈА НА КУЧЕШКАТА ЛАЈШМАНИОЗА ВО КОСОВО И УЛОГАТА НА *PHLEBOTOMUS* SPP. ВО НЕЈЗИНОТО ШИРЕЊЕ

– АПСТРАКТ –

Кучешка лајшманиоза (CanL) е занемарена зооноза предизвикана од *Leishmania* spp. Етиолошките агенси се интрацелуларни протозои кои припаѓаат на родот *Leishmania* (Kinetoplastida, Trypanosomatidae). Животниот циклус на *Leishmania* е хетероксен и се одвива помеѓу женските единки на песочните мушички - флеботомини (Diptera: Psychodidae) како вектори и бројни видови цицачи (вклучувајќи ги и луѓето). *Leishmania infantum* е видот одговорен за зоонозната форма на болеста каде што кучињата се резервоари. Фауната на медитеранските песочни мушички е разновидна, а лајшманиозата, главно предизвикана од *Leishmania infantum*, е ендемична во балканските земји. Со неодамнешните ентомолошки истражувања, само некои области на Косово биле истражени за присуството на песочни мушички, но во мушичките не се барал доказ или потврда за присуството на *L. infantum*. Оваа студија имаше за цел да ја утврди серопреваленцата на CanL кај асимптоматските кучиња во Косово, да даде дополнителни сознанија за видовиот состав на природните популации на песочни мушички во области во кои не се земани примероци и области без извештаи за присуство на лајшманиоза, и да открие присуство на ДНК на *Leishmania* во векторите. Примероците од кучиња беа собрани од 285 кучиња во сите седум области во Косово (35–50 примероци по област) од летото 2021 до пролетта 2022 година. Истражувањето на песочните мушички беше спроведено за време на активната сезона на песочни муви во 2022 година, исто така во сите седум области на Косово. Кучешките серуми беа тестирани со помош на ензимски-поврзан имуносорбентен тест (ELISA), а присуството на IgG против *Leishmania* беше потврдено со индиректен тест на фуоресцентни антитела (IFAT). Вистинската вкупна серопреваленца на CanL кај асимптоматски кучиња во Косово тестирани со ELISA беше 4,21% (95% CI: 2,42-7,21), додека кај тестираните со IFAT беше 3,51% (95% CI: 1,92-6,34). Највисоки проценти беа утврдени во регионот Приштина 8,0% (4/50) со ELISA и 6,0% (3/50) од IFAT, а во регионот Митровица, преваленцата беше 0% (0/40). Женките на песочните мушички беа тестирани

на присуство на ДНК на *Leishmania* со PCR. Позитивните примероци беа секвенционирани и подложени на анализа на максимална веројатност со референтни секвенци за понатамошна молекуларна карактеризација. Активностите за заловување на 114 различни локалитети резултираа со 3272 заловени примероци на песочни мушички кои припаѓаат на седум видови од два рода: *Phlebotomus neglectus, Ph. perfiliewi, Ph. tobbi, Ph. papatasi, Ph. simici, Ph. balcanicus* и *Sergentomyia minuta*. ДНК на *Leishmania infantum* беше откриена во три песочни мушички од видовите *Ph. neglectus* и *Ph. Perfiliewi* заловени во една рурална локација во регионот Призрен (село Семетиш). Во тестираните серуми од кучињата немаше статистички значајни разлики помеѓу регионите, полот, возраста, здравствената состојба и расата. Овие наоди го нагласуваат присуството на CanL во повеќето области на Косово и ја потенцираат важноста на асимптоматските кучиња заразени со *Leishmania*. Од друга страна, ентомолошката студија на векторите е досега најобемното истражување за песочните мушички во Косово кое што известува и за првиот наод на ДНК на *L. infantum* во векторите, што укажува на автохтона циркулација на *L. infantum* и клучната улога на песочните мушички во нејзиното ширење.

Клучни зборови: *Leishmania infantum*, Лајшманиоза, Кучиња, *Phlebotomus* spp., Косово, ELISA, IFAT, PCR.

Acknowledgment

I would like to thank my family, professors, friends, students and
all colleagues who helped me during my PhD study!

Herein, I provide my personal statement for the originality of the stated doctoral dissertation that I duly provide a reference of the quoted sources and that this paper is not used in any other university study for acquiring other knowledge.

Betim B. Xhekaj P.S

I declare that the electronic version of the doctoral dissertation is identical to the printed doctoral dissertation.

Betim B. Xhekaj P.S

TABLE OF CONTENTS **Pages**

LIST OF FIGURES & TABLES

List of Figures **Pages**

List of Tables

LIST OF ABBREVIATIONS & SYMBOLS

ABBREVIATION	MEANING
CanL	Canine Leishmaniosis
ELISA	Enzyme-Linked Immunosorbent Assay
IFAT	Indirect Fluorescent Antibody Test
IFA	Immunofluorescence Assay
HL	Human Leishmaniasis
VL	Visceral Leishmaniasis
CL	Cutaneous Leishmaniasis
ML	Mucosal Leishmaniasis
CVL	Canine Visceral Leishmaniosis
HVL	Human Visceral Leishmaniasis
Spp.	Species
WHO	World Health Organization
WOAH	World Organisation for Animal Health
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
NCBI	National Center for Biotechnology Information
IgG	Immunoglobulin G
AIDS	Acquired Immunodeficiency Syndrome
FML	Fucose-mannoseligand
PBS	Phosphate Buffer Saline
PSG	Promastigote Secretory Gel
FITC	Fluorescein Isothiocyanate
CMCP-10	Colorless non-resinous, water miscible mounting medium
IL - 2	Interleukin - 2
TNF	Tumor Necrosis Factor
IFN	Interferon
FNAC	Fine Needle Aspiration Cytology
MST	Montenegro Skin Test

LAMP	Loop-Mediated Isothermal Amplification
CDC	Centers for Disease Control
HRP	Horseradish Peroxidase
TMB	Tetramethylbenzidine
DMEM	Dulbecco's Modified Eagle Medium
ITS	Internal Transcribed Spacer
cpb	Cysteine Protease B
bp	Base Pair
ML	Maximum Likelihood
Pd	Pairwise Distance
CI	Confidence Interval
CO_2	Carbon Dioxide
°C	Celsius Scale
rpm	Revolutions Per Minute
OD	Optical Density
UV	Ultraviolet
mA	Milliampere
Hz	Hertz
pH	Potential of Hydrogen
ml	Milliliter
µl	Microliter
nm	Nanometer
≈	Approximately equal to

1. INTRODUCTION

The leishmaniases are zoonotic or anthroponotic diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae), transmitted by phlebotomine sand flies of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World). *Leishmania infantum* is a major etiological agent of both canine and human leishmaniosis, prevalent in the Mediterranean region [1]. *Leishmania* parasites undergo a digenetic life cycle, alternating between mammalian hosts, such as humans, dogs, etc., and female sand fly vectors. Out of the approximately 800 recorded sand fly species, roughly 10% are suspected or proven/confirmed vectors for transmitting leishmaniases [2]. Conversely, around 31 out of the 53 identified *Leishmania* species have been documented to infect mammals, with 20 of them leading to diverse clinical presentations in humans. Worldwide, a minimum of 70 species of both wild and domestic animals, encompassing dogs, opossums, bats, rodents, sloths, anteaters, marsupials, and hyraxes, have been identified as confirmed or potential reservoirs of *Leishmania* parasites [3]. *L. infantum* can also be transmitted through alternative routes, such as from mother to child, from a female dog to a puppy, and through the sharing of syringes [4].

Canine leishmaniosis (CanL) due to *Leishmania infantum* is a major worldwide zoonotic disease that can be life-threatening for both humans and dogs. Dogs serve as the primary reservoir of infection for humans. CanL is endemic in over 70 countries globally, including regions in southern Europe, Asia, Africa, and South and Central America, and it has been reported in the United States of America (USA). Additionally, it poses a notable concern in non-endemic countries where imported sick or infected dogs present a veterinary and public health challenge [5]. Human leishmaniasis is considered one of the world's most neglected diseases that predominantly affect the developing countries. The annual incidence of human leishmaniasis caused by different species of *Leishmania* spp. ranges from 700,000 to 1,000,000 cases [6]. Within the Mediterranean region, *Leishmania infantum* is the main species responsible for causing zoonotic cutaneous and visceral leishmaniasis in humans. This species also infects over 2.5 million dogs [7]. Among neglected tropical diseases, visceral leishmaniasis is one of the most lethal parasitic disease in recent history.

Globally, it leads to approximately 300,000 new cases and around ≈20,000 deaths annually. Leishmaniasis has become the hallmark of poverty-related diseases and tropical infections in humans forced to migrate to and from conflict zones [8].

Leishmania parasites are transmitted through the bite of a sand fly carrying the infection. Once they reside in host macrophages, they cause infection due to their ability to evade and attenuate the microbicidal function of the host by the modulation of both innate and adaptive immune responses. Consequently, the inactivated phagocytic macrophages become a secure environment for the proliferation of the parasites. Given that the infection is opportunistic to immune-suppression conditions, it is crucial to initiate a robust immune response for the effective treatment and prevention of visceral leishmaniasis [9]. Various clinical manifestations and immune reactions have been observed in cases of CanL. In fact, *Leishmania infantum* infection in the dog can appear as a chronic subclinical infection, a self-limiting disease, or a non-self-limiting illness. Therefore, to address this variability, a clinical staging system of CanL based on serological status, clinical symptoms, laboratory results, and type of therapy and prognosis for each clinical stage has been suggested. This clinical staging system ranges from stage I, indicating mild disease, to stage IV, signifying very severe disease, with different clinical outcomes, prognosis, and treatment alternatives [10]. During the progression of the disease, various organs, including the skin, kidneys, liver, spleen, and eyes, can be affected. The condition is identified by a variety of related clinical signs, such as skin lesions, generalized lymphadenopathy, muscle atrophy, weight loss, reduced ability to tolerate exercise, loss of appetite, anemia, vomiting, lethargy, splenomegaly, polyuria, polydipsia, ocular lesion, epistaxis, onychogryphosis, and diarrhea [11]. The clinical presentation of this disease relies on the parasite species and the immune response of both animals and humans. The spectrum varies from asymptomatic infection (i.e., 80%–95%) to various clinical and subclinical manifestations (i.e., 5%–20% of cases). In humans, immunosuppressive conditions are considered a main risk factor for visceral leishmaniasis (VL), although asymptomatic infections may be underestimated. Despite the mandatory reporting of human leishmaniasis in most Southern European regions, the low number of cases recorded annually suggests significant underreporting of the disease. In this context, in areas where up to 50% of the canine population is infected with *L. infantum*, most human cases may be asymptomatic [12].

Diagnosis of leishmaniosis exemplifies an instance where infection does not necessarily show clinical signs of the disease due to the high prevalence of subclinical infection. This poses CanL a diagnostic challenge for veterinary clinics, clinical pathologists, and public health officials in both endemic regions and non-endemic countries as well, where imported infections are a concern. Various diagnostic tests are available that can be used to diagnose this infection, and each of them has advantages and disadvantages [13]. The serological analysis serves as a useful method for the detection of specific antibodies and to assess the spread of the disease due to the large proportion of dogs being asymptomatic. Among various serological methods, enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody tests (IFAT) are the most widely used. ELISA, in particular, shows potential as a sensitive tool for large-scale screening in epidemiological studies and is well-suited for use in field conditions [14]. However, serological tests have important limitations, including the potential of cross-reactions, as well as false negative results in dubious reactions. Molecular techniques, including conventional, nested, and real-time PCR offer high sensitivity and specificity, constituting an integral component of veterinary diagnostic routine. These methods are especially useful for follow-up assessments and can be performed on diverse biological samples such as bone marrow aspirate or lymph nodes, peripheral blood, skin fragments, and others. It is crucial to emphasize that information provided by PCR should not be separated from the data obtained from clinicopathological and serological evaluations [11].

The purpose of CanL treatment is to manage clinical symptoms and abnormalities, improve the dog's cell immunity, reduce the parasite load, avoid relapses, and decrease the transmission rate to the vector. Achieving a complete and sterile cure in CanL is extremely difficult, but chemotherapy can effectively reduce the parasite load to asymptomatic levels. The antileishmanial treatment of CanL is similar to visceral leishmaniasis in humans, with some variations. Treatment decisions should be based on the specific stage of disease: stage I (asymptomatic to mild), stage II (moderate), stage III (severe disease associated with chronic kidney disease), and stage IV (very severe disease that includes nephrotic syndrome). Dogs in stage I can be left untreated or treated with allopurinol alone. For those dogs in stages II and III, a combination of allopurinol and antimonials/miltefosine is recommended. Whereas the dogs in stage IV, facing very severe conditions including nephrotic syndrome, should be treated with allopurinol alone to prevent further kidney damage. The therapy approach also includes chronic kidney disease management [15].

Prevention from canine leishmaniosis has a crucial role and importance, and a highly effective prevention approach involves the use of repellents to fight sand fly vectors. Veterinary products containing synthetic pyrethroids, such as permethrin and deltamethrin, demonstrate a repellent effect against these flies. The recommended products are available in two forms: spot-on treatments or collars. Additional preventive measures include keeping the dog indoors from dusk to dawn during the sand fly activity season and trying to minimize microhabitats that are favorable to sand flies. Vaccination options are also available. The most effective vaccines consist of purified fractions derived from *Leishmania* spp. In Brazil, there is a vaccine based on the fucose-mannose ligand (FML) fraction of *L. donovani*. Meanwhile, in Europe, the approved vaccine for dogs is based on the excreted/secreted antigen purified from the specific-medium culture supernatant of *L. infantum*. However, it's important to note that neither of these preventive measures can guarantee 100% protection for the dog [16].

In Kosovo, there is a lack of data on human leishmaniases, and there are no recent records regarding leishmaniosis in dogs or the detection of *L. infantum* in the sand fly vectors. The state of the dog population is challenging to determine due to the large number of stray dogs and the absence of data in the registration system in the Republic of Kosovo. The majority of the dog population consists of stray dogs, those kept in private households, or used as shepherd dogs. Previous data on CanL in Kosovo were primarily focused on a few districts or municipalities, with the first data published in 2008 by Lazri et al. [17]. Samples analyzed in this study mainly originated from three districts, namely Prishtina, Gjakova, and Ferizaj. In another report by Xhekaj et al. [18], anti-*Leishmania* antibodies among stray dogs were screened only in the southwestern region of Kosovo. Data on the distribution of *Leishmania infantum* in dogs across all districts is lacking. Furthermore, information on the distribution of sand flies in Kosovo is limited to a few studies that report the presence of sand fly vectors without conclusive evidence of *L. infantum*. Based on this historical data, this study aimed to determine the seroprevalence of leishmaniosis in dogs across seven districts of Kosovo. Additionally, the study sought to establish associations between seroprevalence and various factors, including geographical location (districts), sex, age, health status, and breed. Furthermore, the study aimed to detect the presence of *L. infantum* in sand fly vectors in Kosovo. Based on the importance of this disease for animal and public health, this dissertation presents the results of seroprevalence and epidemiology of canine leishmaniosis in dogs and the role of *Phlebotomus* spp. vectors in the spread of this disease in Kosovo.

1.1 Motivation and importance

Zoonotic diseases are one of the major public interest and concerns in every country regarding human health. National legislation aims to provide measures that will prevent the spreading of zoonotic diseases. All these measures are based on scientific facts gained through research activities.

Knowledge regarding leishmaniosis in the Republic of Kosovo is scarce, and there are gaps in the data necessary to design national strategies and action plans for monitoring and controlling this dangerous zoonotic disease.

This dissertation aims to provide necessary data related to canine leishmaniosis and the competent vectors in the Republic of Kosovo.

The results from this research will be used by the National Authority to develop strategies and action plans to prevent the spread of leishmaniosis in Kosovo, hence improving public and animal health. Moreover, the results will be used as scientific data for further research in this field of expertise. There are several possibilities for applying the results from this research, but the most important would be the development of a National Program for the control of leishmaniosis in dogs as a risk factor for public health. New programs for managing this disease should be applied such as control of the disease in stray and owned dogs, control of sand flies as vectors of the disease, education and information of the population for presence and risk of this disease, etc.

New data for CanL will be achieved during this study. By knowing the seroprevalence of CanL in all districts of Kosovo, authorities and people will have a clear view of its distribution and take concrete steps for prevention. Control would involve the reduction of the incidence and prevalence of the disease, treatment of infected dogs, development of new strategies and schemes of vaccination against leishmaniosis, preventing dogs from biting from infected vectors by avoiding time when vectors are active, using insecticide as a repellent of vectors, etc. The importance of leishmaniasis in humans has a crucial role in diagnosis to establish early treatment and in evaluating the effectiveness of control programs. Designing an epidemiological surveillance system for leishmaniosis will require the creation of a database of specific prevalence information for each host involved in this protozoa's life cycle.

This doctoral thesis will be the first research of this kind that will cover the whole country, and the results can be used in the future to develop programs and strategies for prevention and reducing the risk of leishmaniosis.

1.2 Working hypotheses and theses

Taking into consideration the objectives of the study, it was expected that:

- *Leishmania infantum* antibodies will be present in the tested samples.
- Different districts will have different seroprevalences.
- Different *Phlebotomus* spp. are going to be detected in Kosovo.
- *Leishmania infantum* and other *Leishmania* spp. will be present in the *Phlebotomus* spp.
- There will be more than one *Phlebotomus* spp. responsible for the transmission of leishmaniosis in dogs and humans.

1.3 Expected scientific contributions

Expected outcomes and scientific contribution of the research activities are the following:

- Estimation of the true seroprevalence of CanL in Kosovo and its distribution in all 7 districts.
- Estimation of the competent vector of CanL in Kosovo among different species of *Phlebotomus* spp.
- Identification of *Leishmania* spp. present in the competent vectors.
- Developing strategies and national action plans for monitoring and controlling the reservoir of CanL (positive dogs) to prevent transmission of leishmaniosis among the human population in Kosovo.
- Dissemination of gained knowledge among veterinary and public health authorities, veterinary and public health professionals, students, and the general public especially pet owners.

1.4 A brief overview of the thesis

The organization of this thesis was performed chronologically as follows:

- The first chapter is the introductory chapter "INTRODUCTION", which is a brief overview of canine leishmaniosis: etiology, distribution, transmission, clinical signs, diagnosis, treatment, and prevention. Then, motivation and importance, working hypotheses and theses, expected scientific contributions, and a brief overview of the thesis.
- Chapter 2 is "LITERATURE REVIEW", the deeper overview of the disease based on different publications and books starting from the history of the disease, etiology, epidemiology, transmission, entomology of vectors, pathogenesis, diagnosis, treatment, prevention and importance of this disease to public health.
- Chapter 3 is "RESEARCH SUBJECT AND OBJECTIVES", this chapter targets the objectives and subject of the thesis, and presents a clear view of the subject of the study including all objectives.
- Chapter 4 is "MATERIALS AND METHODS", it contains a presentation of the research methodology applied in the thesis. It describes the tools and procedures that were applied to reach the results, i.e., selection and targeting groups of the study, sampling and data collection, diagnostic methods of the study, and statistical analysis.
- Chapter 5 titled "RESULTS" presents the results obtained from the study. Here are shown all results of the study reached by different methods and samples starting from dog samples (ELISA and IFAT results and analysis), and vector samples (PCR based methods results and analysis).
- Chapter 6 is "DISCUSSION" which includes a discussion of all data collected in the previous chapters and highlights the importance of those findings and future studies to be developed based on the results of the thesis, and historical data of disease in Kosovo and neighboring countries.
- Chapter 7 is "CONCLUSIONS" which presents all the conclusions of the study.
- Chapter 8 is "REFERENCES" the bibliography of the literature used in the study.
- Chapter 9 is "APPENDIX" where all lists and tables of the thesis, regarding samples, are attached.

2. LITERATURE REVIEW

2.1. History

During the late 1800s, Cunningham, Leishman, Borovsky, Wright, Donovan, Lindenberg, and Vianna all separately identified the parasites that cause leishmaniasis. Ronald Ross later gave the generic name *Leishmania* to this parasite. In 1904, Cathoire and Laveran discovered *Leishmania* in children suffering from infantile splenic anemia. Nicolle, in 1908, named the parasite *L. infantum*, identified its reservoir in dogs in Tunis, and successfully cultured it in the laboratory. In 1912, Carini found *Leishmania* in mucosal lesions of leishmaniasis patients in Brazil. Furthermore, in 1914, the Russians Yakimoff and Shakor distinguished the parasites causing dry, urban, and wet, rural forms of cutaneous leishmaniasis in Central Asia. In the early 1940s, Swaminath, Shortt, and Anderson in India, as well as Adler and Ber in Palestine, demonstrated the transmission of *L. donovani* and '*L. tropica*' (probably *L. major*) by Phlebotomine sandflies. Over time, the clinical and geographical features of the human disease were supplemented by studies of animal reservoirs and vectors, *Leishmania* behavior in experimental animals, and the ecological dynamics of natural leishmaniasis cycles. These studies strengthened the basis for classification and comprehension of transmission to humans. The genetic speciation of *Leishmania* had to await the introduction of iso-enzyme analysis in the 1970s and DNA hybridization in the early 1980s [19].

2.2. Etiology

The leishmaniases are a group of zoonotic vector-borne diseases caused by diverse species of the protozoa *Leishmania*, which is primarily transmitted by sandflies. Globally, approximately 30 species of *Leishmania* exist, with at least 20 species that cause diseases in humans. However, the taxonomy of *Leishmania* spp. has been highly debated. *Leishmania* is divided into two subgenera, namely *Leishmania* and *Vianna*, each developing in either the foregut or the hindgut/midgut of the sandfly vector, respectively. Human disease syndromes associated with leishmaniasis are categorized into cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and visceral leishmaniasis (VL). For dogs, disease syndromes are classified as visceral leishmaniosis and American tegumentary leishmaniosis, which represents a localized cutaneous form that occurs in

regions of South America. The preference for using 'leishmaniosis' instead of 'leishmaniasis' is to make a clear distinction between the disease in animals from that in humans [20].

Leishmaniasis is caused by various protozoan parasites within the family Trypanosomatidae, belonging to the genus *Leishmania*. The modern classification of *Leishmania* parasites is illustrated in Table 2.1 [21].

Table 2.1: Taxonomy of genus Leishmania [21].

Kingdom	-	Protozoa
Phylum	-	Sarcomastigophora
Class	-	Zoomastigophora
Order	-	Kinetoplasitida
Family	-	Trypanosomatidae
Genus	-	*Leishmania*

The classification of *Leishmania* is complex and continually undergoes refinement with ongoing research on the parasites and their interactions with hosts and vectors. Figure 2.1 provides a complete classification of *Leishmania* species. The most relevant *Leishmania* species concerning human and animal health are summarized in four complexes: *L. donovani*, *L. tropica*, *L. mexicana*, and *L. braziliensis*. Within the *L. donovani* complex, both *L. donovani* (syn., *L. archibaldi*) and *L. infantum* (syn., *L. chagasi*) have the potential to cause cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) in both humans and animals [22].

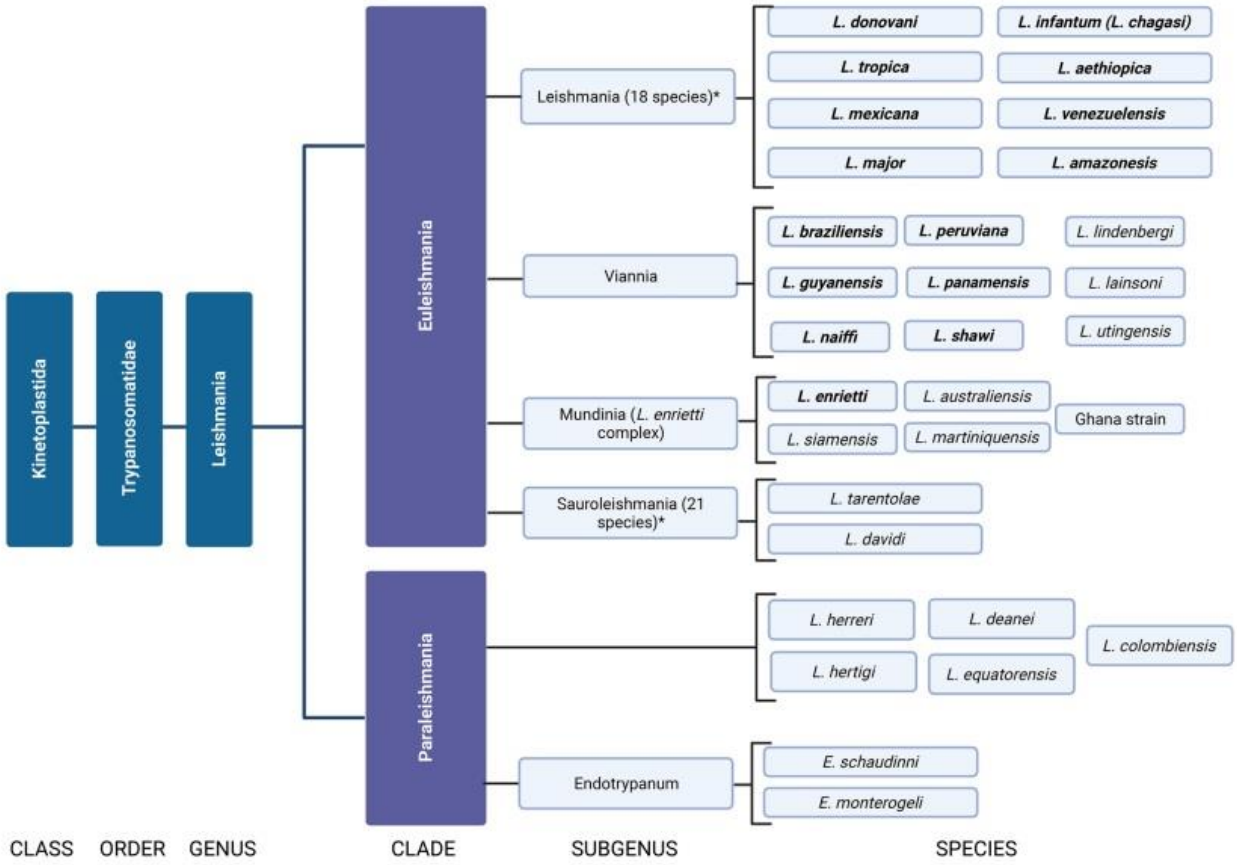


Figure 2.1: Classification of *Leishmania* species [22].

Inside the vertebrate host, *Leishmania* are obligatory intracellular parasites, targeting the macrophages within the lymphatic system and monocytes circulating in the blood. These forms represent the amastigote stage of development. Amastigotes are characterized by a round or oval shape, ranging from 2 to 7 µm in diameter, with a round nucleus, rodlike kinetoplast (mitochondrion), and a rudimentary internal undulipodium (flagellum) (Figure 2.2). Within the macrophages, the parasites undergo binary fission, resulting in the production of 50 to 200 new parasites. Upon the rupture of the host cell, *Leishmania* invades or are taken up by other cells [23].

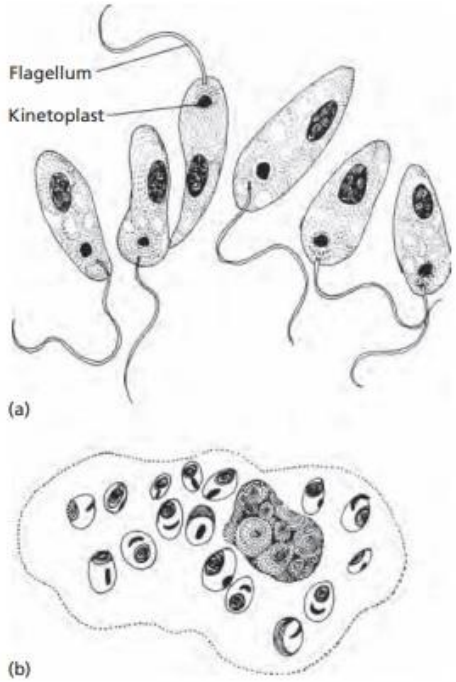


Figure 2.2: *Leishmania*: (a) promastigote form; (b) amastigote form [24].

Basically, amastigote parasites ingested by female sand flies during a blood meal, undergo multiplication in the gut and transform into promastigotes (Figure 2.2). These promastigotes are elongated, have a flagellum, and adhere to the mid-gut or hind-gut wall, and multiply rapidly. After further development, the survivor parasites migrate to the anterior part of the mid-gut and then to the fore-gut. In this stage, some parasites become metacyclic forms. Approximately 4 to 25 days after the sand fly feeds on an infective blood meal, the metacyclic forms are located in the mouthparts and are introduced into a new host during feeding process [25].

In the vertebrate host, *Leishmania* is present in the macrophages and various cells of the reticuloendothelial system, encompassing the skin, lymph nodes, spleen, liver, bone marrow, and mucosa. Additionally, it can be found in the leucocytes within the host's blood. This includes various species such as humans (*Homo sapiens*), dogs (*Canis lupus familiaris*), foxes (*Vulpes vulpes*), black rats (*Rattus rattus*), raccoons (*Nyctereutes procyonoides*), jackals (*Canis aureus*), wolves (*Canis lupus)*, fennec foxes (*Fennecus zerda*), and bush dogs (*Lycalopex vetulus*) [24].

2.3. Biological cycle

Leishmaniasis, also known as 'leishmaniosis' is a complex of mammalian diseases caused by parasitic protozoans *Leishmania* spp. which have a digenetic life cycle altering between animals and sand flies. The natural transmission of leishmaniasis may be zoonotic or anthroponotic, and usually occurs through the bite of sand fly species (order Diptera, family Psychodidae; subfamily Phlebotominae) of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) [26]. The natural life cycle of *Leishmania* infection involves a sand fly vector and a vertebrate host (Figure 2.3). Within vertebrate hosts, *Leishmania* exists in its nonflagellate form, known as the amastigote, residing in macrophages. Amastigotes are ovoid or round in shape, measuring 2.5 to 5 μm in length and 1.5 to 2 μm in width (Figure 2.2). With Wright's or Giemsa stain, in addition to a basophilic-staining nucleus, a rod-shaped, darker-staining kinetoplast is visible. Amastigotes undergo multiplication through binary fission, subsequently rupturing out of the macrophage to infect new cells. When sandflies feed on an infected host's blood and become engorged, they can ingest amastigotes. Inside the sand fly gut, amastigotes are released from their host cells. Subsequently, they undergo a series of morphological changes, transforming into the extracellular, flagellated procyclic promastigote form and undergo replication. Within an appropriate vector, there is significant replication accompanied by genetically regulated molecular alterations in the parasite's cell surface, leading to detachment from the midgut epithelium. This process results in the anterior migration of now-infectious metacyclic promastigotes into the foregut and mouthparts of the vector. During a subsequent feeding episode, female sand flies inject promastigotes with saliva into the skin of a vertebrate host. Following inoculation, the promastigotes lose their flagella and transform back into amastigotes [27].

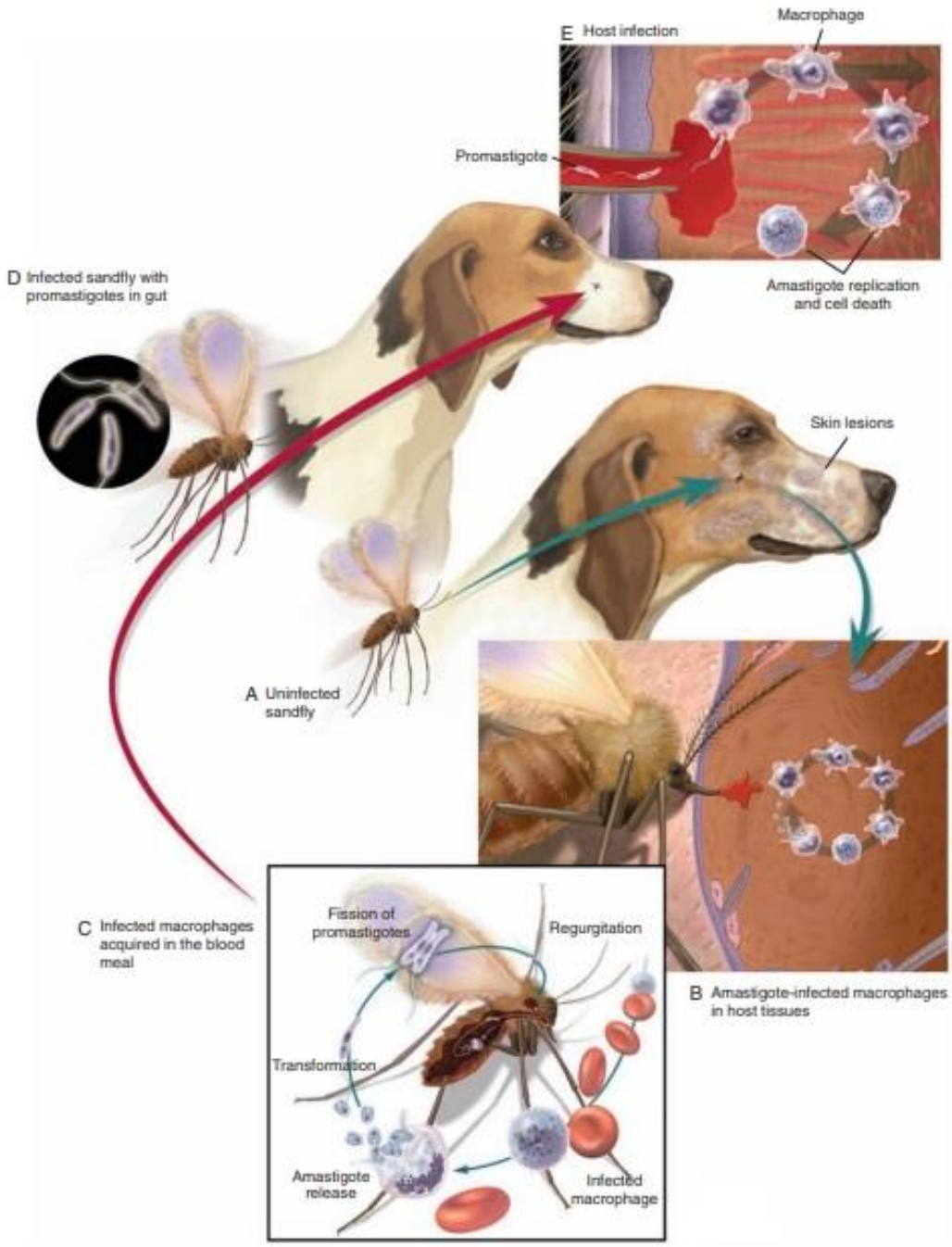


Figure 2.3: The life cycle of *L. infantum*. In (A), an uninfected sand fly feeds on an infected host and (B) ingests infected macrophages from the host tissues. Subsequently, (C) the organisms are released from the infected macrophages into the sand fly gut, where they (D) transform into motile promastigotes and undergo replication in the sand fly gut. During feeding, (E) the promastigotes are regurgitated by the infected sand fly, thereby transmitting the infection to a new host [27].

2.4. Epidemiology

Leishmaniasis has a significant clinical and epidemiological diversity, posing a substantial global public health challenge. It stands as one of the most neglected tropical diseases and is prevalent in 98 countries worldwide, with the exception of Antarctica. The geographic distribution of leishmaniasis is global but is primarily endemic in tropical and sub-tropical regions, including Africa, parts of Asia, the Middle East, Latin America, and the Mediterranean region. In Europe, the disease seems to be gradually spreading northward from its traditional foci in the south. In recent times, there have been instances of the disease emerging or re-emerging in various geographical areas, raising concerns about its impact on global health and the economy, and fear of affecting humans, domestic animals, and wildlife [28]. Leishmaniases are predominantly concentrated in tropical and temperate regions, mainly in areas characterized by a low Human Development Index. In these countries, leishmaniases are considered endemic, with approximately 12 million reported cases and an annual occurrence of 1.5–2 million new cases among a population of 350 million people at risk of infection. In Europe, dogs serve as the primary reservoir for the zoonotic forms of both visceral and cutaneous leishmaniasis. The estimated annual incidence of visceral leishmaniasis (VL) in the WHO European Region ranges from 1100 to 1900 cases. Italy, along with Georgia, Spain, Albania, Turkey, Tajikistan, and Azerbaijan, is among the most affected countries, with an estimated annual VL incidence ranging from 160 to 240 [29]. In Europe, canine leishmaniosis is primarily caused by *Leishmania infantum*, which encompasses various enzymatic types known as zymodemes. Other species, such as *L. tropica* and *L. major* have rarely been diagnosed. Reports of leishmaniosis have been documented in animals and/or humans in nearly all or the majority of territorial subdivisions in Spain, Portugal, Southeast France, Corsica, Malta, Italy, Albania, Greece, Cyprus, the Adriatic coast of Croatia and Montenegro, and southern Bulgaria. In contrast, infections have been reported from only one subdivision in Hungary, Austria, Slovenia, North Macedonia, and Ukraine, two in Kosovo, four in Romania and Germany, six in Serbia, and eight in Bosnia and Herzegovina [30]. In Figure 2.4, the regional distribution of autochthonous *Leishmania infantum* infections in both humans and animals across European countries is depicted. Outside this region, numerous imported cases of canine leishmaniosis and a limited number of cases in cats have been diagnosed and treated. Nevertheless, there are few reports of isolated cases in dogs that have not travelled through or reside in endemic areas. It is

likely that focal transmission can take place for a limited duration when there is a significant infection pressure from imported infected dogs and competent vectors [30,31].

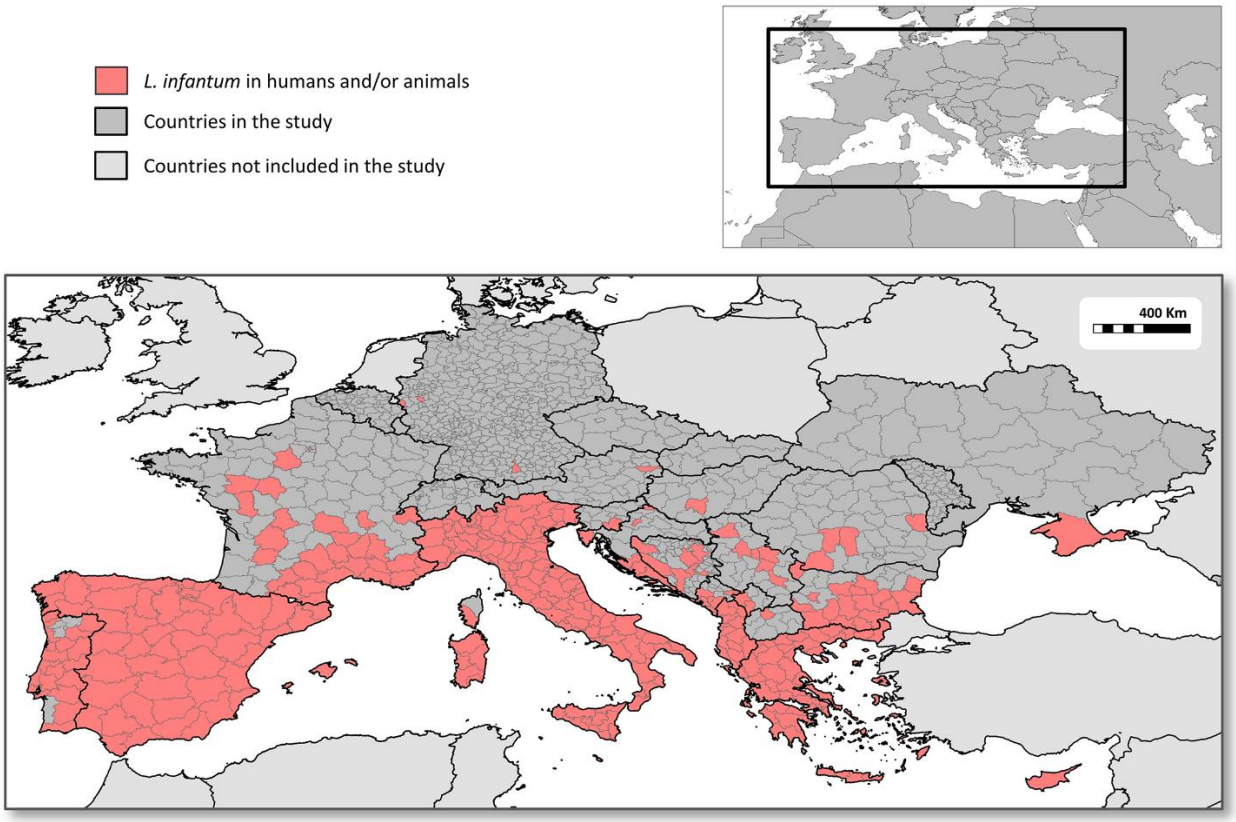


Figure 2.4: Geographical distribution of autochthonous *L. infantum* infections in humans and animals across European countries, as documented in scientific literature and reports from health ministries of some countries, between 2009 and 2020 [30].

The majority of available data on CanL in Europe is derived from countries where CanL is endemic, such as Portugal, Spain, France, and Italy. Recently, information on CanL has started to emerge from non-endemic countries, including the Netherlands, Germany, Denmark, Sweden, and the United Kingdom. The Balkan region has a history of being an endemic area for leishmaniasis, with the first recorded CanL cases dating back to the early 20th century. During the endemic period of leishmaniasis in the 20th century, the focus was primarily on human cases, leading to a lack of diagnosis for canine cases. At that time, the disease in the animal population was largely ignored, resulting in an unknown number of cases and a lack of understanding of the extent of disease [32].

2.5. Transmission

Leishmaniasis, as a vector-borne disease, caused by an obligatory parasitic protozoan, which is transmitted through the bite of infected female sandflies belonging to the *Phlebotomus* genus in the Old World and the *Lutzomyia* genus in the New World. There are at least 93 proven or potential vector species of sandflies worldwide. The *Leishmania* genus is wide, leading to a complex life cycle that involves two main stages—promastigote and amastigote—adapting to the host, either an invertebrate vector (sand fly) or a vertebrate host (human or animal), respectively. Transmission occurs through the sand fly and can be either anthroponotic or zoonotic, depending on the geographical region [22].

2.5.1. Role of the host

Since the identification of CanL, dogs have been identified as a major reservoir of the causative agent of Visceral Leishmaniasis (VL), playing a crucial role in its transmission. While other infected mammals are suspected to contribute epidemiologically to the transmission, their confirmation as reservoirs and their impact on the transmission cycle remains unknown. The domestic dog, due to its close relationship with humans, has been consistently identified as the main reservoir of *L. infantum*, and it has been proven that infected dogs serve as sources of infection for phlebotomine sand flies [33]. Maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) have the potential to transmit infection to sand fly vectors even in the absence of observable clinical signs, but the significance of these findings in epidemiological terms remains uncertain. The susceptibility of domestic cats (*Felis catus*) to *L. infantum* infection, the clinical outcomes, and their role in maintaining the parasite's life cycle are not well understood. It appears that the immune response in cats is generally effective in controlling the infection and providing a certain level of resistance unless there are immunosuppressive events such as retroviruses, autoimmune diseases, cancer, and other factors. While domestic cats infected with *L. infantum* may have the potential to transmit the infection to competent vectors, additional research is needed to confirm their role as accidental hosts and secondary or alternative reservoirs. When a sand fly bites an infected host, it takes in macrophages containing rounded and nonmotile amastigote forms. When the infectious promastigote forms are inoculated into the host's skin from the vector's

proboscis, macrophages phagocytize them. Subsequently, these forms transform into the amastigote stage, where they undergo continuous asexual reproduction within macrophages until rupture occurs. The parasites spread by infiltrating mononuclear phagocytes in various organs, mostly the spleen, lymph nodes, liver, bone marrow, and other tissues. Interestingly, the occurrence of autochthonous cases of VL in areas where the presence of phlebotomine has not been confirmed suggests alternative transmission routes. While the non-sand fly transmission is often considered to be low, various studies have highlighted the potential impact of alternative transmission routes of CanL. Notably, sexual (venereal) and transplacental (vertical) transmissions have been identified, indicating their epidemiological importance in the spread and maintenance of the disease, especially when the natural insect vector is absent. *Leishmania* transmission through sexual and transplacental ways has already been reported in mice, humans, and dogs. In dogs, there is evidence of efficient transmission from infected males to susceptible females, especially dogs with genital lesions associated with VL. The presence of *Leishmania* spp. has been detected in many biological samples from stillborn or newborn puppies, symptomatic or asymptomatic naturally infected female dogs, often linked to necrotizing placentitis, abortion, or various gross/microscopic observable changes in the placenta. Collectively, these studies provide robust support for the hypothesis that CanL is transmitted vertically. Other potential forms of transmission, such as infection through blood transfusion or its derivatives from infected donors, organ transplantation, and the sharing of contaminated needles, should be carefully considered, especially in humans and dogs. Additionally, a suspected mode of transmission is the direct transfer of the parasite among dogs through wounds or bites. There have been suspicions about other blood-feeding arthropods, like ticks or fleas, being involved in *Leishmania* transmission, based on the association of the presence of CanL with the presence of these alternative vectors. Although there is no conclusive evidence regarding the definitive role of these ectoparasites in the disease's transmission cycle, it is still advisable to take preventive measures and treat dogs for fleas, ticks, and mosquitoes [34].

2.5.2. Role of the vectors

The development of *Leishmania* parasites within the sand fly vector is a highly complex process, involving distinct differentiation stages crucial for the establishment of a successful infection (Figure 2.5). The organism undergoes multiplication in the insect gut and then migrates to the proboscis, where it waits to be inoculated into a new host. The transmission can also occur even if the fly is crushed onto abraded skin [35]. When a sand fly ingests an amastigote within a macrophage of any host, these amastigotes undergo differentiation into promastigotes (stages in which the kinetoplast is near the anterior portion of the cell, from which the free flagellum extends). Inside the fly, the promastigotes undergo multiplication, generating very large numbers [36]. According to Cecilio et al. [37] the development of *Leishmania* parasites is confined to the digestive tract of the sand fly, without any crossing or disruption of the epithelial barrier. This tract can be simplistically divided (excluding the crop) into three sections: (I) the foregut, encompassing the most anterior portion from the mouth to the cardia, which includes the stomodeal valve; (II) the midgut (thoracic and abdominal), extending from the cardia to the pylorus; and (III) the hindgut, covering the most posterior portion from the pylorus to the rectum. The initial differentiation step occurs shortly after the sand fly ingests the infected blood meal. Due to alterations in environmental conditions, such as a decrease in temperature and an increase in pH, the amastigotes transform into procyclic promastigotes, which are weakly motile forms. The procyclic promastigotes represent the initial replicative form found in the sand fly vector, facilitating an increase in parasite numbers and playing a crucial role in the subsequent stage of the vector infection cycle. Approximately 48 hours after the ingestion of the blood meal, the replication of procyclic promastigotes slows down, and they undergo differentiation into highly motile and long forms known as nectomonad promastigotes. These parasite forms are essential for attaching to the sand fly midgut, constituting another critical step in completing the *Leishmania* cycle within the vector. This attachment prevents the parasites from being eliminated along with the remnants of the blood meal during defecation. The life cycle then progresses as nectomonad promastigotes migrate towards the anterior midgut and undergo further differentiation into leptomonad promastigotes. These shorter forms of the parasite represent another phase of replication within the insect, contributing to the population of the sand fly's anterior midgut as well as an important role in secreting the promastigote secretory gel (PSG), which is crucial for the transmission process. Eventually, the leptomonads undergo a further differentiation process known

as metacyclogenesis, leading to the development of the parasite stage that is infective to vertebrate hosts—the metacyclic promastigote. Metacyclic parasites exhibit a smaller body size and a long flagellum, responsible for their extremely rapid motility. It is noteworthy that, beyond their morphological distinctions, all these stages of the parasite have been demonstrated to exhibit varying degrees of transcriptional distinctiveness. In nature, sand flies typically acquire a blood meal every five to six days to complete as many gonotrophic cycles as possible. A reconsideration of the life cycle within the vector includes a new parasite stage termed retroleptomonad, resulting from the de-differentiation of metacyclic parasites in the presence of newly available nutrients following the ingestion of blood by infected sand flies. As the name suggests, this recently identified parasite form closely resembles leptomonad promastigotes in morphology and appears to be functional. In contrast to metacyclic forms and similar to leptomonad promastigotes, retroleptomonads represent replicative forms in the life cycle. Eventually, when the stressful conditions are reinstated in the midgut through the defecation of remnants from the second blood meal, retroleptomonad parasites undergo a process of "redifferentiation" into metacyclic promastigotes. Significantly, the replication of retroleptomonads has a consequence, better vector infections, both quantitatively (with increased parasite numbers per midgut) and qualitatively (yielding more uniform populations of metacyclic promastigotes). These findings, when considered in the ecological context, suggest that the development of a successful infection in wild sand flies is a gradual process reliant on the amplification of parasites facilitated by the ingestion of multiple blood meals by the sand fly vector. Even if the initial parasite numbers are minimal, the consumption of a second blood meal acts as a boost for the replication of leptomonads, resulting in an increase in parasite numbers to a level conducive to their differentiation into metacyclics. Depending on the initial infectious inoculum, a third blood meal and another round of parasite replication may be required. Nevertheless, the de-differentiation of metacyclic promastigotes into replicative retroleptomonads upon subsequent blood meals by infected sand flies enhances the development of more potent infections and elevates vector competence up to a point where transmission becomes the most probable scenario, provided the sand fly survives for a sufficient duration [37].

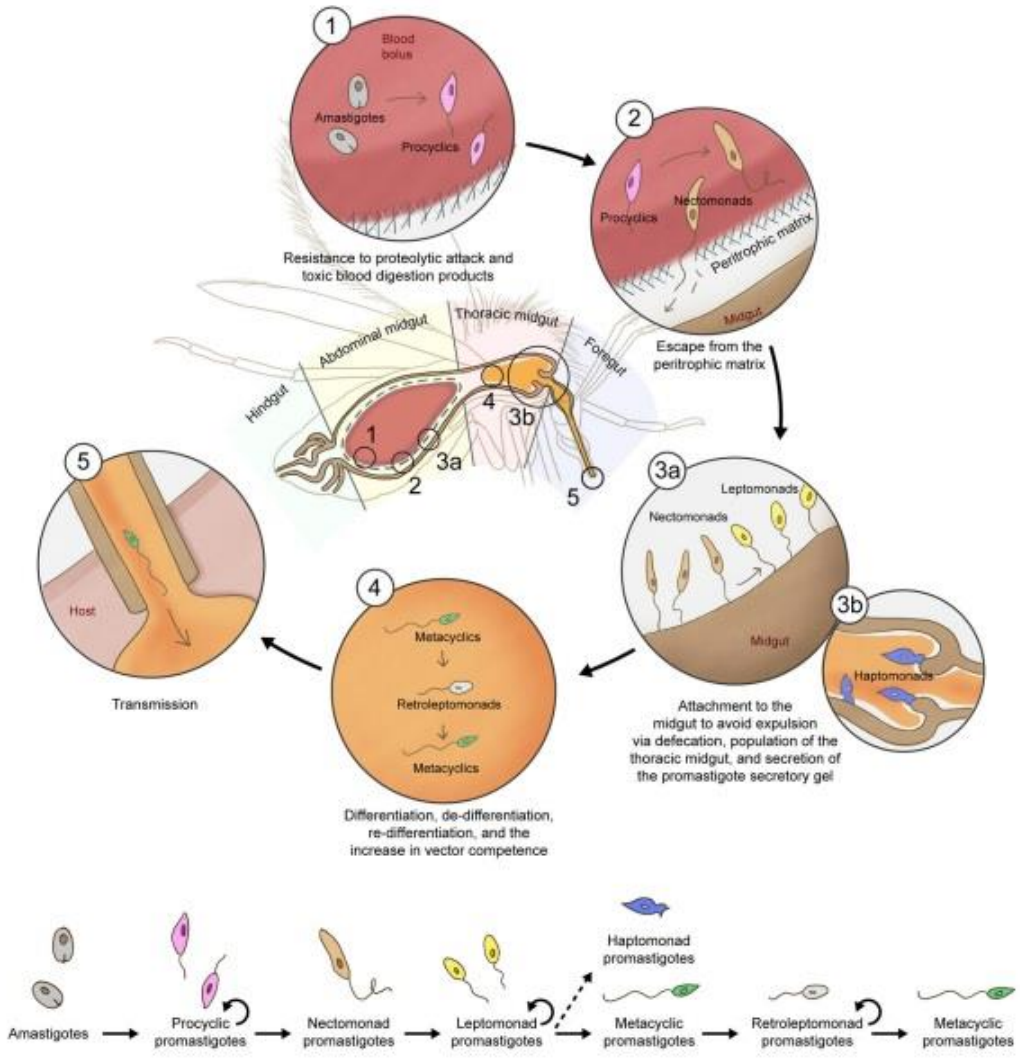


Figure 2.5: *Leishmania* development in the sand fly midgut. This schematic illustration depicts the various stages of *Leishmania* parasites within the sand fly vector and the primary obstacles they must overcome to establish a successful infection. These include resistance to proteolytic attack and toxic byproducts from blood digestion (1), escape free from the peritrophic matrix (2), adhering to the midgut to prevent expulsion (3a), attaching to and influencing the stomodeal valve (3b), and the dynamics of (de-)differentiation and replication (4). The ultimate goal is to ensure transmission (5) of the parasites to a new host [37].

2.6. Entomology and surveillance of sand flies

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) play an important role in the field of medical and veterinary sciences. These blood-feeding insects are significant vectors, transmitting numerous pathogens such as protozoa, bacteria, and viruses to both animals and humans [38]. The geographical distribution of sand flies is a crucial factor for the determination of the geographical distribution of the diseases. Although their activity is primarily during warmer months, favorable climatic changes enable sand flies to extend their geographical range and remain active longer. Phlebotomine sand flies, characterized by their delicate nature, small size, ectothermic attributes, and hairy appearance are unable to colonize new distant areas through either passive transportation or individual mobility (Figure 2.6). So far, no sand fly species have been classified as invasive. The transmission of pathogens by sand flies in endemic regions follows a distinct seasonality, influenced by environmental factors affecting the abundance of both vectors and reservoir hosts. In Europe, sand flies are typically identified between April and November, with the specific timing varying based on latitude [39]. They have excellent ability to adapt and possess a flexible response to alterations in environmental and climatic conditions. Typically, sand flies are mostly found in rural and peri-urban environments, avoiding high altitudes due to their preference for warm temperatures. However, the potential for increasing genetic variation, influenced by shifts in developmental temperatures, could significantly impact the distribution and expansion of sand fly species, particularly in warmer environments. Ecological studies indicate that both altitude and bioclimatic structure play a crucial role in the distribution of sand fly species. While altitude itself may not be a direct ecological factor, it can influence sand fly distribution through habitat diversity, relief features, and the climate gradient that it offers [40].

Figure 2.6: Sand flies under a stereomicroscope.

2.6.1. Life cycle of sand flies

Adult sand flies are small insects, measuring approximately 3 mm in length, and unique golden, brownish, or gray colored. They have elongated, piercing mouthparts, that are proficient at sucking blood from their chosen hosts. When at rest, sand flies position their wings in a distinctive vertical V-shape, a characteristic that distinguishes them apart from other small fly species. Additionally, the six legs of adults are extremely long, surpassing the insect's body in size (Figure 2.6) [41]. Upon closer inspection, it becomes evident that vein two of the wings in phlebotomines branches twice towards the middle or tip of the wing. The male sand fly can be easily distinguished from the female by the presence of prominent claspers at the tip of its abdomen. In regions with temperate climates, sand flies are present only in the summer months, whereas in tropical areas, their presence may extend throughout the entire year. Mating of sand flies is linked to the host rather than occurring through swarming. Male sand flies jostle together and await females in mating gatherings known as 'leks,' near or on the host. Both male and female sand flies feed on sugars from plants or aphid honeydew, but only the females feed on blood [42]. Following mating, females deposit 30 to 60 eggs per batch in small cracks or holes in damp ground and around the roots of forest trees. These eggs typically hatch within a span of 4 to 20 days. The larvae feed on decaying organic matter, fungi, and associated microorganisms, and undergo four developmental stages (instars) over a period that ranges from 30 to 60 days, depending on temperature and food quality. In climates characterized by a prolonged hot or dry season or cold winter, diapause or estivation may occur during the egg stage or the fourth larval instar, enabling the sandflies to endure for up to a year (Figure 2.7). Whereas, the pupal stage lasts approximately one week [43]. Knowing the fact that only females have functional mouthparts and are blood feeders, the adult females of most species are anautogenous. Species belonging to the *Phlebotomus* genus primarily feed on mammals and are found in savannah and desert regions. Adults often gather in the burrows of rodents or in other shelters like caves that offer a suitable microclimate. In these shelters, adults remain refugia, preferring to feed during the night, dawn, or dusk on the occupants or mammals in close vicinity. Adult sandflies exhibit limited flight capabilities, with a range of only 100-200 meters. Their movement is characterized by short hops, and they can only fly under conditions of low wind speed. The life-cycle development rate is generally slow, taking a minimum of 7-10 weeks, with numerous Palaearctic species having just two generations per year [44].

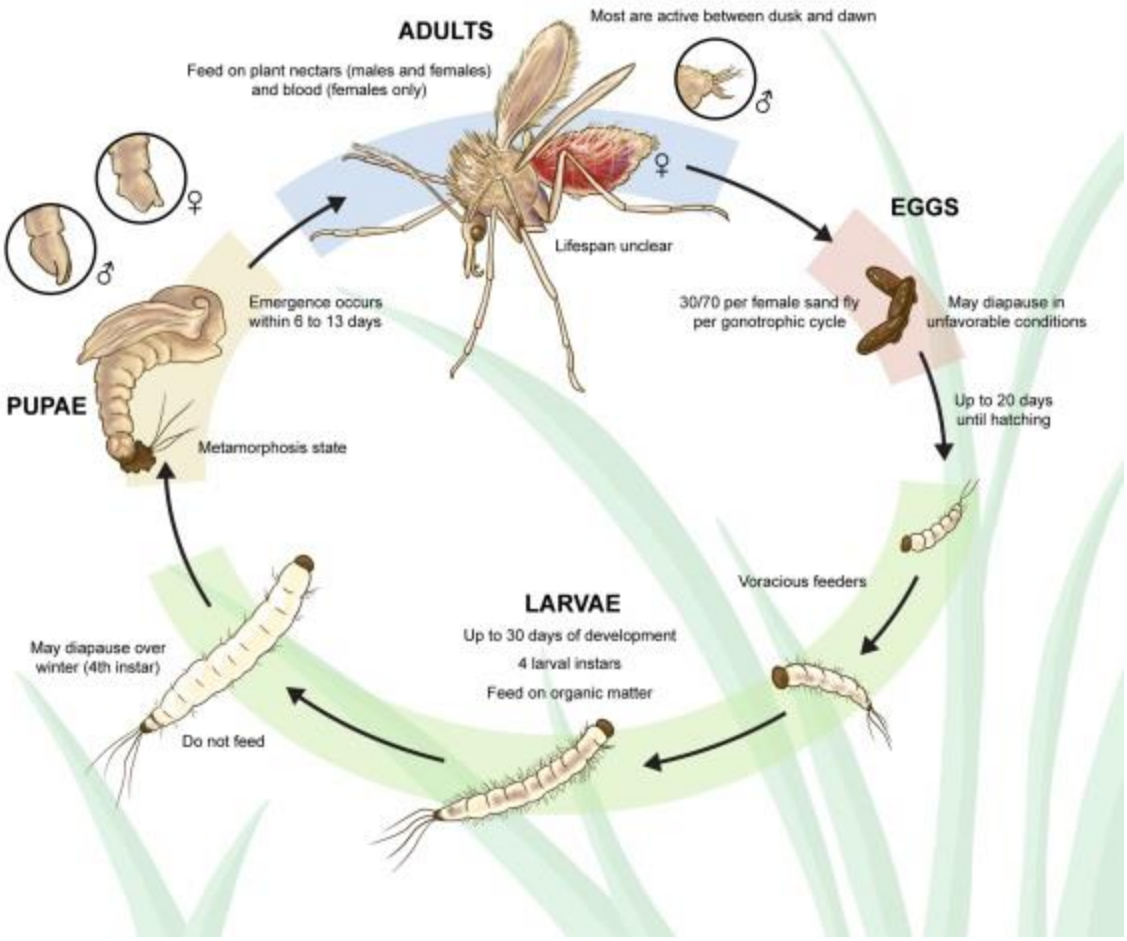


Figure 2.7: Life cycle of sand flies. The life cycle consists of four major stages: eggs (orange background), larvae (four instars: green background), pupae (yellow background), and adults (blue background). The most important characteristics for each stage (and sub-stage) are listed near the images, along with average development timings [37].

2.6.2. Surveillance of sand flies

Studies of the relationships between sand flies and parasites, as well as the study of vector behavior, taxonomy, and ecology, require sampling techniques that ensure the insects remain alive or adequately preserved for transportation to the laboratory and subsequent processing. Various methods used for sand fly collection, the interpretation of field data, and specimen preservation techniques associated with each collection method present both advantages and disadvantages concerning practicality, safety, and cost. Sand fly adults of both genders can be collected through

various methods, whether they are actively foraging at night or resting during the day. However, locating immature stages proves challenging, and there is still much to uncover about sand fly breeding sites. This knowledge gap not only constrains options for vector control but also limits the number of species that can be successfully cultivated in the laboratory. The choice of sand fly collection methods depends on the study's objectives, the level of familiarity with the local fauna, and the ecology of the study site. If the intent is to collect live sand flies for colonization or experimentation, specific techniques will be necessary. In situations where there is limited information about sand fly populations, it is advisable to use a mix of sampling methods to comprehensively identify the species spectrum. However, if the habits and ecology of the specific sand fly population are well known, one or two methods customized to that situation may be adequate [45]. The majority of field investigations related to sand flies are based on the necessity to identify potential vectors, particularly those associated with human and canine leishmaniasis. The primary objective is to find out the locations and timings of *Leishmania* transmission. This involves the sampling and identification of anthropophilic sand fly species found in close proximity (within a few hundred meters) to the suspected sites of infection cases. Collections from resting sites typically provide more comprehensive samples of the entire fauna, enabling the capture of substantial numbers of sand flies across various species [46]. Light traps, carbon dioxide (CO_2) traps, sticky traps, and aspirators are the most common methods for trapping sand flies (Figure 2.8). However, each type of trap has advantages and disadvantages, as well as variations in effectiveness. Therefore, it may be advisable to combine different traps when conducting field studies intended to estimate the quantity and variety of sand flies. Depending on the specific research goals, various other traps and techniques are also at one's disposal for use in the field [47]. Light traps are extensively used in sand fly studies. The predominant type of trap is a battery-operated light suction trap, notable for its ability to be left operational overnight for sand fly collection. This trap functions by drawing sand flies into a funnel equipped with a mesh screen adapted to the sand flies' size, leading to a killing bottle. For special investigations where live sand flies are required, the killing bottle is substituted with a holding cage, exchangeable multiple times within the same night as needed. Although the trap's range for attracting sand flies is not extensive, its effectiveness is notable when strategically placed near resting or breeding sites, using the appropriate light source. Light traps attract host-seeking sand flies while also capturing gravid females and male sand flies [48].

Sticky traps offer an alternative method for capturing sandflies through interception rather than attraction. Standardized pieces of thin cardboard or unwaxed paper (such as 20 x 20 cm, 25 x 20 cm, or A4 plain paper) are soaked in castor oil and positioned in resting spots or areas where sandflies are likely to be active. To achieve quantitative outcomes, either one or both sides of the paper must be fully exposed. The paper can be strategically placed near resting spots and secured, using materials like wood, bamboo, hooks, wire, staples, or paper clips, depending on the local conditions. These traps are generally cost-effective and can be easily produced in large quantities. They can be prepared in advance for field studies and stored until needed. Castor oil-impregnated sticky traps can prove effective even in dry regions when shielded from the wind [49]. Sand flies can be collected using an aspirator, either from animals or while at rest on walls in structures or animal shelters, offering the key benefit of collecting live specimens. Based on the delicate nature of sand flies, it's essential for the aspirator's body to be wider than the opening. Plastic aspirators, although light and strong, may pose a risk of damaging sand flies due to static electricity. Therefore, a glass aspirator is recommended for most studies, both in field and laboratory environments. The Castro aspirator, a modification of the mouth aspirator, consists of a length of rubber tubing connected to the glass/plastic tube, with fine mesh gauze sealing the junction between the two parts. This design allows sand flies to be sucked quickly, followed by their immediate release into holding containers. The primary advantage lies in the ability to separate insects collected from different microhabitats in a single session, providing more detailed data rather than storing them together in a single aspirator [48].

A  B 

Figure 2.8: Different traps used for sand flies: A) CDC light trap, B) sticky traps [48].

2.7. Pathogenesis of canine leishmaniosis

The parasite is transmitted through the bites of female sandflies, which inject metacyclic promastigotes into the dog's skin. After encountering the skin's immune system and transforming into amastigotes, they are transported by infected macrophages to the regional lymph nodes. The manifestation of clinical disease depends on the immune response of the individual host, and two distinct patterns are observed: firstly, a T cell-mediated protective immune response, wherein dogs remain infected but do not progress to clinical illness; and secondly, a marked humoral non-protective immune response with reduced or lacking T cell-mediated immunity, resulting in dogs developing evident clinical disease [50]. From this stage onward, the infection's outcome will vary based on numerous factors related to the vector (such as repeated infectious bites and intradermal injection of sand fly saliva), the parasite (its virulence), and the host (including genetic background, cell-mediated and humoral immune response, cytokine milieu, and concurrent diseases). Parasites might undergo local elimination (resulting in a self-limited infection), be sequestered in the skin and lymph nodes (indicating a non-disseminated and usually asymptomatic infection), or spread throughout the body (resulting in a disseminated infection), leading (symptomatic infection) or not (disseminated, asymptomatic infection) to the manifestation of clinical signs and/or clinicopathological abnormalities of CanL (Figure 2.9) [51]. In regions where the parasite is endemic, the majority of dogs infected do not exhibit clinical signs or clinicopathological abnormalities. When clinical signs arise, they tend to vary based on the dog's immune response and the parasitic load. Dogs that launch a predominant T helper-1 (Th1) cell-mediated response to *L. infantum* infection are more likely to overcome illness and/or remain apparently healthy. The apparently healthy infected dogs can result in sand fly infection, potentially contributing to transmission to others. On the contrary, those with a more pronounced Th2 humoral antibody-mediated response often develop moderate to severe disease manifestations. Dogs affected by CanL often present with non-specific signs during evaluation, including lethargy, weight loss, reduced appetite, diarrhea, vomiting, polyuria, and/or polydipsia. Physical examination of these dogs may reveal fever, generalized lymphadenomegaly, splenomegaly, and pale mucous membranes [52].

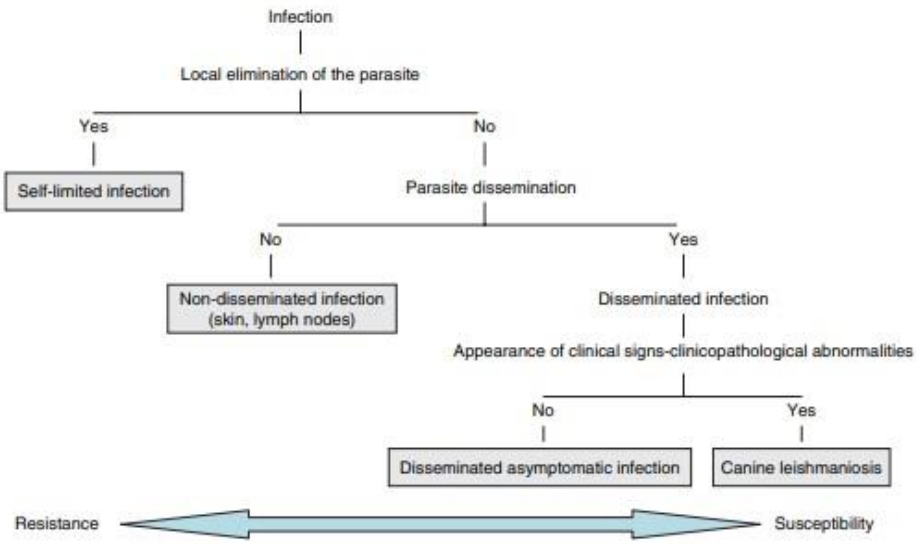


Figure 2.9: Possible outcomes of the canine infection by *L. infantum* [51].

2.7.1. Macroscopic lesions and clinical signs

Diagnosing and controlling CanL continues to pose challenges for veterinary practitioners. Identifying infected dogs is complex due to several reasons. Dogs acquiring the infection may remain without symptoms for extended periods, spanning months or even years, and in some instances, for their entire lifespan. Symptomatic dogs exhibit a broad range of nonspecific clinical signs, ranging from mild local skin lesions to severe systemic syndromes. This variability is attributed to the diverse pathogenic pathways of the disease and the distinct immune responses of the hosts [53]. In a typical case of CanL, the patient's history and physical examination may reveal symptoms such as anorexia or increased appetite, lethargy, skin lesions, ocular lesions, cachexia, splenomegaly, peripheral lymphadenomegaly, epistaxis, emaciation, exercise intolerance, temporal muscle atrophy, polyuria/polydipsia, onychogryphosis, lameness, vomiting/diarrhea etc. These symptoms can manifest individually or, more commonly, in various combinations. The clinical signs of CanL are diverse and nonspecific, leading to an extensive list of differentials. The complexity is further heightened because the clinical diversity of CanL may also be induced by other vector-borne organisms thriving in the same geographical regions, potentially infecting any dog with *Leishmania* [54]. Internal organs like the spleen, kidney, liver, and lymph nodes may also be affected. Some clinical signs are more prevalent than others; skin lesions are the most common,

affecting 50 to 90% of symptomatic dogs. Weight loss is 25 to 80%, along with onychogryphosis in 30 to 75%, and ocular abnormalities in 16 to 24%. Figure 2.10 illustrates the most frequent clinical signs of CanL. The primary biochemical findings in CanL include hyperglobulinemia, primarily resulting from increased antibody production, and hypoalbuminemia, attributed to chronic inflammation, as well as renal and hepatic failure. These alterations lead to a decreased albumin/globulin ratio and hyperproteinemia. Moreover, in cases of severe CanL, there are alterations in hematological parameters, including severe anemia and leukopenia, associated with lymphopenia, eosinopenia, and monocytopenia. Immune-mediated thrombocytopenia is also a factor, contributing to instances of bleeding such as epistaxis, hematuria, etc [55].

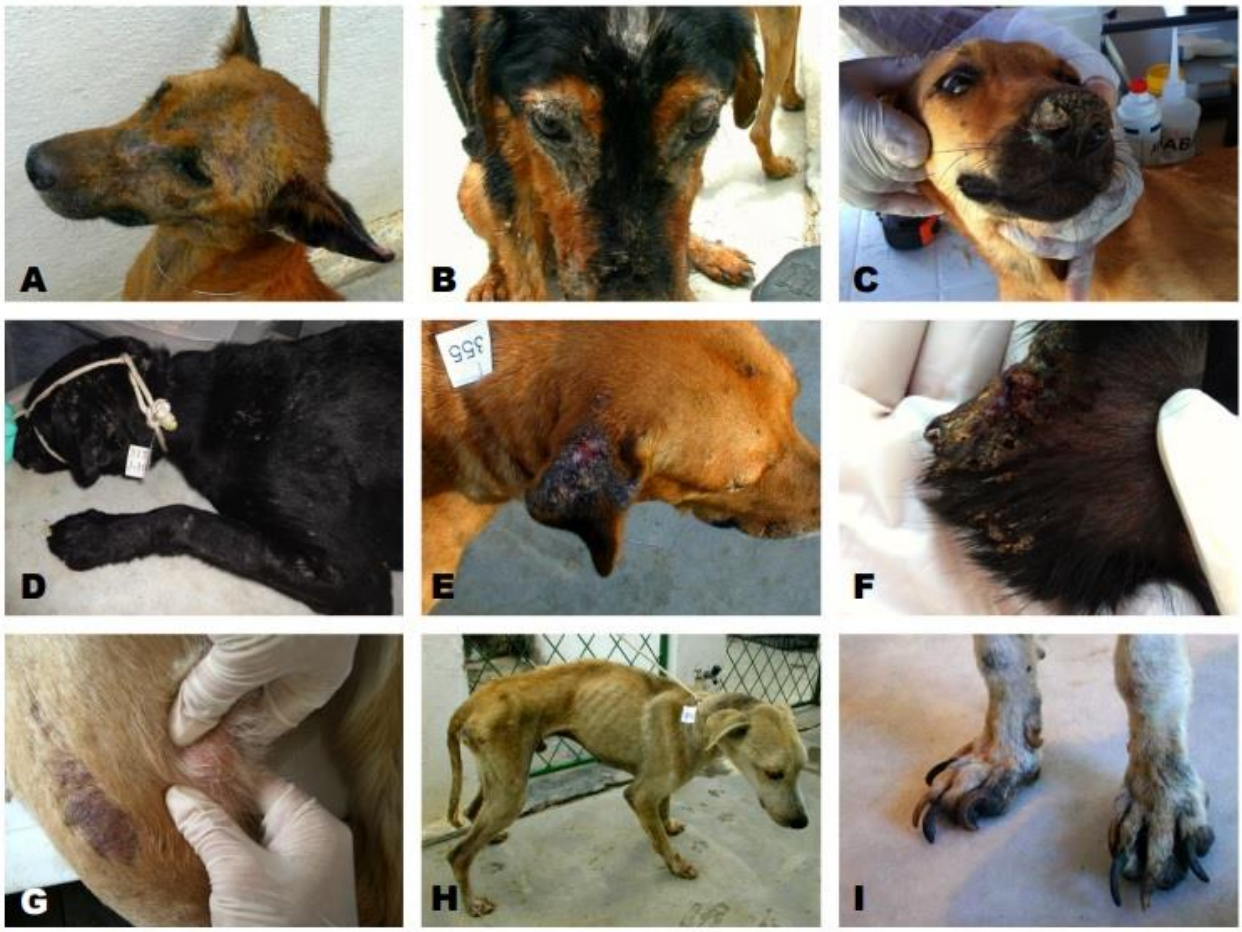


Figure 2.10: Major clinical signs associated with CanL. A: alopecia on the muzzle, B: periocular dermatitis with keratoconjunctivitis and hyperkeratosis; C: hyperkeratosis of the nasal mucosa; D: generalized non-pruritic exfoliative dermatitis; E: ulcerated lesions in the ear; F: crust with vascular injury on the tip of the ear; G: lymphadenomegaly of the popliteal lymph node; H: cachexia; I: onychogryphosis [55].

2.7.2. Microscopic lesions

The immune response generated after *L. infantum* infection can vary among hosts, determining the disease's severity. After the sandfly bite introduces the parasite, the host's macrophages lead the *L. infantum* antigen to undifferentiated Th0 lymphocytes, initiating two possible types of responses. One involves the activation of Th1 lymphocytes, initiating a cellular response characterized by the synthesis of free oxygen radicals and the destruction of the parasite by macrophages. Key cytokines in this pathway include interleukin 2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ). This response makes it possible to control the infection, and the host remains asymptomatic. Studies have indicated that animals with low parasite loads and no clinical signs have higher levels of IFN-γ compared to sick dogs. The second response is the pathway mediated by Th2-type lymphocytes, stimulating humoral immunity through the stimulation of interleukins 4, 5, and 10 (IL-4, IL-5, and IL-10), resulting in an excess of antibodies. This humoral response proves ineffective in killing parasites since they reside within macrophages. As a result, the disease progresses, and clinical signs finally appear [56]. A common histopathological observation in dogs with Canine Visceral Leishmaniosis (CVL) is a granulomatous inflammatory reaction associated with the presence of *Leishmania* amastigotes within macrophages. These dogs often experience renal involvement due to the deposition of immunocomplexes in the basal membranes of the renal parenchyma, a consequence of persistent infection associated with prolonged antigenemia. Both asymptomatic and symptomatic dogs may exhibit varying degrees of renal lesions, as some studies indicate that nearly 100% of dogs with CVL have micro or macroscopic renal lesions. Clinical signs of renal dysfunction, however, only become apparent with significant tissue damage. CVL is typically associated with hyperimmunoglobulinemia, characterized by high IgG levels, circulating immune complexes, and high titers of rheumatoid factor and cryoglobulin. The presence of immunoglobulins in the glomeruli suggests the crucial role of polyclonal B-cell activation and "classical" B-cell activation in the pathogenesis of leishmanial nephritis [57]. Dermatological alterations in CanL include exfoliative, ulcerative, nodular, and pustular dermatitis. Analysis of the extracellular matrix in the skin of symptomatic dogs revealed a decrease in collagen type I and an increase in collagen type III fibers, corresponding to the intensity of skin lesions and tissue destruction. The significant enlargement of lymph nodes in CanL results from an increased number and size of lymphoid follicles, along with notable hypertrophy and hyperplasia of medullary macrophages in the cords and sinuses. The

parasitic load in enlarged lymph nodes often lacks correlation with the type and severity of lesions in other organs. The spleen enlargement in CanL is associated with increased monocyte and macrophage cellularity, alterations in microvascular structure featuring abundant pulp venules and veins, and heightened reticular fibers. Joint and bone abnormalities are commonly found in dogs affected by the infection. Both erosive and nonerosive polyarthritis have been identified, with *Leishmania* amastigotes observed through microscopy in the synovial fluid. Affected bones typically have proliferative lesions in the periosteum and intramedullary regions, often accompanied by cortical and medullary osteolysis. Progressive muscle atrophy is associated with chronic polymyositis, characterized by mononuclear infiltrates containing *Leishmania* amastigotes, neutrophilic vasculitis, and the presence of IgG immune complexes in muscle tissues, along with serum anti-myofiber antibodies. Ocular lesions are present in 16% to 80.5% of dogs with symptomatic CanL. These include anterior uveitis, conjunctivitis, dry keratoconjunctivitis, blepharitis, or a combination of these conditions. In some cases, eye lesions are the sole presenting complaint, accounting for up to 16% of symptomatic cases. In dry keratoconjunctivitis, inflammatory infiltrates surrounding the lacrimal ducts lead to secretory retention and a reduction in tear production. Bleeding from the nose (epistaxis), hematuria, and hemorrhagic diarrhea in CanL are linked to tissue ulceration and disruptions in both primary and secondary hemostasis. Hemostatic disorders observed in CanL involve abnormalities in platelet aggregation, resulting in platelet dysfunction, a reduced platelet number, decreased coagulation factor activities, and fibrinolysis. Anemia is prevalent in most symptomatic dogs, attributed to chronic renal disease or decreased erythropoiesis induced by chronic disease, and it may be aggravated by blood loss or immune-mediated destruction of red blood cells [58]. In addition to the mentioned organs, there is evidence suggesting the involvement of many others. The parasite has the capability to affect mucosal tissues, with observed lesions in the tongue, penis, and oral cavity. Amastigotes produce vascular lesions affecting the small arteries in various organs such as the skin, intestinal tract, kidneys, eyes, lungs etc. Ocular lesions result from the infiltration of plasma cells and macrophages containing amastigotes, leading to damage in different ocular structures. Amastigotes are also detected in muscle cells (myofibers), accompanied by deposits of IgG, inducing necrosis and atrophy of muscular tissue. Meningitis caused by *L. infantum* has been documented, with antibodies against the parasite detected in the cerebrospinal fluid [59].

2.8. Diagnostic methods

In recent years, there have been significant changes in *Leishmania* diagnostics, especially in Europe. While many endemic regions still consider microscopy as the gold standard method, central European laboratories, with limited experience in *Leishmania* microscopy, mostly prefer molecular techniques for diagnosis. This approach offers the advantage of requiring less invasive sampling, with usually high sensitivity. Additionally, molecular tests have the potential to identify the *Leishmania* species and strain, a task traditionally accomplished through multilocus enzyme electrophoresis (zymodemes), which requires prior parasite isolation and culture. The diagnostic methods used for both humans and animals are generally similar, although specific guidelines exist for the diagnosis and practical management of canine leishmaniosis (Figure 2.11) [60]. Valid diagnostic tests are crucial for the detection of *Leishmania* infection in dogs, even though they may not achieve 100% specificity and sensitivity. Each diagnostic method comes with its own set of advantages and disadvantages. Canine leishmaniosis diagnosis often involves identifying specific serum antibodies (IgG), preferably through quantitative serological techniques such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody test (IFAT). While immunochromatography-based assays offer convenient and quick qualitative results on-site, their performance is still not optimal. It is essential to submit samples to a laboratory equipped with quantitative serological assays capable of providing an endpoint titer (IFAT) or an optical density reading (ELISA), along with a classification of antibody levels. The sensitive and specific diagnosis of infection can be achieved by detecting *Leishmania* DNA in tissues by PCR [5]. Nevertheless, the most commonly used methods for detecting the parasites involve PCR and real-time quantitative polymerase chain reaction (qPCR). The qPCR method can detect and measure the parasitic load during treatment, evaluate potential therapeutic shortcomings, and monitor relapse cases. It can support the screening of asymptomatic individuals, given that the maintenance of the disease involves the circulation in anthropozoonotic reservoirs, such as domestic animals and humans. Consequently, this molecular tool is essential for both the diagnosis and advancing our comprehension of epidemiology in regions where the disease is endemic. Early diagnosis is very important to direct the most appropriate treatment, preventing the deterioration and progression of the clinical condition, and thereby reducing mortality [61].

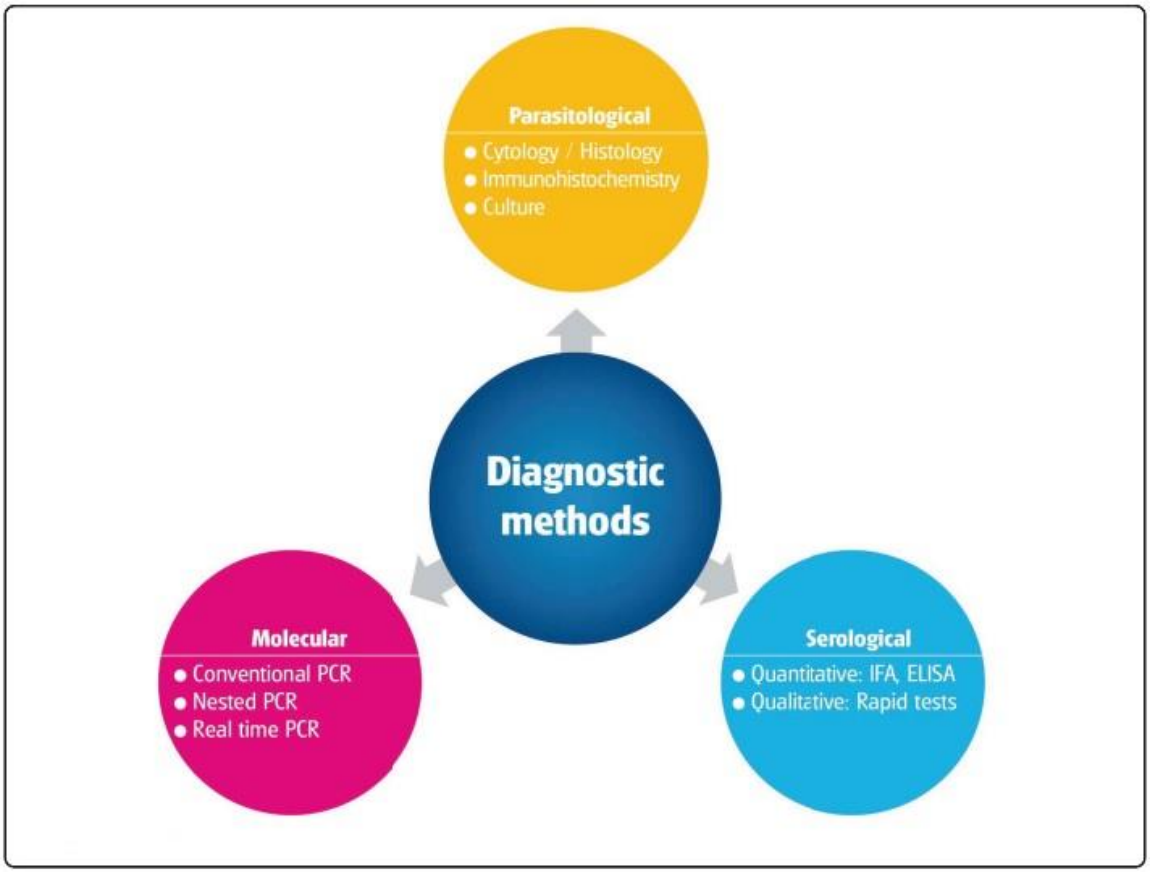


Figure 2.11: The most common diagnostic methods for CanL [5].

2.8.1. Clinical examination

Clinical signs appear after a widely variable incubation period, typically occurring between three months and a year after infection. Consequently, dogs returning from areas endemic to *Leishmania* may exhibit clinical signs of the infection several months, and sometimes several years after their visit. Leishmaniosis presents with a diverse range of clinical manifestations, causing a variety of clinical signs in both general and cutaneous forms. The appearance of any single sign should raise concerns about the disease, especially in enzootic regions. The intensity and duration of clinical signs can vary, with some being more or less pronounced and exhibiting differing timelines for development [62].

According to Beugnet et al. [62] common clinical indicators include:

• Character change: This is a relatively consistent sign frequently observed and reported by owners. Dogs may display apathy, reduced playfulness, and signs of depression. In severe cases, this state can escalate to torpor. Additionally, there is a decrease in appetite.

• Muscle atrophy, specifically amyotrophy, is evident in dogs, impacting the head initially, particularly the temporal and jaw muscles. This results in the deepening of the temporal fossae, giving the animal a distinctive appearance reminiscent of an "old dog's head." Subsequently, there is a noticeable thinning of the limbs and prominence of the hips.

• Weight loss is concurrent with muscle wasting, leading the dog to look like a sad and old dog.

• Irregular hyperthermia is observed in young dogs, specifically those under the age of 2 years.

• Blood and biochemical changes typically include anemia, leukopenia, and thrombocytopenia. Leukopenia is often accompanied by monocytosis, and an increase in hyperproteinemia becomes apparent. Globulins increase, leading to a reversal of the albumin/globulin ratio from 1 to 0.3–0.1.

• Hair loss is characterized by diffuse alopecia and thinning of the coat, without distinctly localized baldness, and never nummular. The loss of hair is more conspicuous on the limbs and head, including the area around the eyes, ears, and tail.

• Onychogryphosis, a result of *Leishmania* multiplying in the claw matrix, sometimes causes a characteristic sign marked by continuously and rapidly growing claws. This phenomenon is associated with the presence of infected macrophages in the inguinal matrix and chronic inflammation, which stimulates the growth of the horn.

• Keratogenic disorders involve significant scaling, characterized by a lot of large and bright scales. This dandruff quickly reappears upon grooming the dog. Hyperkeratosis may accompany this issue, leading to the thickening of the epidermis and pigmentation (melanosis), resulting in a pleated and graying appearance, typically on the nose and ears before spreading to other areas.

• Ulcers manifest as a consequence of mucosal damage, resulting in the discharge of a serous fluid rich in *Leishmania*. In some instances, ulcers may temporarily heal. The ulcers are commonly found in the inner ear (corresponding to the primary site of sand fly inoculation), nose, paw pads

(causing intense pain and reflex lameness), pituitary mucosa (leading to nosebleeds and epistaxis, indicative of leishmaniosis in endemic areas), oral mucosa, digestive mucosa, etc.

• Subcutaneous nodules are the result of the proliferation of macrophagic cell lines in the dermis that may form nodules, reaching several centimeters in diameter. These nodules are palpable and painless. Certain breeds, like Boxers, appear to be more susceptible to this type of nodules.

Other clinical signs may manifest with differing frequencies. This is exemplified by ocular manifestations such as blue keratitis, conjunctivitis, anterior uveitis, and chorioretinitis. Motor and sensory nervous disorders have also been documented. The abundance of immune complexes largely accounts for most clinical disorders, including the gradual onset of chronic renal failure due to glomerulonephritis and polyarthritis. Leishmaniosis is a chronic disease, and animals can sustain a satisfactory condition for several months, but it typically advances to cachexia and eventual death. Treatment does not eliminate or clear the parasite from the host, making relapses possible. The progression of the disease can be accelerated by the development of polyarthritis or immune-mediated glomerulonephritis. The diagnosis of leishmaniosis relies on differential considerations, primarily drawing from epidemiological and clinical factors [62].

2.8.2. Parasitological methods

Parasitological methods, relying on either direct microscopic observation or parasite culture, are considered gold standard methods of diagnosing the disease due to their 100% specificity. However, their sensitivity varies significantly, and they are dependent on obtaining biological samples through invasive procedures such as aspiration biopsy of the spleen, bone marrow, or lymph nodes. That makes them risky and impractical for field use [63]. Fine-needle aspiration cytology (FNAC) is an economical and reliable diagnostic approach for demonstrating the presence of *L. infantum* amastigotes in dogs showing clinical signs or laboratory abnormalities indicative of CanL. This method, performed on mucocutaneous lesions or enlarged lymph nodes, is easy and cost-effective. However, it's important to note that obtaining sufficient lymphoid tissue material may be challenging when palpable nodes are not enlarged. Additionally, lymph node enlargement may not always be an evident clinical sign, becoming noticeable only several months after *L. infantum* infection. While bone marrow FNAC is considered one of the most sensitive

techniques for a reliable diagnosis of CanL, it is not without complications. The procedure can potentially cause pain, hemorrhage, and infection. In comparison, histology offers the presence of *L. infantum* along with additional information on the cytoarchitectural pattern of the lesions. However, it is more expensive and time-consuming, and identifying amastigotes may be more challenging than in cytologic samples. Parasite culture and xenodiagnoses are challenging to apply routinely. These methods are impractical and restricted to specialized reference centers [64].

2.8.3. Serological methods

The primary challenge in assessing diagnostic tests for CanL is the absence of a definitive diagnostic reference test or gold standard against which alternative diagnostic assays can be compared. No diagnostic test kit guarantees 100% sensitivity and 100% specificity for detecting *L. infantum* infection. As a result, it is crucial to be aware of the terms and limitations associated with each diagnostic test and carefully choose the most suitable tests for the specific diagnostic purpose [65]. Serological tests play a crucial role in diagnosing leishmaniosis. Considering that a high number of infected dogs are without evident clinical signs, the detection of anti-*Leishmania* antibodies serves as the first indicator of infection. High antibody titers can be predictive of the potential development of clinical disease. However, it's important to note that low antibody titers are not necessarily indicative of the disease, as they could result from cross-reactivity with American trypanosomes or *Ehrlichia* spp. There are both qualitative and quantitative serological methods available for diagnosis. Qualitative methods, such as immunochromatographic strips, and quantitative methods, like immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), each have their advantages and disadvantages in terms of clinical usefulness [66]. For the diagnosis of CanL, it is recommended to use an indirect immunofluorescence assay (IFA) associated with either the Montenegro skin test (MST) or a parasitological technique to establish a differential diagnosis. However, a limitation of IFA is its inability to correlate the levels of circulating antibodies with the staging of the disease. The majority of immunological techniques for detecting anti-*Leishmania* antibodies have relied on reactions such as ELISA. The sensitivity and specificity of ELISA depend on the antigen used. In this context, various antigens with different molecular weights have been identified for potential use in diagnosis. One notably important and widely used antigen is the recombinant *Leishmania*

protein K39 (rK39), which has demonstrated 100% specificity and 96% sensitivity in the diagnosis of VL [67]. Among quantitative methods, IFAT stands out as the most commonly used method. However, quantitative ELISAs are progressively becoming preferred as the serological methods of choice for diagnosing *Leishmania* infection in both the Old and New World. This preference is attributed to their improved sensitivity and specificity compared to IFAT. Additionally, a recently modified direct agglutination test (DAT) has been validated as a sensitive and specific tool, proving particularly valuable for large-scale epidemiological and ecological studies [66].

2.8.4. Molecular methods

Molecular techniques can be easily applied to various biological samples. In addition to specifically injured tissues, samples with the highest chance of containing leishmanial DNA include, in descending order of sensitivity, bone marrow or lymph nodes, skin, conjunctiva, buffy coat, and whole peripherally obtained blood. However, in dogs with efficient immune responses against *Leishmania*, infection may not result in parasite dissemination. Therefore, in regions endemic to the disease, positive PCR assay results for skin samples without apparent lesions may not necessarily indicate established infection or the development of disease. Similarly, positive PCR assay results for bone marrow samples obtained during or shortly after a dog's exposure to the organism may be followed by a return to negative values. For PCR analyses, fresh or frozen samples are preferred, and samples fixed in 95% ethyl alcohol are also suitable. In contrast, formalin-fixed, paraffin-embedded samples are less effective as diagnostic specimens. It is advisable to consider requesting a PCR test when cytologic and histologic evaluations produce negative results despite compelling clinical evidence of leishmaniosis [68]. PCR methods are accessible for diagnosing and/or identifying *Leishmania* in various human and animal samples. PCR is particularly sensitive, especially when targeting "multi-copy" genomic sequences, which are abundantly present in every individual parasite, such as the kinetoplast minicircles DNA. According to WOAH [69], the three primary techniques commonly used are:

i) Conventional or traditional PCR: In this method, *Leishmania* DNA is amplified using a pair of primers, which are complementary base sequences to the target sequence contained in *Leishmania* DNA;

ii) Nested PCR: This technique is a modification of traditional PCR, and is more sensitive but less specific. Increasing the number of steps tends to elevate the risk of contamination by foreign DNA, leading to a higher likelihood of false positive results.

iii) Real-time PCR: This method involves the use of fluorescent molecules or probes, allowing the quantification of the number of DNA copies present in a biological sample. Real-time PCR exhibits a sensitivity similar to nested PCR. However, when performed with 'closed' systems, it is more specific, as the sample undergoes fewer manipulations, reducing the risk of contamination. Real-time PCR can also provide valuable information during the monitoring phase, such as the number of parasites.

Studies have indicated the potential of loop-mediated isothermal amplification (LAMP) as a point-of-care diagnostic method for CanL, particularly in resource-limited endemic areas [69].

2.9. Treatment of canine leishmaniosis

Managing leishmaniosis in dogs through drug treatment poses a difficulty for veterinary professionals. The infection can progress with varying results, from an asymptomatic phase to diverse clinical stages characterized by a wide range of clinical signs and alterations in clinical pathology [70]. The immune response plays a crucial role in the progression, outcome, and treatment response of *Leishmania* infection in dogs. All known drugs used to combat *Leishmania* in dogs can bring about temporary or permanent relief from clinical signs, but none are capable of completely eradicating the infection. It's worth noting that these anti-*Leishmania* drugs currently used in dogs were initially developed for treating leishmaniasis in humans. Many therapeutic protocols were formulated based on human clinical studies and later adapted for use in dogs, often lacking sufficient pharmacokinetic information specific to dogs. In the case of dogs, the primary objectives of anti-*Leishmania* treatment typically involve reducing the overall parasite load, restoring effective immune responses, treating organ damage caused by the parasite, maintaining drug-induced clinical improvement, and managing clinical relapses [71]. The most frequently used drugs and current therapeutic protocols for treating CanL are listed in Table 2.2. [72] In Europe, meglumine antimoniate, aminosidine, and miltefosine are licensed drugs specifically intended for CanL treatment. The combination of meglumine antimoniate with allopurinol is recognized as the

most effective therapy and serves as the primary protocol against the disease. However, numerous therapeutic protocols are suggested, in terms of dosage, dose interval, and treatment duration. The pharmacokinetics of antimonials can vary considerably in dogs with naturally occurring CanL, and the presence of renal failure may prolong their half-life. Consequently, the risk of toxicity may be elevated in dogs with a reduced glomerular filtration rate. Miltefosine has recently been proposed as an alternative treatment for CanL, in combination with allopurinol, replacing the traditional meglumine antimoniate and allopurinol combination. Amphotericin B has demonstrated good efficacy in various clinical trials; however, this drug has significant disadvantages such as intravenous administration and nephrotoxicity, especially considering CanL adverse impact on the kidneys. Aminosidine, presents severe side effects like nephrotoxicity and ototoxicity, discouraging its use as a primary therapy for CanL. Beyond these drugs, several potential medications against CanL have been studied in vitro or in laboratory animals, including pentamidine, ketoconazole, aminosidine, metronidazole, spiramycin, and marbofloxacin, but controlled clinical trials with naturally infected dogs are needed to validate their therapeutic efficacy [72].

Table 2.2: Current treatment protocols applied in canine leishmaniosis [72].

Protocol	**Drugs and dosages**	**Main side effects**
1st line	N-methylglucamine antimoniate[a] (75–100 mg/kg/SID) for 4–8 weeks, S.C. + allopurinol (10 mg/kg/BID) for at least 6–12 months P.O.	Potential nephrotoxicity and cutaneous abcesses/cellulitis (N-methylglucamine antimoniate) Xantine urolithiasis (allopurinol)
2nd line	Miltefosine[a] (2 mg/kg/SID) for 4 weeks P.O. + allopurinol (10 mg/kg/BID) for at least 6–12 months P.O. Allopurinol (10 mg/kg/BID) for at least 6–12 months P.O.	Vomiting, diarrhea (Miltefosine) Xantine urolithiasis
3rd line	Amphotericin B[b] (0.5–0.8 mg/kg, I.V/SID/twice per week) for 2 months Liposomal amphotericin B[b] (3 mg/kg/SID) for 5 consecutive days, I.V. Metronidazole (25 mg/kg/SID) + spiramycin (150,000 U/SID) for 3 months P.O. Marbofloxacin (2 mg/kg/SID for 1-month P.O.)	Nephrotoxicity Transient nephrotoxicity Not described Not described

[a] Registered for veterinary use in Europe.

[b] 1st line drug for Human Visceral Leishmaniasis (HVL) in EU; not recommended for veterinary use, to avoid drug parasite resistance.

Treatment with antileishmanial drugs, particularly the meglumine antimoniate/allopurinol combination or allopurinol alone, often results in clinical improvement. However, some dogs that initially respond well to therapy may experience a recurrence of clinical symptoms either after the treatment is discontinued or during its course, indicating that the infection might not have been completely eradicated. Additionally, achieving a parasitological cure is uncommon, and even treated dogs on a prolonged allopurinol regimen may still harbor the parasite and remain infectious to sand flies, although to a lesser extent. Therefore, early detection of the disease is crucial for the patient. Consequently, it is recommended to regularly screen dogs residing in endemic areas for *Leishmania* antibodies, at least every 6–12 months, to facilitate prompt initiation of both therapeutic and preventive measures [72].

2.10. Prevention from canine leishmaniosis

Preventive measures against *L. infantum* infection have expanded in recent decades. However, there are primarily two approaches for preventing this infection: (i) using physical barriers and insecticides against the vector, and (ii) using immunoprophylaxis. In terms of vector prevention, it is advisable to avoid outdoor activities during dawn and dusk, periods when the vectors are highly active. Additionally, using fine mesh nets in windows and applying topical insecticides, such as synthetic pyrethroid-based compounds, is recommended due to their repellent and anti-feeding effects. Topical insecticides are available in various forms, including impregnated collars, spot-on treatments, and sprays, each with distinct onset and maximum [73]. Controlling immature sand flies in the environment is impractical due to the highly variable microhabitats of larvae and pupae, such as tree roots, animal burrows, decaying foliage, and tree holes. Likewise, evidence suggests that spatial fogging for adult sand fly control is ineffective, and the short residual effect of house wall spraying makes this method impractical and inefficient, especially in rural areas. In regions where *L. infantum* is prevalent, the majority of dogs and people exposed to sand flies may encounter the parasite but remain asymptomatic. Using repellents like synthetic pyrethroids has emerged as the most efficient method for preventing *L. infantum* infection in dogs. These repellents exert a toxic and irritating impact on sand flies, inducing insect disorientation and prompt abandonment of the host, ultimately leading to the insect's death shortly after landing on the treated animal's coat. As a result, blood feeding is typically prevented, and infection is avoided. The

duration of the synthetic pyrethroids effect in spot-on formulations or collars can range from approximately 1 to 8 months, respectively [74]. As stated by WHO [19], vaccination is probably the most effective strategy for managing a vector-borne disease such as leishmaniosis. While vaccines for CanL have undergone trials and obtained licenses in Brazil and Europe, a globally available and efficacious vaccine is still lacking [19]. Four vaccines have been developed or are in use to prevent CanL: Leishmune® and Leish-Tec® in Brazil, and CaniLeish® and LetiFend® in Europe. Leishmune® (Fort Dodge Wyeth, later Zoetis, Brazil) became the first officially licensed CanL vaccine, receiving approval in Brazil in 2004. Classified as a second-generation vaccine, it comprises the fucose-mannose ligand (FML) of *L. donovani* and a saponin adjuvant. The vaccination protocol involves three subcutaneous doses administered every 21 days to dogs aged four months or older, followed by annual boosters. Leish-Tec® (Hertape Calier Saude Animal, later Ceva, Brazil) is formulated with a recombinant protein A2 from *L. donovani* amastigotes and saponin as a vaccine adjuvant. Licensed in Brazil in 2007, it is currently the only authorized CanL vaccine in the country. The vaccination process entails administering three doses subcutaneously at 21-day intervals for dogs aged four months or older, followed by annual boosters. CaniLeish® vaccine (Virbac, France), was introduced to the European market in 2011. The vaccine is made up of purified excreted–secreted proteins of *L. infantum* (LiESP) and is adjuvanted with a purified fraction of the Quilaja saponaria saponin (QA-21). The recommended vaccination protocol involves administering one subcutaneous dose to dogs older than six months, repeated every 21 days for a total of three doses, followed by annual single-dose boosters. LetiFend® (Laboratorios LETI, Spain) received European licensure in February 2016. This recombinant vaccine contains a chimerical protein (protein Q) formed by combining five antigenic fragments from four distinct *L. infantum* proteins (ribosomal proteins LiP2a, LiP2b, and LiP0, and the histone H2A), with no added adjuvant. The vaccination protocol consists of one initial vaccine dose, followed by annual boosters, and it is specifically recommended for dogs aged six months or older [75]. Considering the ample tolerance and safety margins exhibited by the insecticides and vaccines currently available, it is possible to provide targeted recommendations for optimizing control to achieve the desired repellent effect in specific local contexts when determining the most effective approach for *Leishmania* control in dogs. It is essential to view control measures not in isolation but as integral components of a comprehensive prevention program for leishmaniosis in dogs, whether they are healthy, clinically healthy despite being infected, or infected and sick [76].

2.11. One-health approach

According to the World Health Organization (WHO), leishmaniasis is categorized as a neglected tropical disease, as it affects public health and is concealed negligence in the development of an adequate and affordable cure. With millions of cases reported annually worldwide, leishmaniasis remains a prevalent and pervasive disease [77]. Globally, leishmaniasis affects nearly 12 million people, with an annual incidence of over 58,000 cases of VL and more than 220,000 cases of CL. It ranks as the ninth largest burden among infectious diseases. The infection is prevalent in approximately 90 countries across tropical, subtropical regions, and southern Europe [78]. Around 350 million individuals globally face the risk of infection. In regions where the disease is prevalent, numerous natural reservoirs of the parasites exist. Dogs are the primary reservoir for human infections [79]. Canine leishmaniosis has been documented in 50 out of almost 90 countries where human leishmaniasis is prevalent, with the Mediterranean basin standing out as one of the most heavily affected regions. In southwestern Europe alone, a minimum of 2.5 million dogs are infected with *L. infantum*, along with various species of sylvatic hosts [80]. Over the years, millions of dogs have been killed as part of government policies aimed at controlling human VL caused by *L. infantum*, also known as zoonotic VL. Some Central Asian, Caucasian, and some Balkan countries still adhere to national public health policies recommending the culling of any *L. infantum*–positive dog. In rural regions of China, the Maghreb countries (North Africa), and parts of the Middle East, dog culling persists as a common practice, although medical therapy is usually allowed for owned dogs. In Central and South America, several countries, including Argentina, Brazil, Colombia, Uruguay and Venezuela, have historically advocated and implemented dog culling. However, more effective approaches have gradually replaced this practice, even in countries like Brazil, where thousands of dogs used to be culled annually [81]. The World Health Organization recommends several measures for leishmaniasis control in humans, including treating the human disease, culling wild and feral dogs that test positive for the infection, and using insecticidal treatments in human homes, such as pyrethroid sprays. The effectiveness of culling seropositive or infected dogs is a matter of controversy. Concerns have been raised regarding the ethical implications of euthanizing pet dogs, delays between detecting seropositive dogs and implementing culling, and the continued presence of other subclinically or clinically infected canine and wildlife reservoirs. Vaccinating dogs may prove to be more effective than culling seropositive dogs and could address ethical concerns associated with culling [20].

3. RESEARCH SUBJECT AND OBJECTIVES

The research subject of this doctoral thesis is canine leishmaniosis in Kosovo, the competent vectors from the genus *Phlebotomus*, and the causative agent in the competent vector. For this purpose, the epidemiology of CanL was assessed by a serological survey of dogs in all seven districts of Kosovo. Identification of the vectors (*Phlebotomus* spp.) and the causative agent in the vectors led to the conclusion of the role of the competent vectors toward the spreading of the CanL in Kosovo. Finally, the risk of spreading leishmaniosis among dogs and the human population was assessed.

The main objectives of this doctoral dissertation were:

- To perform a serological survey of CanL in Kosovo.
- To perform an epidemiological analysis of CanL in Kosovo.
- To identify the *Phlebotomus* spp. present in Kosovo.
- To detect the *Leishmania* spp. in *Phlebotomus* spp. by molecular techniques.
- To determine which species of the genus *Leishmania* are circulating in Kosovo
- To identify the competent vector of CanL in Kosovo
- To assess the role of *Phlebotomus* spp. in spreading CanL in Kosovo
- To perform a risk assessment for Public Health.

4. MATERIALS AND METHODS

4.1. Study area

The study was conducted in the Republic of Kosovo, a landlocked country located in the center of the Balkan Peninsula in Southeastern Europe. It lies between latitudes 41 and 43° N and longitudes 20 and 22° E. Kosovo has a total land area of 10,900 km² and an estimated 1.8 million people [82]. Kosovo is divided into seven districts: Prishtina (01), Mitrovica (02), Peja (03), Prizreni (04), Ferizaj (05), Gjilani (06), and Gjakova (07) (Figure 4.1). The study area has a continental climate with Mediterranean and Alpine influences. There is no available data regarding the number of dogs in Kosovo. The dogs are either stray, kept in private households, or used as shepherd dogs. In the countryside, various agricultural activities include farming various animals such as cattle, sheep, goats, poultry, etc. Agriculture is widely present, with 53 % of Kosovo's land dedicated to agricultural use, 44 % as forest land, 1 % as water surface, and 5 % as other surfaces (e.g., roads, urban, and other land) [83].

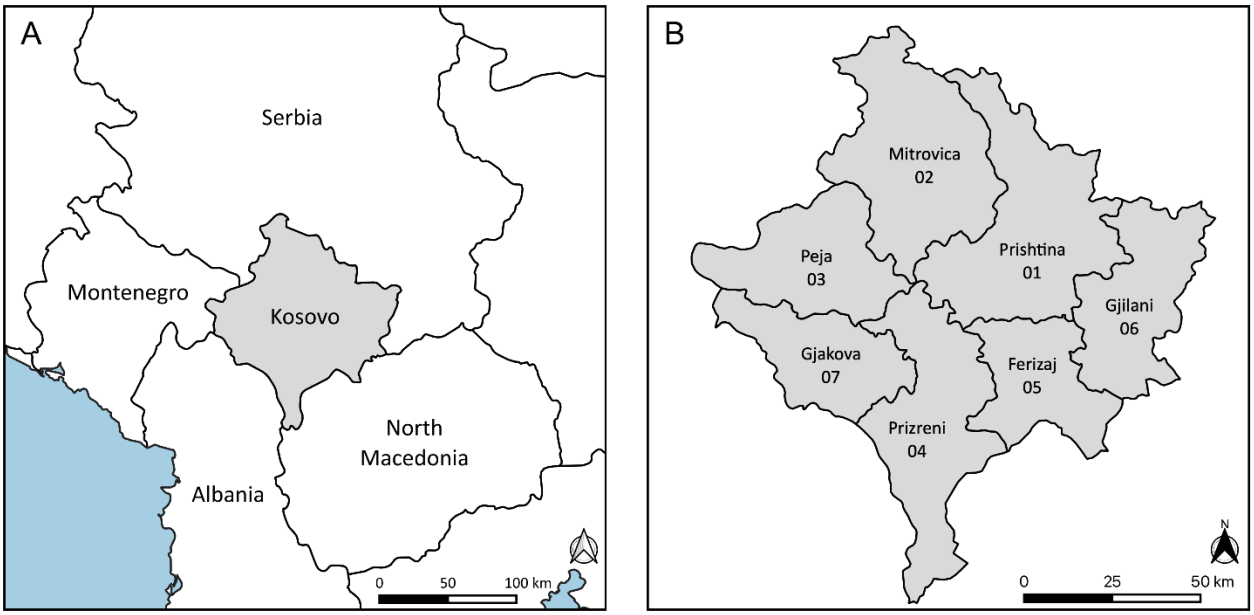


Figure 4.1: Map of Kosovo. The geographic position of Kosovo in the Balkan (a), seven districts of Kosovo (b). The Mediterranean Sea is shown in blue on the left map.

4.2. Selection and target group of the study

Dog samples were collected through a randomized sampling approach, encompassing dogs from private households, stray dogs (housed in shelters), and shepherd dogs. A total of 285 samples were collected from dogs across all seven districts of Kosovo, with 35-50 samples per district, over a period of 1 year from summer 2021 to spring 2022. All samples were collected following the basic ethical principles and were labeled with the dog's name or chip number, location, age, breed, sex, and health status (Figure 4.2).

Vector samples were collected from July to September 2022 in all seven districts of Kosovo. A comprehensive survey covered a total of 114 different locations, resulting in 323 trap nights. The number of collection sites ranged between 15-20 per district. Sampling locations were selected randomly, targeting various sites such as dog shelters, cow farms, chicken farms, goat farms, sheep farms, and pigeon farms. Detailed data including coordinates, farm type, and trap placement (outdoor and indoor) were consistently recorded (Figure 4.2).

4.3. Sampling and data collection

All samples were collected in accordance with basic ethical principles. Verbal consent was obtained from dog owners, where the importance of the disease and the study's results were explained and reported. Regarding sand fly data, agreements were reached with homeowners to place traps on their properties.

A

B

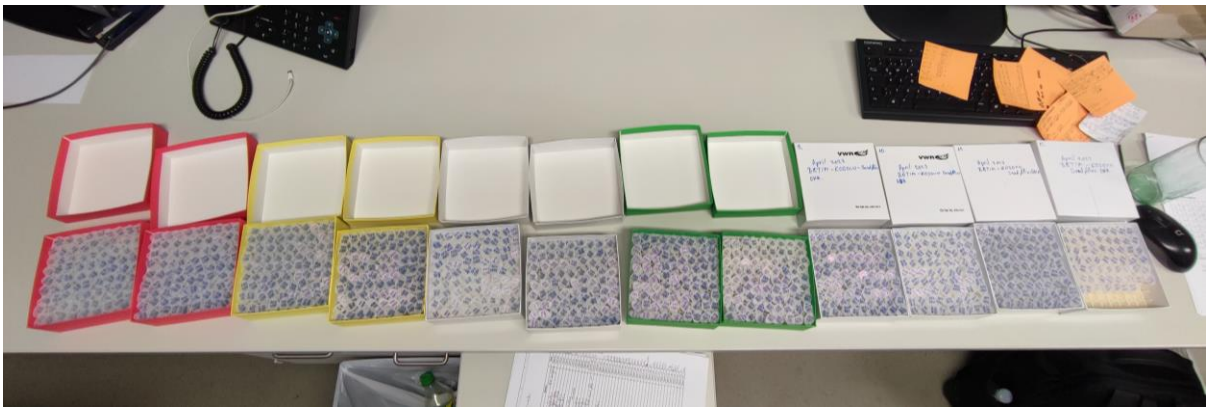


Figure 4.2: Samples included in the study. A) Dog samples, B) Sand fly samples.

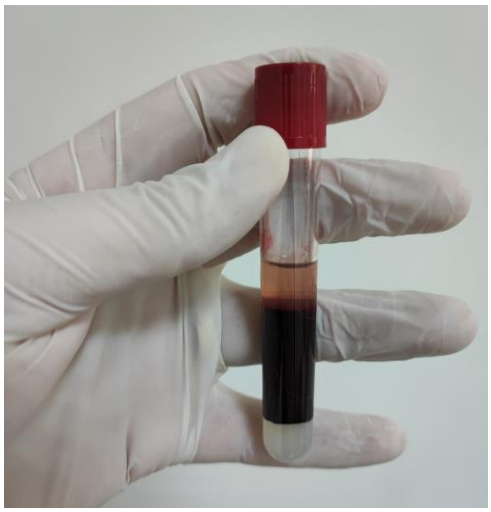
4.3.1. Dog samples

From each dog, 5–7 ml of blood samples was collected from the cephalic vein, as depicted in Figure 4.3. Each sample was labeled with the dog's name or chip number, location, age, breed, sex, and health status. Thirty-five to fifty samples were collected from each district (Prishtina n=50, Mitrovica n=40, Peja n=35, Prizreni n=40, Ferizaj n=40, Gjilani n=40, and Gjakova n=40). Dogs were categorized by gender, resulting in 141 males and 144 females. Based on age, dogs were classified into the following groups: 1–2 years (n=41), 2–3 years (n=64), 3–4 years (n=60), 4–6 years (n=67), 6–8 years (n=23), 8–10 years (n=14), and >10 years (n=16). In terms of health status, dogs were classified as normal (n=245) or disrupted (n=40), including those with random pathologies unrelated to CanL (dermatitis, arthritis, tumor, vasculitis, etc.). Regarding breed status, 176 dogs were identified as a mixed breed, and 109 were classified as pure-breed dogs (refer to

Annex 1). The collected samples were transported from the field to the laboratory under cool conditions and allowed to clot at room temperature. Subsequently, the blood was centrifuged for 5 minutes at 800 rotations per minute (rpm), and the sera were separated, transferred into a microtube (1.5 ml), and stored at −20 °C until further serological examination.

1 

2 

3 

4 

Figure 4.3: Preparation of dog sera.

4.3.2. Entomological sampling

The entomological survey was conducted between July 1st and September 1st, 2022, covering all seven districts of Kosovo. A total of 114 different locations were surveyed using up to three CDC miniature light traps (John W. Hock Company, Gainesville, FL, USA), resulting in 323 trap nights (Figure 4.4). The distribution of collection sites was as follows: 20 in Prishtina, 16 in Mitrovica, 16 in Peja, 15 in Prizreni, 15 in Ferizaj, 16 in Gjilani, and 16 in Gjakova district. Due to restricted funding, sites that tested positive during the first collection were not resampled, whereas some negative dog shelters were resampled. Sampling locations were randomly chosen, focusing on areas such as dog shelters, cow farms, chicken farms, goat farms, sheep farms, and pigeon farms. Relevant data, including coordinates, farm type, and trap placement (outdoor and indoor), were consistently recorded (refer to Annex 2). Traps were operated overnight, set at sunset, and collected before sunrise the next day. Placed one meter above the ground, traps were positioned both outside and inside farms. After collection, the nets were immediately placed on dry ice, transported to the laboratory under cool conditions, and stored at −80 °C until dissection.

Figure 4.4: Sand fly sampling.

4.4. Diagnostic methods for dog samples

All collected dog samples were subjected to testing using two distinct diagnostic methods: a competitive enzyme-linked immunosorbent assay (ELISA), with confirmation achieved through an indirect fluorescent antibody test (IFAT).

4.4.1. ELISA test

The ELISA used in this study was obtained from the company ID Screen® Leishmaniasis Indirect Test (VET-Innovate ID Diagnostics, France). The assay was conducted following the manufacturer's instructions. The diagnostic sensitivity of this kit, as provided by the manufacturer, was 98.54% (95% CI: 94.29–99.75%), and the diagnostic specificity was 99.18% (95% CI: 98.22–99.62%). The optical density (OD) for each sample was read at a wavelength of 450 nm. The results were calculated as a percentage of the positive or negative control, respectively, as indicated by the manufacturer.

General information about the diagnostic kit:

This diagnostic kit was designed for the detection of antibodies directed against *Leishmania infantum*. Wells were coated with *L. infantum* purified antigen prepared according to the OIE manual (OIE Terrestrial Manual 2008, CHP 2.1.8, part B-2-b). Samples were tested, and controls were added to the microwells. In seropositive dogs, anti-*Leishmania* antibodies formed an antibody-antigen complex. An anti-dog IgG-horseradish peroxidase (HRP) conjugate was added to the microwells, binding to the anti-*Leishmania* antibodies and forming an antigen-antibody-conjugate-HRP complex. After washing to eliminate excess conjugate, the substrate solution (TMB) was added. The resulting coloration depended on the number of specific antibodies present in the specimen tested: in the presence of antibodies, a blue coloration appeared, which turned yellow after the stop solution was added. In the absence of antibodies, no coloration appeared. The components of the kit included microplates coated with *L. infantum* purified antigen, concentrated conjugate (10X), positive control, negative control, dilution buffer 2, dilution buffer 3, wash concentrate (20X), substrate solution, and stop solution (Figure 4.5).

Figure 4.5: The ELISA kit used in the study.

Before starting the testing procedures, all reagents were brought into the laboratory and allowed to reach room temperature (21°C ± 5°C) before use. Subsequently, all reagents were homogenized using a vortex, and reagents requiring dilution were prepared. The Wash Solution (1X) was prepared by diluting the Wash Concentrate (20X) to 1/20 in distilled/deionized water. Additionally, the conjugate 1X was prepared before the administration procedure in accordance with the protocol, by diluting the Concentrated Conjugate 10X to 1/10 in Dilution Buffer 3.

<u>Testing procedure:</u>

1. The first step was to add:

- 190 µl of Dilution Buffer 2 to each microwell.

- 10 µl of the Negative Control to wells A1 and B1.

- 10 µl of the Positive Control to wells C1 and D1.

- 10 µl of each tested sample to the remaining wells.

2. The plate was covered and incubated 45 min ± 5 min at 37°C (± 2°C).

3. The wells were emptied, and each well was washed 3 times with at least 300 µl of the Wash Solution, taking care to avoid the wells from drying between washes.

4. Conjugate 1X was prepared.

5. A 100 µl of the Conjugate 1X was added to each well.

6. The plate was covered and incubated for 30 min ± 3 min at 37°C (± 2°C).

7. The washing step was repeated as described in step 3.

8. A 100 µl of the Substrate Solution was added to each well.

9. The plate was covered and incubated 15 min ± 2 min at 21°C (± 5°C) in the dark.

10. A 100 µl of the Stop Solution was added to each well, following the same order as in step No. 8, to stop the reaction.

11. The optical density (O.D) was read and recorded at 450 nm.

The validation of the test was made by ensuring that the mean value of the Positive Control O.D.(OD_{PC}) was greater than 0.350. $\mathbf{OD_{PC} > 0.350}$ and that the ratio of the mean values of the Positive and Negative Controls (OD_{PC} and OD_{NC}) was greater than 3. $\mathbf{OD_{PC}/OD_{NC} > 3}$.

The interpretation of results was made by calculating the S/P percentage (S/P%) for each sample:

$$S/P\% = \frac{ODsample - ODNC}{ODPC - ODNC} x100$$

Results less than or equal to 40% were considered negative, those greater than 40% and less than 50% were considered doubtful, and those greater than or equal to 50% were considered positive.

4.4.2. IFAT test

After the ELISA test, all sera were tested using the MegaFLUO® LEISH (MEGACOR Diagnostic GmbH, Austria) (Figure 4.6) test kit for indirect fluorescent antibody detection. This test kit was designed for the semiquantitative immunofluorescence detection of IgG antibodies against *Leishmania infantum* in the plasma or serum of dogs. The testing was conducted at the Faculty of Veterinary Medicine—Skopje in North Macedonia. The diagnostic sensitivity of this kit, as indicated by the manufacturer, was 98.2%, and the diagnostic specificity was 92.3%. Anti-*Leishmania* antibodies were detected using FITC anti-dog IgG conjugate, following the manufacturer's instructions. The sera were classified as positive if promastigote cytoplasmic or membrane fluorescence was observed at a serum dilution of 1:160 or higher.

Figure 4.6: The IFAT kit used in the study.

Each test kit comprised 10 slides coated with *Leishmania infantum*, 1 dropper bottle with 3.0 ml FITC anti-dog IgG conjugate, 1 dropper bottle with 0.5 ml Positive Control, 1 dropper bottle with 0.5 ml Negative Control, and 1 dropper bottle with 3.0 ml Mounting Medium. To perform the test, additional materials were required, including PBS (phosphate buffered saline) with a pH of 7.2–7.4, a washbasin for PBS, test tubes for serum dilutions, microliter pipettes, 24 x 50 mm cover

slips, a fluorescence microscope with a filter system for FITC (fluorescein isothiocyanate, excitation wavelength of 465–495 and a barrier filter of 515–555) and 400× magnification, a 37°C incubator, and a humid chamber.

The test principle involved diluting dog sera in PBS (pH 7.2–7.4) and applying them to the slide wells to facilitate an antigen-antibody reaction at 37 °C in the case of a positive sample. Subsequent washing with PBS was performed to remove non-bound, unspecific serum proteins. In the following step, fluorescein-marked FLUO FITC anti-dog IgG conjugate was added, which binds to the antigen-antibody complexes. After a 30-minute incubation, the non-bound conjugate was washed off with PBS. Finally, the wells were covered with Mounting Medium and a cover slip. Evaluation was carried out using a fluorescence microscope with a filter system for FITC and 400× magnification.

The test procedure (Figure 4.7):

- The test-kit components (excluding the conjugate) and the sera to be tested were brought to room temperature at the time of testing.

- Dilutions with PBS for all sera were prepared appropriately.

- The slides were removed from their foil pouch shortly before use and placed into the humid chamber. Then, 1 drop (20 µl) of the Positive and Negative Control was applied to each slide in separate antigen wells, along with 20 µl of every serum dilution in separate antigen wells.

- Incubation time was set at 30 minutes at 37 °C.

- Washing step: The remaining serum dilutions were gently removed from the slides, and the slides were shaked gently for 5 minutes in PBS. This step was repeated for another 5 minutes with fresh PBS. Afterward, the slides were briefly rinsed with distilled water. Any remaining water was gently removed from the slides, taking care not to allow the antigen wells to dry out.

- The slides were then placed back into the humid chamber, and immediately, 1 drop of FLUO FITC anti-dog IgG conjugate was added to each used well.

- Incubation time was set for 30 minutes at 37 °C in the dark to protect the photosensitive conjugate.

- The washing step was repeated as described above.

- Drops of Mounting Medium were added on the cover slips and carefully placed on the slides.

- Evaluation of the slides was made using a fluorescence microscope at 400× magnification, comparing each well to the fluorescence pattern observed in the Positive and Negative Controls.

- Sealed slides could be stored at 2–8 °C in the dark for up to 7 days.

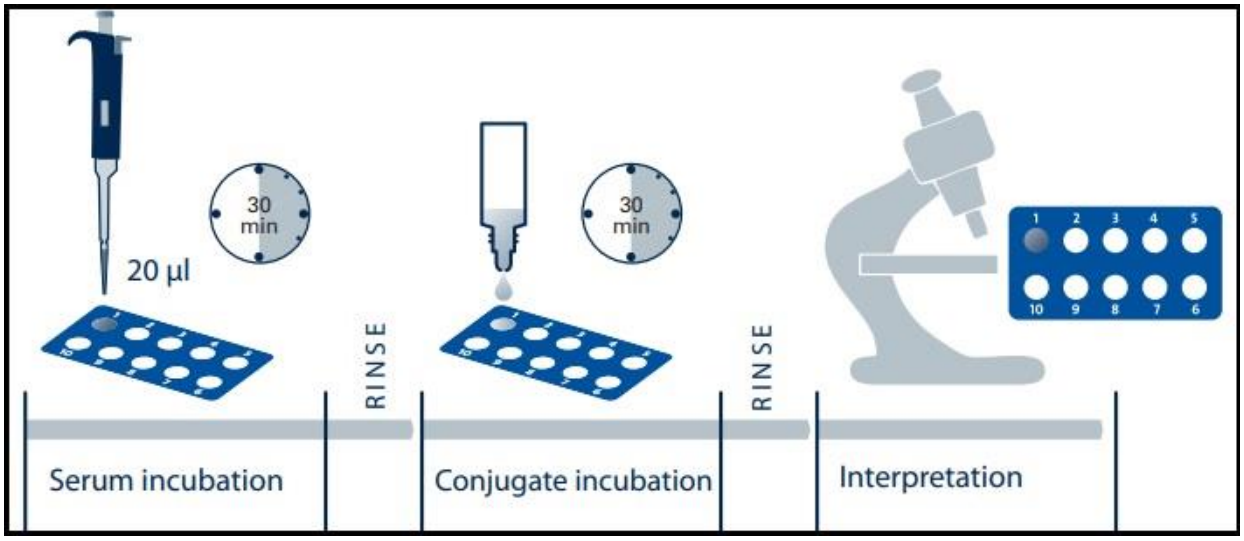


Figure 4.7: Test procedures in IFAT method.

For the test evaluation, a fluorescence microscope with a FITC filter system (excitation wavelength 465–495, barrier filter 515–555) and 400× magnification was necessary. The fluorescence pattern (form, density, etc.) of the Negative and Positive Controls served as a reference pattern. Any reactivity patterns differing from those observed in the controls should be considered non-specific.

A Positive fluorescence pattern was defined as ≥ 1:160, *Leishmania* promastigots exhibited a distinct yellow-green fluorescence in their membrane and flagella area. It was advisable to perform further dilutions of positive samples to establish the endpoint titre (the highest dilution that is still positive).

The cut-off fluorescence pattern or recommended cut-off was set 1:160. *Leishmania* promastigots (membrane, flagella) showed a weak yellow-green fluorescence at this dilution.

A Negative fluorescence pattern was indicated by < 1:160. In this case, *Leishmania* promastigots did not show any yellow-green fluorescence; instead, they appeared greyish-red.

4.5. Diagnostic methods for vector samples

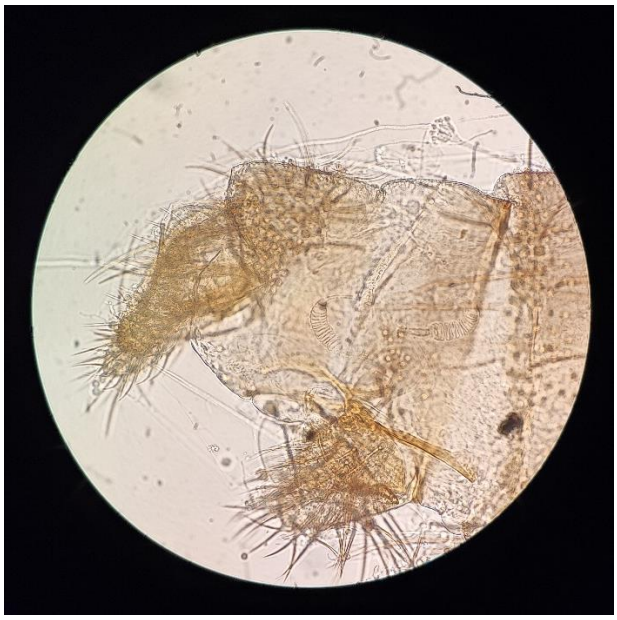
After collection of the sand fly specimens, various methods for morphological and molecular identification, as well as pathogen screening, were applied. Initially, specimens were individually identified based on morphology and confirmed by applying a barcoding PCR targeting a fragment of the cytochrome c oxidase subunit I (COI).

To detect *Leishmania* DNA, all samples were screened with a sensitive nested-PCR protocol, targeting the small subunit ribosomal ribonucleic acid (ssu) rRNA gene. In sand fly specimens that tested positive for *Leishmania* DNA, molecular characterization was performed for further clarification of species boundaries and to determine the discriminatory power of the applied PCRs.

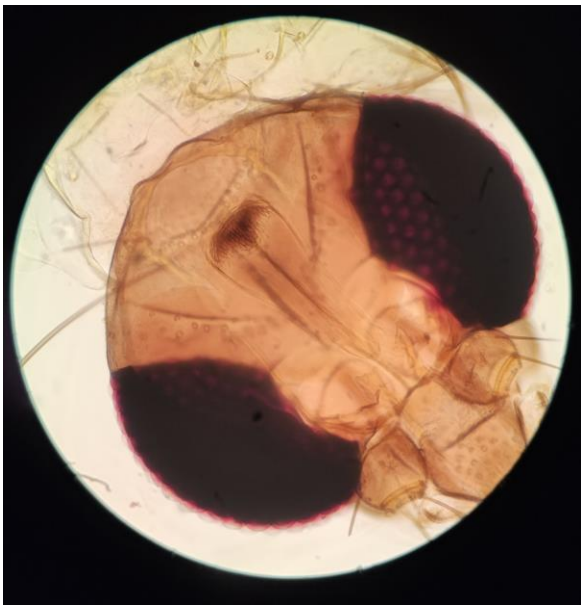
4.5.1. Morphological identification of sand flies

The sand flies were individually identified by morphology, with few exceptions. Bloodfed specimens were exclusively identified through molecular analysis. In locations where more than 100 individuals were collected (specifically, five locations: 02/7, 03/7, 03/8, 04/9, and 07/8), at least 8% of the catch was individually identified, depending on the collection size. Initially, the sex was determined under a stereomicroscope based on the sand flies' genitalia. Subsequently, the head and genitalia of the sand flies were cut with a small sterile needle and placed in CMCP-10 mounting medium (Polysciences, Inc., Warrington, PA, USA). The slide was then covered with a cover glass, marked with a unique number, and left to dry for at least 24 hours. After this period, the morphological identification was performed within 24–48 hours. The identification was conducted under a stereomicroscope using the 10x objective and the 40x objective for accurate distinction (Figure 4.8) and relied on published morphological keys and descriptions of male genitalia, female spermatheca, and pharyngeal armature [84,85]. The remaining body parts were transferred to individual tubes for homogenization and nucleic acid extraction.

Male genitalia 40x



Female spermathecae 40x



Pharyngeal armature 40x

Figure 4.8: Morphologic identification of sand flies based on different parameters.

4.5.2. Molecular analysis by PCR

For nucleic acid extraction, the specimens were homogenized in 500 µL (individual sand flies and pools with up to 15 specimens) or 1000 µL (pools with more than 15 specimens) of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% bovine serum albumin, 1% penicillin/streptomycin, 10 µg/mL gentamicin, and 0.25 µg/mL amphotericin B (all from Gibco, Thermo Fisher Scientific). Two metal beads (3 mm diameter) were added to each 2.0 mL tube and homogenized with a TissueLyser bead mill (QIAGEN GmbH, Hilden, Germany) for 1 minute at 30 Hz. The homogenate was cleared via centrifugation in a 4°C benchtop centrifuge for 5 minutes at 14,000 rpm (rounds per minute). All morphologically identified specimens were homogenized individually. The remaining unidentified specimens (at locations with more than 100 individuals) were sorted by sex, feeding status, and location, and homogenized in pools of up to 30 specimens. For DNA isolation, 200 µL of supernatant was taken from an individual or pooled female, and DNA isolation was performed using the QIAmp® DNeasy Blood and Tissue kit 250 (Qiagen, Hilden, Germany) by strictly following the manufacturer's protocol with final elution in 100 µL. The remaining supernatants were stored at -80°C for prospective RNA-based screenings.

<u>Protocol of DNA isolation in individual specimens (Figure 4.9):</u>

- Two metal beads (3 mm diameter) and 500 µL of Dulbecco's Modified Eagle Medium (DMEM) were added to a 2 mL safe-lock tube containing individual sand fly specimens kept at -80°C. Homogenization was performed with a TissueLyser bead mill (QIAGEN GmbH, Hilden, Germany) for 1 minute of shaking at 30 Hz.

- The homogenate was cleared via centrifugation, and 200 µL of supernatant was taken from each individual sample and placed in a new 2 mL microcentrifuge tube, which was prepared and labeled accordingly.

- Twenty µL of proteinase K and 200 µL of buffer AL were added to the tube containing supernatant and vortexed for 15 seconds (sec) at high speed.

- Incubation of the prepared mixture was done in a Thermomix incubator at 56°C for 10 minutes at 500 rpm.

- After incubation, 200 µL ethanol (96–100%) was added to the tube, vortexed at high speed for 1 minute, followed by centrifugation to clear the tubes for 30 seconds at 4,000 rpm.

- The mixture was pipetted and placed into a DNeasy Mini spin column placed in a 2 mL collection tube and centrifuged for 1 minute at 8,000 rpm. Discard the flow-through and collection tube.

- The spin column was placed in a new 2 mL collection tube, and 500 µL of buffer AW1 was added, and centrifuged for 1 minute at 8,000 rpm. The flow-through was discarded.

- The spin column was placed in a new 2 mL collection tube, and 500 µL of buffer AW2 was added, then centrifuged for 3 minutes at 14,000 rpm. The flow-through was discarded.

- The spin column was placed in a new 2 mL collection tube and centrifuged for 1 minute at 14,000 rpm to clear the contents.

- The spin column was transferred to a new 1.5 mL microcentrifuge tube, and the DNA was eluted by adding 200 µL buffer AE to the center of the spin column membrane, incubated for 1 minute at room temperature (15–25 °C), and centrifuged for 1 minute at 8,000 rpm.

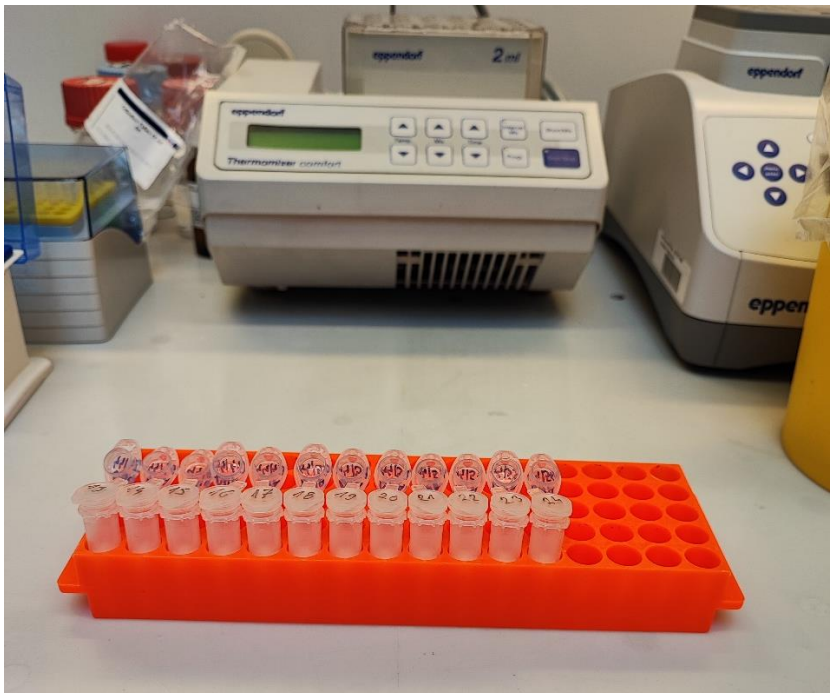


Figure 4.9: Nucleic acid extraction process.

4.5.3. Detection of *Leishmania infantum* DNA

Extracts of all individual and pooled females were screened for the presence of *Leishmania* spp. DNA. Initially, a sensitive nested-PCR protocol targeting the small subunit ribosomal ribonucleic acid (ssu) rRNA gene was applied. For further species typing, two additional PCRs were performed, targeting the internal transcribed spacer 1 (ITS1) gene region and the cysteine protease B (cpb) gene region. Furthermore, all samples positive for *Leishmania* DNA were subjected to analysis using a probe-based qPCR protocol targeting the kinetoplast DNA (kDNA) to obtain CT values. Primers and PCR protocols are provided in Table 4.1.

Table 4.1: PCR-based protocols for the detection of Leishmania DNA were used in this study.

Target	Primer and Probe (5' -3')	Fragment Size (bp)	Protocol	Reference
ssu rRNA[a] (1st round)	R221(GGTTCCTTTCCTGATTTACG) R332(GGCCGGTAAAGGCCGAATAG)	~600 bp	94 ◦C/5 min; 15 cycles: 94 ◦C/30 s, 53 ◦C/30 s, 72 ◦C/30 s; 72 ◦C 10 min	[86]
ssu rRNA[a] (2nd round)	R223 (TCCCATCGCAACCTCGGTT) R333 (AAAGCGGGCGCGGTGCTG)	~350 bp	94 ◦C 5 min; 32 cycles: 94 ◦C/30 s, 65 ◦C/30 s, 72 ◦C/30 s; 72 ◦C 10 min	[87]
ITS1[a]	LITSR (CTGGATCATTTTCCGATG) 5.8S (TGATACCACTTATCGCACTT)	~320 bp	94 ◦C/5 min; 34 cycles: 94 ◦C/20 s, 53 ◦C/30 s,72 ◦C/1 min;72 ◦C/10min	[88]
Cpb[a]	cpfF (CGTGACGCCGGTGAAGAAT) cpbR (CGTGCACTCGGCCGTCTT)	~740–780 bp	94 ◦C/5 min; 34 cycles: 94 ◦C/30 s, 62 ◦C/1min,72 ◦C/1 min;72 ◦C/10 min	[89]
kDNA [b]	F (CTTTTCTGGTCCTCCGGGTAGG) R (CCACCCGGCCCTATTTTACACCAA) Probe (FAM-TTTTCGCAGAAC GCCCCTACCCGC-TAMRA)	-	50 ◦C/10 min; 95 ◦C/10 min; 45 cycles: 95 ◦C/15 s, 60 ◦C/1 min	[90]

[a]PCR, [b]qPCR

All PCRs were performed using a 2× EmeraldAmp® GT PCR Master Mix (Takara Bio Europe AB, Göteborg, Sweden) in a final volume of 25 µL with an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Bands were analyzed using a Gel Doc™ XR + Imager (Bio-Rad Laboratories Inc., Hercules, CA, USA), cut out from the gel, and purified with an Illustra™ GFX™ PCR DNA and Gel Purification Kit (GE Healthcare, Buckinghamshire, UK). The samples were sent to Microsynth (Microsynth Austria GmbH, Vienna, Austria) for Sanger sequencing, and the obtained sequences from both strands were aligned with ClustalX 2.1 and edited with GeneDoc 2.7.0. The consensus sequences were blasted and uploaded to the NCBI sequence database (accession numbers: OR344780, OR345127–OR345132) for comparison with reference sequences. The qPCR was conducted using a TaqMan Universal PCR Master Mix (Applied Biosystems Inc., Waltham, MA, USA) in a final volume of 25 µL with a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Leishmania DNA amplification by sensitive nested-PCR protocol (Figure 4.10):

- First, I prepared a list and a plate with the numbers of the samples to be screened.

- The master mix was prepared in two different tubes (two rounds of PCR) based on the number of samples being run. An appropriate amount of primers was mixed (Takara MM + Primer F + Primer R + H2O) and vortexed.

- Twenty µL of master mix was pipetted into the 96-well plate according to the number of analyzed samples, and 5 µL of extracted DNA from the samples. One negative control (well G12) and one positive control (well H12) were added. The negative control was added and the plate was sealed, followed by the pippeted of the positive control after sealing all sample wells to avoid contamination.

- The plate was then placed in an Eppendorf Mastercycler for DNA amplification using a specific program for the first round.

- After the first round of amplification, the second plate was prepared as described above. Twenty µL of the master mix was added to all wells, and 5 µL of samples were transferred from the first plate, which was amplified in the first round including negative and positive controls. The plate was then sealed with 8-strip caps (Figure 4.10).

- The plate was placed in an Eppendorf Mastercycler for DNA amplification using a specific program for the second round.

- After the second amplification, the samples were put on an agar gel.

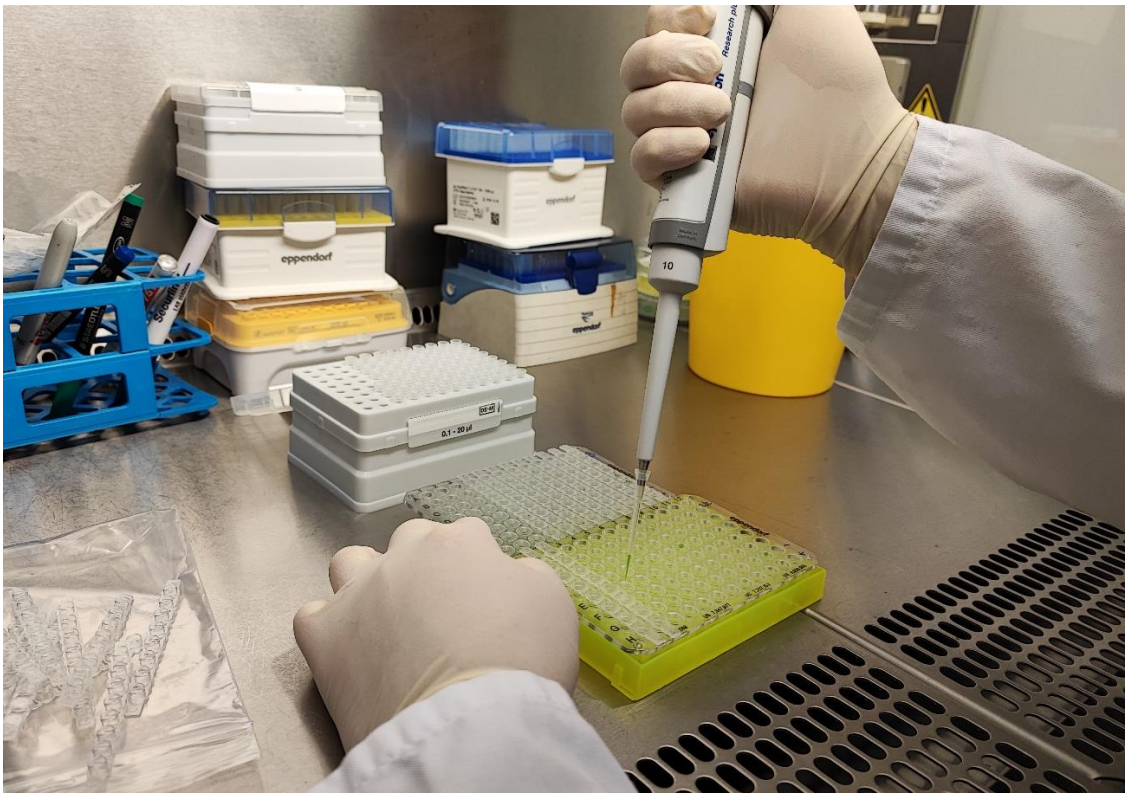


Figure 4.10: *Leishmania* DNA amplification process by sensitive nested-PCR.

<u>Preparation of a 2% agar gel (Figure 4.11):</u>

- Agar gels were prepared according to the respective sample size. In this case, an agar gel with 20 slots was prepared.

- First, 100 mL of 1x TAE Buffer solution was added to a 250 mL flask and 2.0 g of agarose. A magnet flea was added for mixing, and the mixture was boiled.

- After boiling, the mixture was allowed to cool for 2 minutes at room temperature, and then 5 µL of GelRed (Nucleic acid stain, 10,000x) was added.

- The content was poured into the gel plate, and a comb with respective slots was inserted. The gel was allowed to cool for a minimum of 30 minutes at room temperature until it hardened.

- Twenty-five µL of the step marker was added to the first slot of the plate, and the remaining slots were filled with 25 µL of the previously amplified samples.

- Gel electrophoresis: The plate was placed into 1x TAE buffer and run at 120–130 volts and 300 mA (milliamperes) for 20–30 minutes.

- The gel was then photographed under UV light. Therefore, the plate and gel were placed under a UV camera.

- In the case of positive samples, a band can be observed on the gel at the height of the respective positive control.

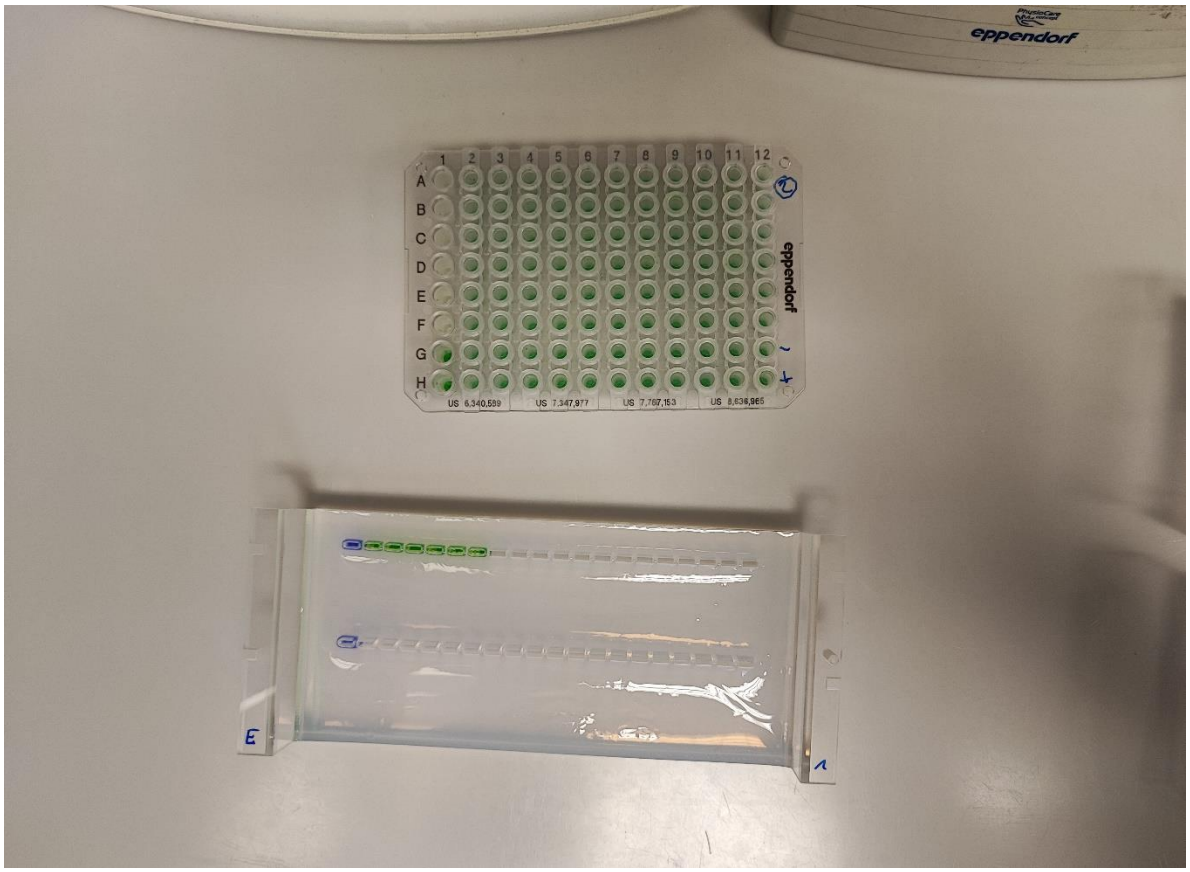


Figure 4.11: Agar gel preparation and transfer of the samples.

Purification of DNA from the gel:

- The positive bands in the gel were cut out under UV light and purified for sequencing.

- Using a clean razor blade or scalpel, the visible band was cut out from the agarose gel containing the amplified target DNA. The positive bands were cut closely around the observed bands and transferred to a 1.5 mL microcentrifuge tube.

- Three hundred µL of capture buffer was added and placed in an Eppendorf thermomixer at 60°C and 650 rpm for 20 minutes until the agarose was completely dissolved.

- Centrifugation of the tubes for 30 seconds at 4,000 rpm was performed to avoid spillage when opening the tubes.

- During the incubation, the GFX columns were placed in a collection tube in preparation for each sample.

- The samples were transferred to the GFX spin column and incubated for 1 minute at room temperature.

- The centrifugation process was carried out for 30 seconds at 14,000 rpm.

- The collection tubes were then discarded, and the GFX spin column was placed in a new collection tube.

- Five hundred µL of wash buffer was added to the column and centrifuged for 30 seconds at 14,000 rpm.

- The collection tubes were discarded, and the GFX spin columns were transferred to fresh 1.5 mL microcentrifuge tubes.

- Thirty-five µL of elution buffer was added directly to the center of the GFX spin column to elute DNA.

- The samples were incubated at room temperature for 2 minutes, then centrifuged at full speed (14,000 rpm) for 1 minute to recover the purified DNA.

- Purified DNA (including primer) was sent to Microsynth (Microsynth Austria GmbH, Vienna, Austria) for Sanger sequencing, and the obtained sequences from both strands were aligned using ClustalX 2.1 and edited with GeneDoc 2.7.0. The consensus sequences were then blasted and uploaded to the NCBI sequence database (accession numbers: OR344780, OR345127–OR345132) and compared to reference sequences (Figure 4.12).

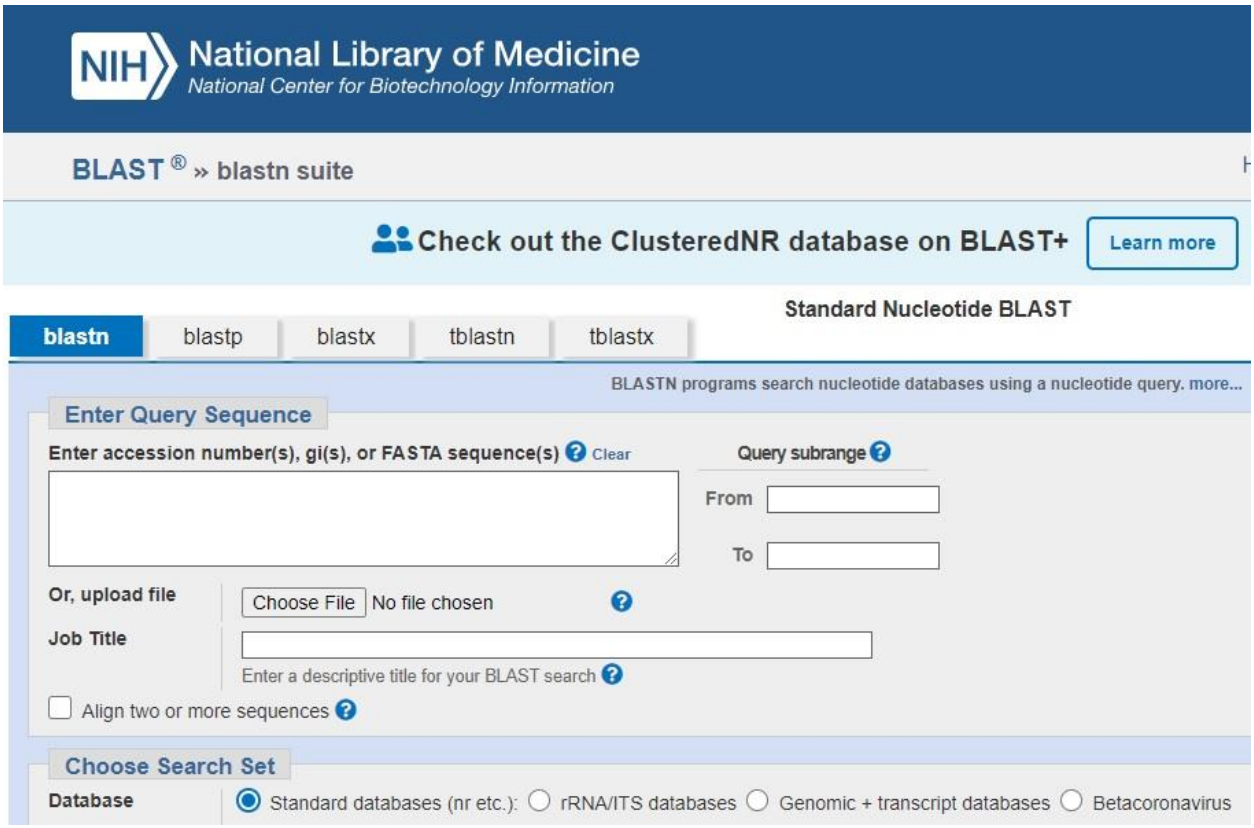


Figure 4.12: The NCBI sequence database.

4.5.4. Molecular identification of *Leishmania* positive sand flies

To confirm the morphological identification of sand fly specimens that tested positive for *Leishmania* DNA, a barcoding PCR targeting a 658-base pair (bp) fragment of the cytochrome c oxidase subunit I (COI) gene was performed using the primers LCO1490/HCO2198 following the protocol of Folmer et al. [91].

The extracted DNA from sand flies tested for *Leishmania* DNA was also used for the molecular identification of sand flies that tested positive for *Leishmania*. The protocol for this identification was similar to the *Leishmania* DNA screening protocols, albeit with different primers and master mix.

All PCRs were performed using a 2x EmeraldAmp® GT PCR Master Mix (Takara Bio Europe AB, Göteborg, Sweden) in a final volume of 25 µL with an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Bands were analyzed using a Gel Doc™ XR + Imager (Bio-Rad Laboratories Inc., California, USA), cut out from the gel, and purified using an Illustra™ GFX™ PCR DNA and Gel Purification Kit (GE Healthcare, Buckinghamshire, UK). The samples were sequenced, and the obtained sequences from both strands were aligned using ClustalX 2.1 and edited with GeneDoc 2.7.0 (Table 4.2). The consensus sequences were blasted against the NCBI sequence database (GenBank) and compared to reference sequences.

Table 4.2: Sample of obtained forward sequence from sand flies by molecular identification.

>4_78_COI_F
NTNATTTTTGGAGCCTGAGCTGGAATAGTAGGAACTTCATTAAGAATTCTTATTCGAGCAGA ATTAGGACATCCTGGAGCATTAATTGGAGATGATCAAATTTATAATGTAATTGTAACAGCTC ATGCATTTGTAATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGTTTTGGAAATTGAC TTGTTCCTTTAATATTAGGAGCCCCAGATATAGCATTCCCACGAATAAATAATATAAGATTTT GATTACTTCCTCCCTCTCTAACCTTACTTTTAACTAGAAGTATAGTTGAAACTGGGGCTGGAA CTGGATGAACTGTTTATCCCCCTCTTTCCAGAAATATTGCTCATAGAGGAGCTTCTGTTGACT TAGCAATTTTTTCACTTCACTTAGCTGGAATTTCCTCAATTTTAGGAGCAGTTAACTTTATTA CTACTGTTATTAATATACGTGCTACCGGAATTACCTTAGATCGAATACCCTTATTTGCATGAT CTGTAGTAATTACAGCTATTTTATTACTTCTTTCTTTACCTGTTTTAGCAGGAGCTATTACAAT ACTATTAACAGATCGAAATTTAAATACATCATTTTTTGATCCTGCAGGAGGAGGAGATCCAA TTTTATACCAACACTTATTTTGATTTTTTGGTCACCNGGAAAA

4.5.5. Molecular characterization of detected *Leishmania* DNA

Available *Leishmania* spp. reference sequences for comparison were downloaded from GenBank and aligned with the obtained sequences using ClustalX 2.1 for multiple alignment [92], and GeneDoc 2.7.0 [93] for manual editing and data analysis. For further clarification of species boundaries and to determine the discriminatory power of all three applied PCRs, pairwise distances and maximum likelihood (ML) analyses were calculated in MEGA X [94]. Based on the best-fit evolutionary model selection, the Kimura-2-parameter model with bootstrap support of 1000 replications was applied.

4.5.6. Mapping of sand fly data

Coordinates of sampling sites were georeferenced into a distribution map using Quantum GIS 3.4.11 [95]. Data on country, district, and municipality borders were sourced from Natural Earth (naturalearthdata.com). First-level administrative divisions for Kosovo in 2015 were obtained from https://earthworks.stanford.edu/catalog/stanford-zh532mm5047 (accessed on 26 January 2024).

4.6. Statistical analyses

For the dog samples, two different statistical analyses were performed.

First, a statistical analysis was conducted to test for significant differences among the different districts, gender, age, health status, and breed. A power analysis (GPower v.3.9.1.2, developed by Franz Faul at Kiel University, Germany) was conducted a priori to assess the power of the chi-square analysis (Pearson or Fisher's exact test) (SPSS, IBM®). The preset effect size of 0.3, α probability of 0.05, appropriate sample size (n=273–285), and degrees of freedom (df=1–14) were used for the analysis. Chi-square results were considered relevant if the obtained power was ≥0.95 and $p<0.05$.

The second statistical analysis involved a comparison of results using Kappa statistics on two different methods, ELISA and IFAT, for the detection of antibodies against *Leishmania infantum*. This analysis aimed to determine the level of agreement between both tests and to assess the validity and reliability of these tests. The comparison of the methods is presented in Table 4.3.

Table 4.3: Table used for distribution of results.

	IFAT test		
ELISA test	**Positive**	**Negative**	**Total**
Positive	**a**	**b**	**a+b**
Negative	**c**	**d**	**c+d**
Total	**a+c**	**b+d**	**n**

a=number of samples positive in both tests, b=number of samples positive in ELISA test, but negative in IFAT test, c=number of samples negative in ELISA test, but positive on IFAT, d=number of samples negative on both tests.

To assess the agreement between two different tests, I used the following statistics:

The observed proportion agreement between the two tests,

$OP = (a + d)/n$, where $n = (a + b + c + d)$.

Expected proportion agreement by chance (both positive):

$EP + = \{(a + b)/ n\} \times \{(a + c)/n\}$

Expected proportion agreement by chance (both negative):

$EP - = \{(c + d)/n\} \times \{(b + d)/ n\}$

Thus: $EP = (EP +) + (EP-)$

Observed agreement beyond chance: $OA = OP-EP$

Maximum possible agreement beyond chance: $MA= 1-EP$

The Kappa coefficient was calculated as the ratio of the observed agreement beyond chance (OA) to the maximum possible agreement beyond chance (MA):

$Kappa=OA/MA$,

OA and MA were calculated using the following formula:

$OA = [(a+d)/n] – \{[(a+b)/n * (a+c/n] – [(c+d)/n * [b+d)/n]\}$

$MA = 1 - \{[(a+b)/n * (a+c/n] – [(c+d)/n * [b+d)/n]\}$

Given the nature of our sand fly dataset and the analyses conducted, we refrained from detailed descriptive statistical analysis. For analyzing sand fly presence by district and *Leishmania* positivity, total numbers and precentages were calculated.

5. RESULTS

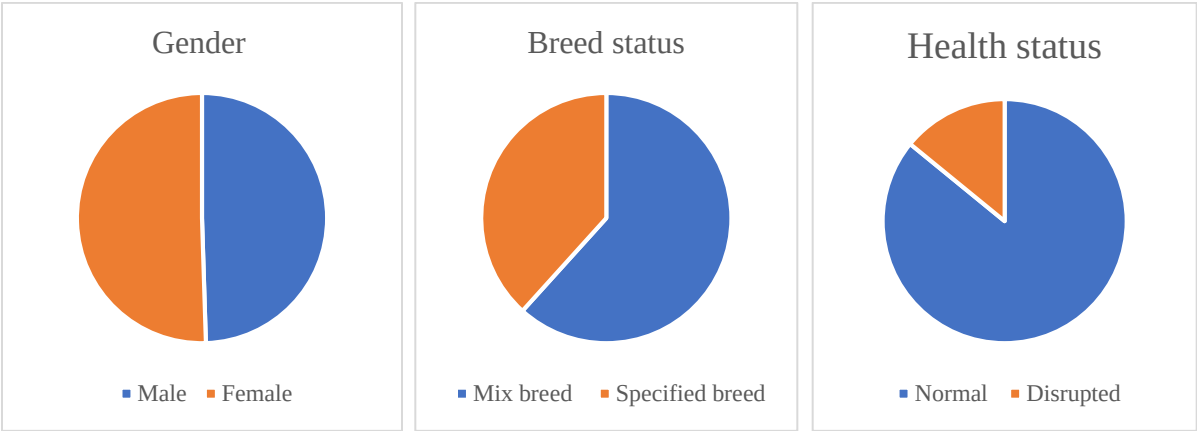
The results of the study are divided into two parts to reach a single conclusion: findings from the dog samples and findings from the vectors. They both play a crucial role in the circulation of canine leishmaniosis.

5.1. Results of dog samples

The overall prevalence rate of CanL antibodies among asymptomatic dogs in all seven districts of Kosovo was 4.21% (95% CI: 2.42–7.21%), representing 12 out of 285 samples in the ELISA test and 3.51% or 10 out of 285 samples, (95% CI: 1.92–6.34%) in the IFAT test.

Out of the 285 tested dogs, 141 (49.47%) were male and 144 (50.53%) were female. Additionally, 176 (61.75%) were mixed breeds, and 109 (38.25%) were purebred dogs. Among them, 245 (85.96%) were healthy dogs with no clinical signs, while 40 (14.04%) exhibited various pathologies such as dermatitis, arthritis, tumors, vasculitis, etc.

The age was categorized from 1 to 10+ years old. Among the 285 dogs, 41 were 1-2 years old (14.39%), 64 were 2-3 years old (22.46%), 60 were 3-4 years old (21.05%), 67 were between 4-6 years old (23.51%), 23 were between 6-8 years old (8.07%), 14 were between 8-10 years old (4.91%), and 16 were more than 10 years old (5.61%) (Figure 5.1).



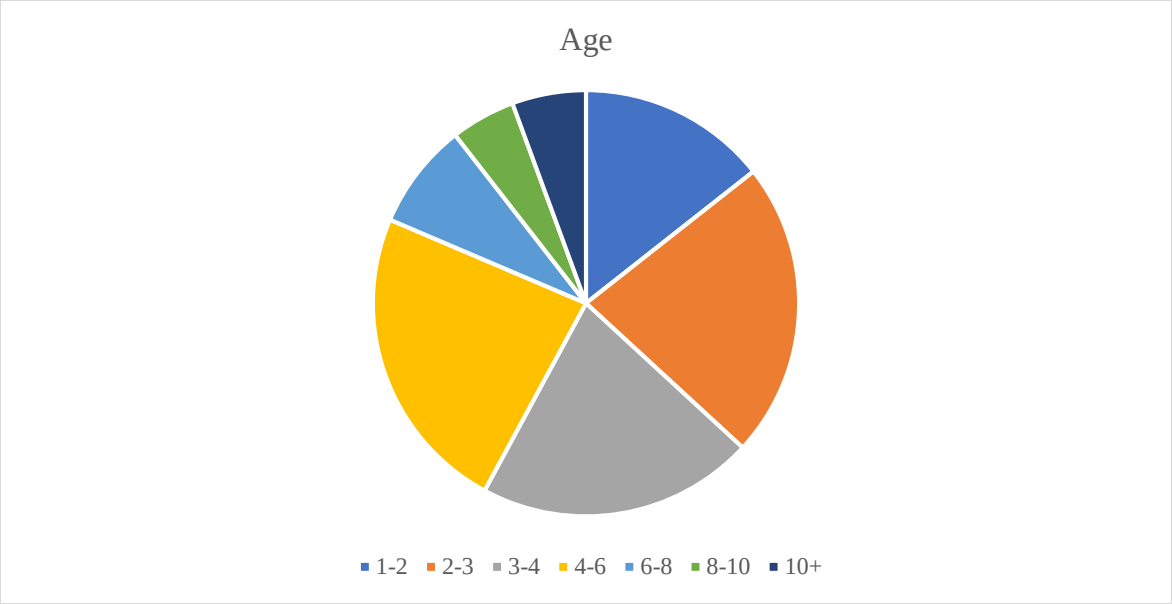


Figure 5.1: Schematic view of different parameters of the dogs involved in the study.

The distribution of positive ELISA and IFAT samples by districts was as follows: Prishtina (33% and 30%), Gjakova (17% and 30%), Prizreni (17% and 10%), Ferizaj (17% and 10%), Peja (8% and 10%), and Gjilani (8% and 10%). The Mitrovica district showed negative results (refer to Figure 5.2).

The seropositive samples by gender were distributed as follows: 67% and 33% for ELISA and 70% and 30% for IFAT in males and females, respectively.

The seropositive samples by age on ELISA and IFAT were distributed as follows: 50% and 60% for 4–6 years, 17% and 20% for 8–10 years, 17% and 10% for 3–4 years, 8% and 10% for 6–8 years, 8% and 0% for the >10 years group, and 0% for 1–2 and 2–3 years, respectively.

The seropositive samples by health status on ELISA and IFAT were distributed as follows: 92% and 8% for normal, and 90% and 10% for disrupted health status, respectively.

The seropositive samples by breed status on ELISA and IFAT were distributed as follows: 67% and 33% for the specified breed, and 70% and 30% for the mixed-breed dogs (refer to Table 5.1 and Table 5.2).

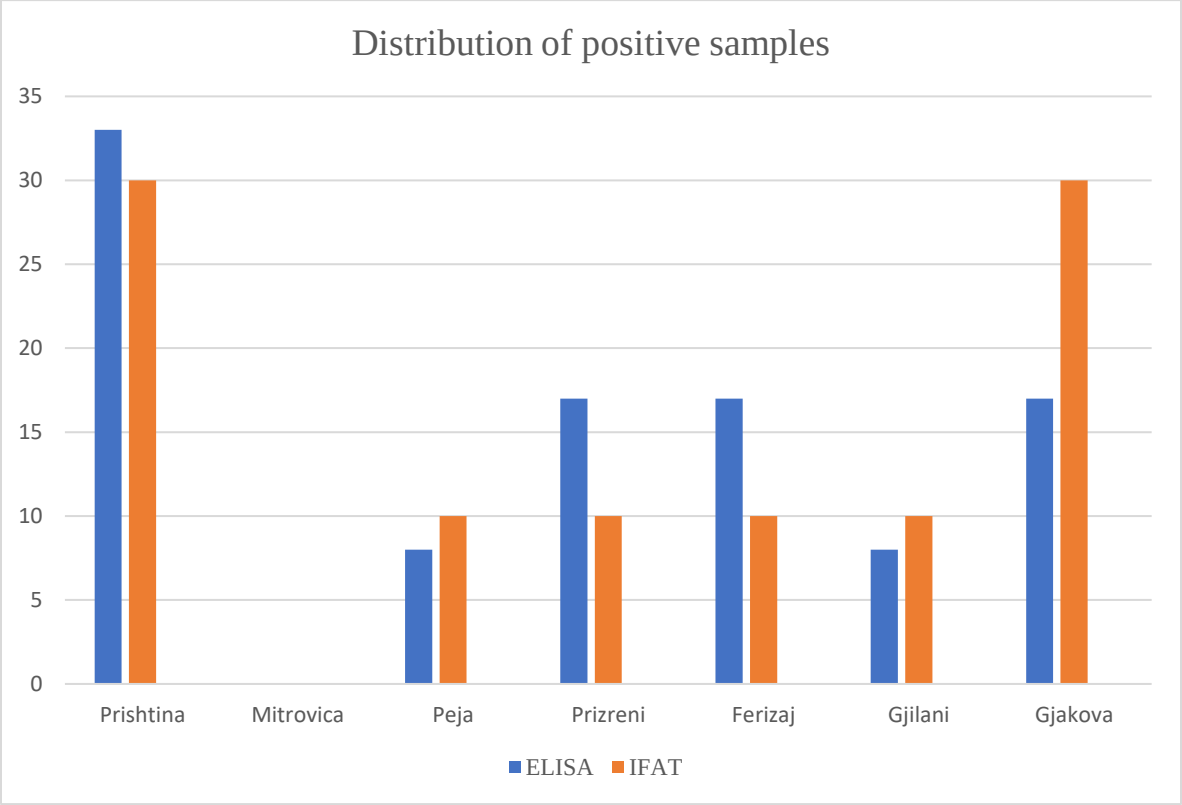


Figure 5.2: Distribution of positive ELISA and IFAT samples based on different districts.

5.1.1. Results of ELISA

The overall prevalence of the ELISA testing for anti-*Leishmania* antibodies among dogs in the seven districts of Kosovo was 4.21% (95% CI 2.42–7.21), representing 12 out of 285 samples.

The highest number of positive samples was detected in the Prishtina district, with a prevalence rate of 8.0% (4 out of 50), followed by the Gjakova district at 5.0% (2 out of 40), the Prizreni district had a prevalence rate of 5.0% (2 out of 40), the Ferizaj district also had a prevalence rate of 5.0% (2 out of 40). The Peja district showed a prevalence rate of 2.86% (1 out of 35), the Gjilani district had a prevalence rate of 2.5% (1 out of 40), and the Mitrovica district had no positive samples (0 out of 40) (Table 5.1 and Figure 5.3).

Table 5.1: Seroprevalence of CanL in Kosovo by ELISA related to different variables.

Parameter	Factor	No. samples	No. of positive ELISA	No. of negative ELISA	% ELISA	95% CI ELISA
Location	Prishtina 01	50	4	46	8.0	3.15–18.84
	Mitrovica 02	40	0	40	0	0–8.76
	Peja 03	35	1	34	2.86	0.51–14.53
	Prizreni 04	40	2	38	5.0	1.38–16.5
	Ferizaj 05	40	2	38	5.0	1.38–16.5
	Gjilani 06	40	1	39	2.5	0.44–12.88
	Gjakova 07	40	2	38	5.0	1.38–16.5
Sex	Male	141	8	133	5.67	2.9–10.8
	Female	144	4	140	2.78	1.1–6.92
Age	1–2 years	41	0	41	0	0–8.57
	2–3 years	64	0	64	0	0–5.66
	3–4 years	60	2	58	3.33	0.92–11.36
	4–6 years	67	6	61	8.96	4.17–18.19
	6–8 years	23	1	22	4.35	0.77–20.99
	8–10 years	14	2	12	14.29	4.01–39.94
	>10 years	16	1	15	6.25	1.11–28.33
Health status	Normal	245	11	234	4.49	2.53–7.86
	Disrupted	40	1	39	2.5	0.44–12.88
Breed status	Mix-breed	176	8	168	4.55	2.32–8.71
	Specified-breed	109	4	105	3.67	1.44–9.06

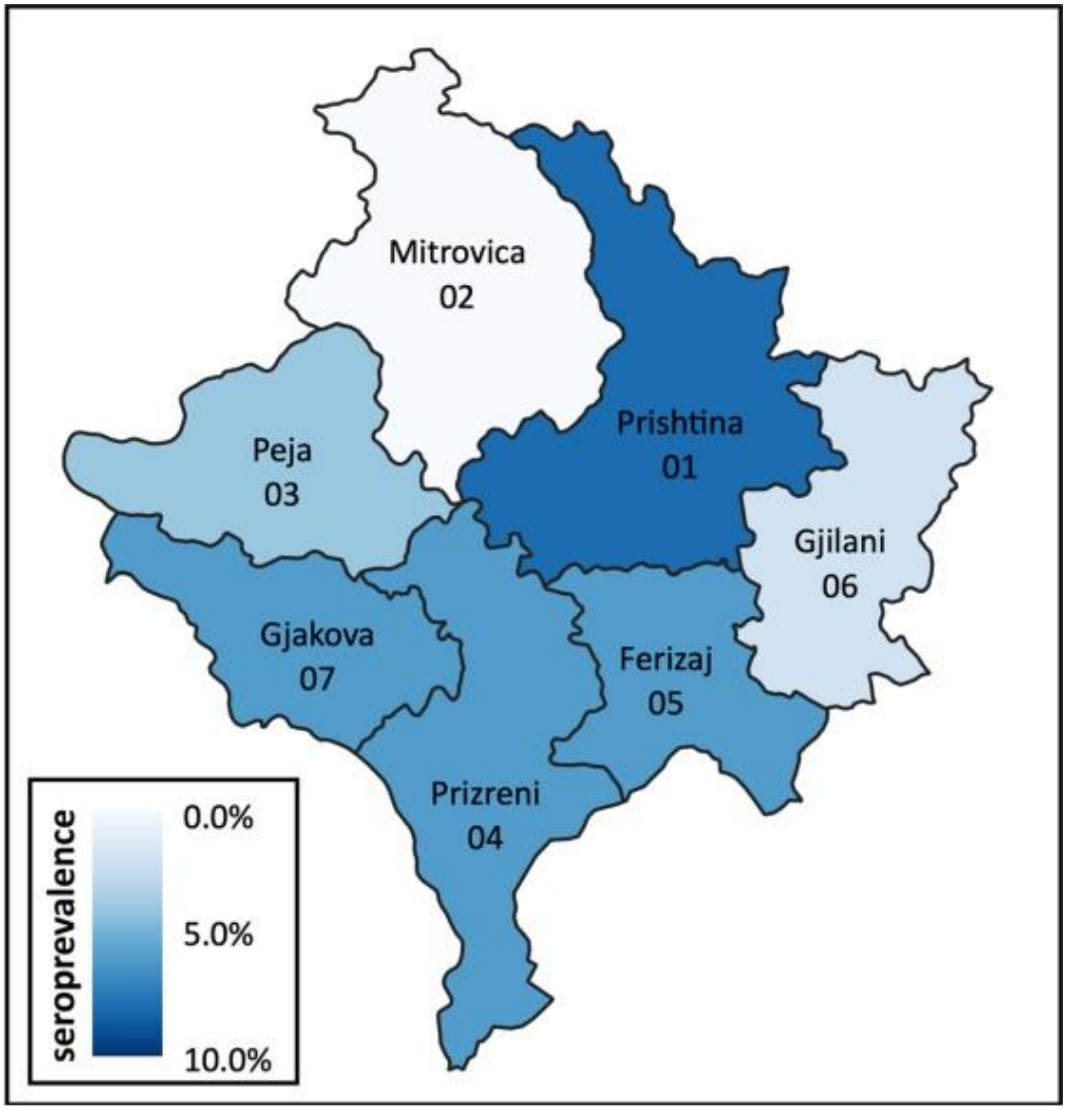


Figure 5.3: *Leishmania infantum* seroprevalence by ELISA in the seven districts of Kosovo.

All ELISA plates used in the study resulted valid, based on the mean value of the Positive Control O.D.(OD_{PC}) being greater than 0.350. (**OD_{PC} > 0.350),** and the ratio of the mean values of the Positive and Negative Controls (OD_{PC} and OD_{NC}) being greater than 3 (**OD_{PC}/OD_{NC} > 3)** (Figure 5.4).

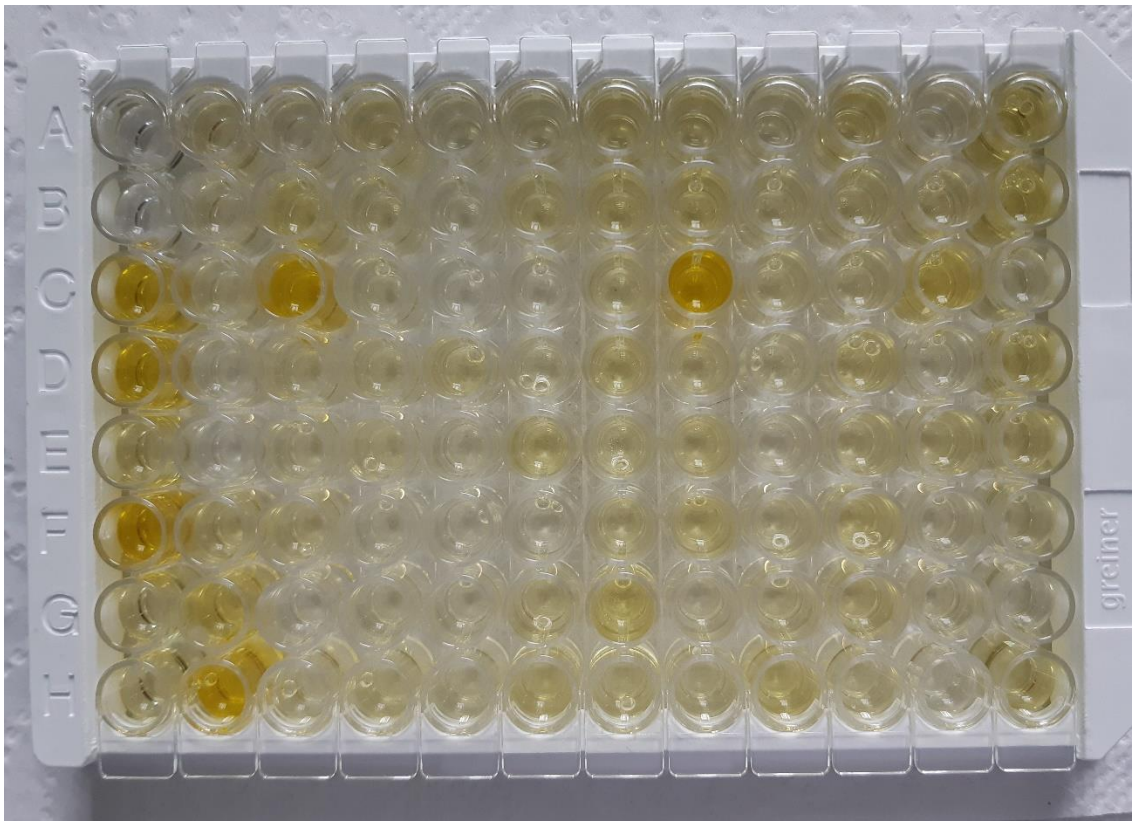


Figure 5.4: The ELISA plate used in the study (before the reading step).

All doubtful samples were retested and remained within the doubtful range. After the second test, all samples that remained within this border were considered negative on the ELISA test.

5.1.2. Results of IFAT

The prevalence of canine leishmaniosis tested by IFAT in the seven districts of Kosovo was 3.51%, representing 10 out of 285 samples.

The highest number of positive samples was detected in the Gjakova district, with a prevalence of 7.5% (3 out of 40), followed by the Prishtina district, which had a prevalence of 6.0% (3 out of 50). Additionally, the Peja district showed a prevalence of 2.86% (1 out of 35), the Prizreni district had a prevalence of 2.5% (1 out of 40), and both the Ferizaj and Gjilani districts also had a prevalence of 2.5% each (1 out of 40). The Mitrovica district had no positive samples (0 out of 40) (Table 5.2 and Figure 5.5).

Table 5.2: Seroprevalence of CanL in Kosovo by IFAT related to different variables.

Parameter	Factor	No. samples	No. of positive IFAT	No. of negative IFAT	% IFAT	95% CI IFAT
Location	Prishtina 01	50	3	47	6.0	2.06–16.22
	Mitrovica 02	40	0	0	0	0–8.76
	Peja 03	35	1	34	2.86	0.51–14.53
	Prizreni 04	40	1	39	2.5	0.44–12.88
	Ferizaj 05	40	1	39	2.5	0.44–12.88
	Gjilani 06	40	1	39	2.5	0.44–12.88
	Gjakova 07	40	3	37	7.5	2.58–19.86
Sex	Male	141	7	134	4.96	2.43–9.89
	Female	144	3	141	2.08	0.71–5.95
Age	1–2 years	41	0	0	0	0–8.57
	2–3 years	64	0	0	0	0–5.66
	3–4 years	60	1	59	1.67	0.29–8.86
	4–6 years	67	6	61	8.96	4.17–18.19
	6–8 years	23	1	22	4.35	0.77–20.99
	8–10 years	14	2	12	14.29	4.01–39.94
	>10 years	16	0	16	0	0–19.36
Health status	Normal	245	9	236	3.67	1.94–6.83
	Disrupted	40	1	39	2.5	0.44–12.88
Breed status	Mix-breed	176	7	169	3.98	1.94–7.98
	Specified-breed	109	3	106	2.75	0.94–7.78

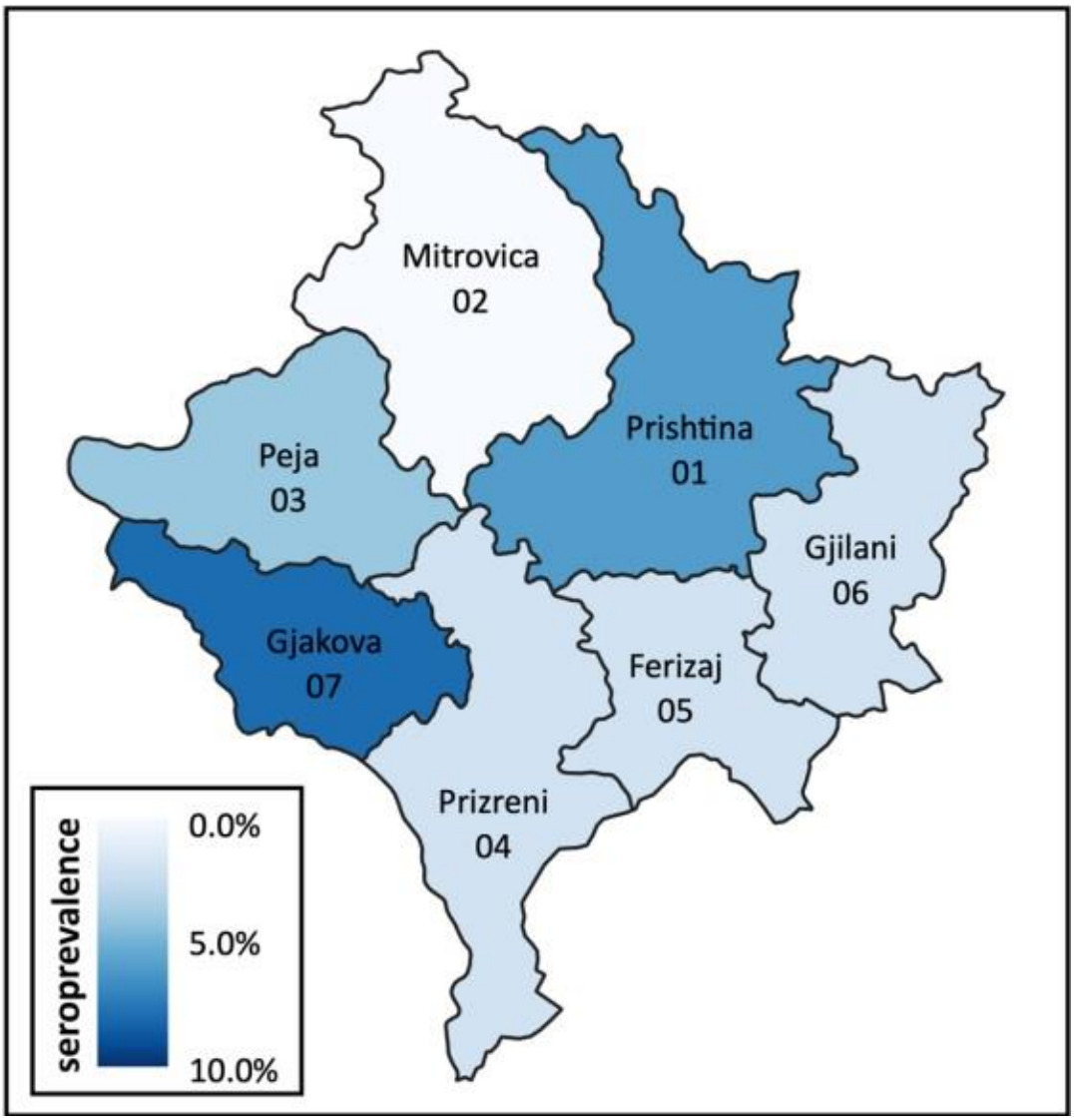


Figure 5.5: *Leishmania infantum* seroprevalence by IFAT in the seven districts of Kosovo.

In positive fluorescence pattern: ≥ 1:160. The *Leishmania* (membrane, flagella) showed a clear, yellow-green fluorescence on its membrane and flagella area (Figure 5.6).

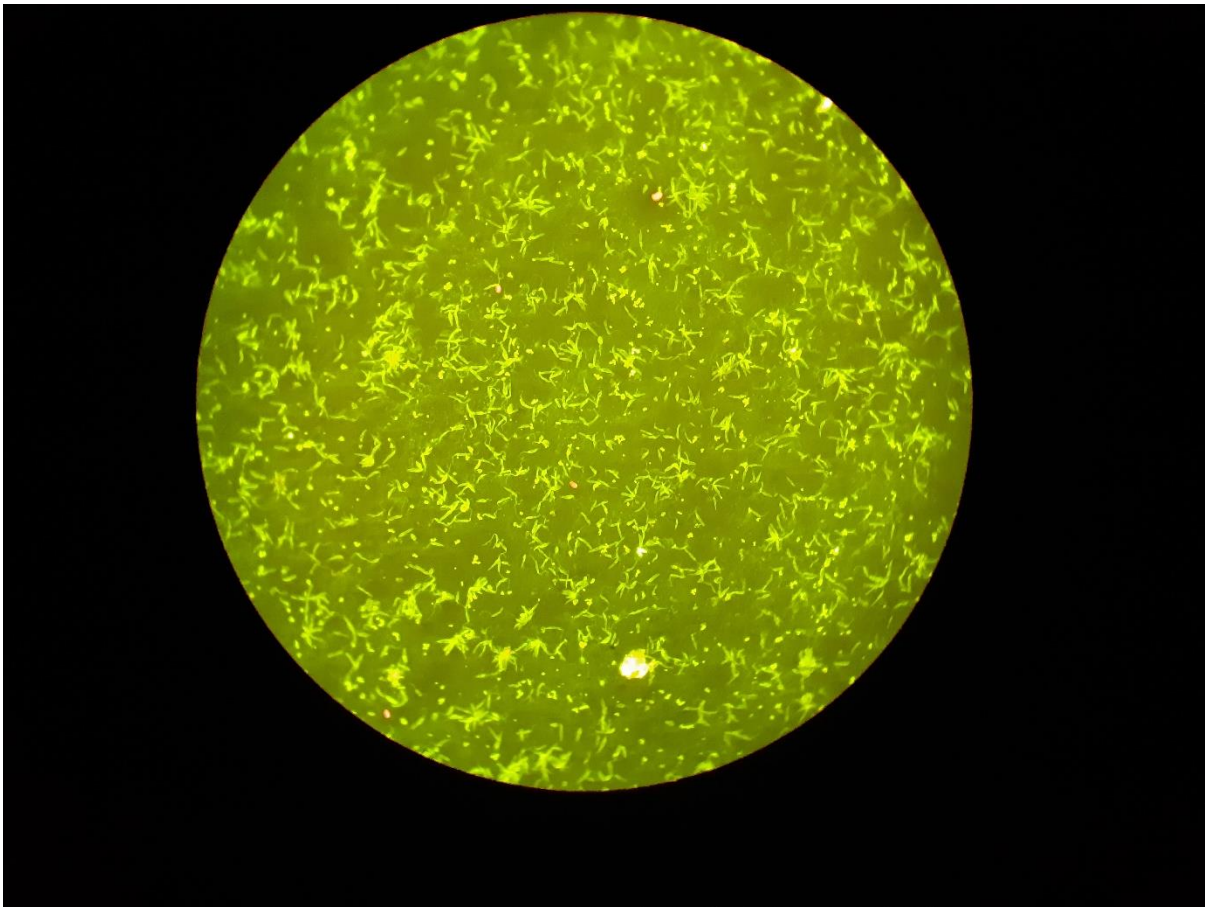


Figure 5.6: The IFAT positive sample under a microscope.

Samples were classified as positive if promastigote cytoplasmic or membrane fluorescence was observed at a serum dilution of 1:160 or higher.

5.2. Result comparison of serological tests

A power analysis, performed a priori to assess the power of the chi-square analysis, indicated that there were no significant differences among the different districts, genders, ages, health statuses, and breeds.

The Kappa analysis revealed a perfect agreement between the tests, suggesting that the obtained results for the determination of seroprevalence of CanL in dogs in Kosovo using the aforementioned commercial tests were reliable and valid.

Out of all sera tested, 3 samples (1.05%) were positive on ELISA but negative on IFAT, and 1 sample (0.35%) was positive on IFAT but negative on the ELISA test. Both tests yielded identical results for 281 samples (98.5%), resulting in an observed agreement of 0.986. The results are presented in Table 5.3.

Table 5.3: Comparison of results obtained on both ELISA and IFAT tests.

	IFAT test		
ELISA test	**Positive**	**Negative**	**Total**
Positive	**a (9)**	**b (3)**	**a+b (12)**
Negative	**c (1)**	**d (272)**	**c+d (273)**
Total	**a+c (10)**	**b+d (275)**	**N (285)**

The expected agreement by chance (EP) was 0.925. The maximum possible agreement beyond chance was 0.075, while the observed agreement beyond chance was 0.061. This yields a kappa ratio of 0.81.

These results were calculated as follows:

- The observed proportion agreement between the two tests,

OP = (9 + 272)/285

= 0.986.

- Expected proportion agreement by chance (both positive):

EP + = {(9 + 3)/ 285} × {(9 + 1)/ 285}

= 0.0421 × 0.0350

= 0.00147.

- Expected proportion agreement by chance (both negative):

EP − = {(1 + 272)/ 285} × {(3 + 272)/ 285}

= 0.957 × 0.965

= 0.92350.

Thus: EP = (0.00147) + (0.92350)

= 0.925

- Observed agreement beyond chance:

OA = 0.986 − 0.925

= 0.061

- Maximum possible agreement beyond chance:

MA = 1− 0.925

= 0.075

- The kappa coefficient was calculated as the ratio of the observed agreement beyond chances (OA) to the maximum possible agreement beyond chance (MA):

Kappa = 0.061/0.075

= 0.81

5.3. Results of vector samples

In total, 114 locations were sampled, of which 77 (67.5%) were positive for sand flies (Figure 5.7). The number of sampling locations per district ranged from 15 to 20; the lowest capture rate was observed in Ferizaj 05 (7/15, 46.6%), whereas the highest was observed in Gjakova 07 (14/16, 87.5%).

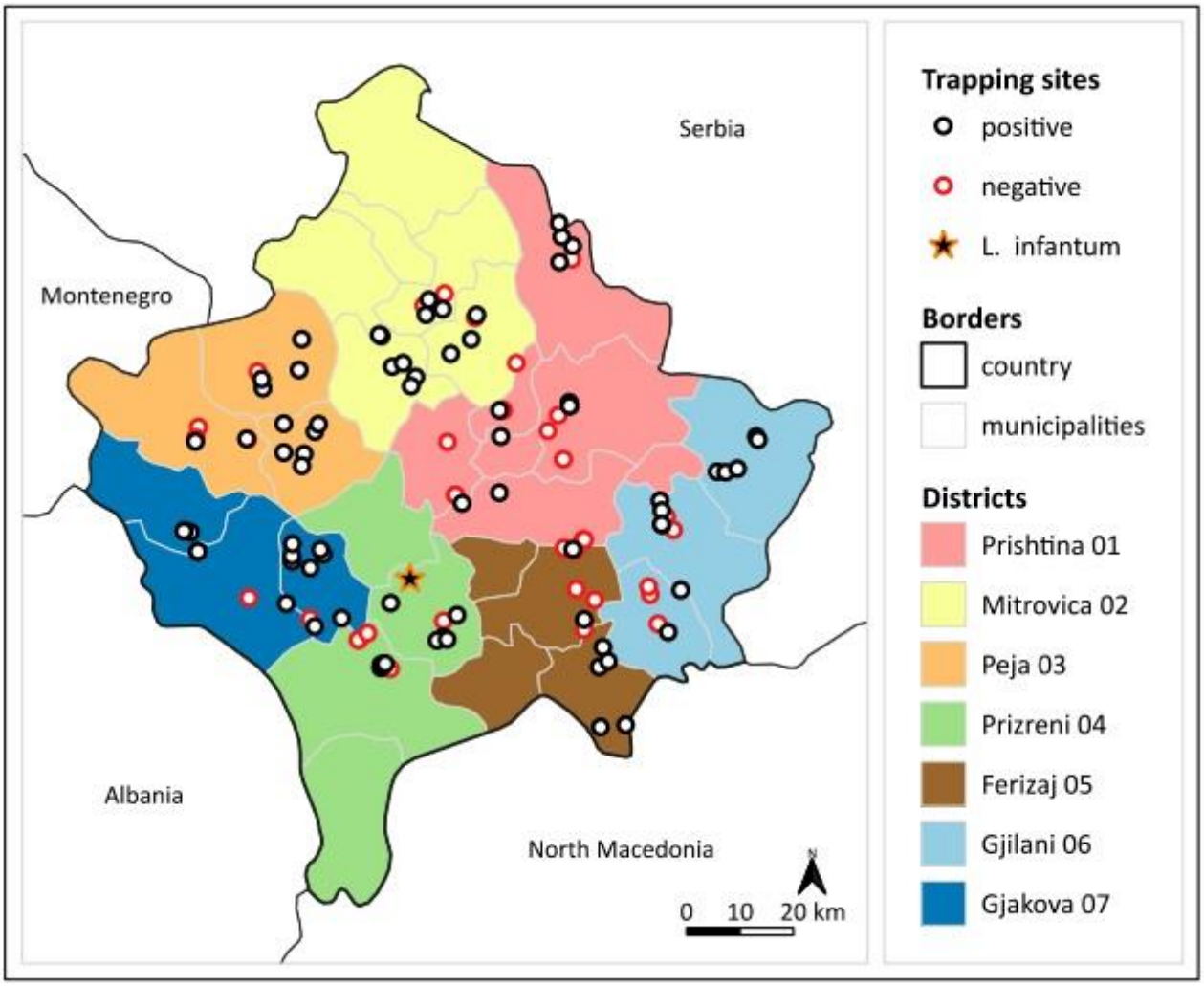


Figure 5.7: Map of positive and negative sand fly trapping sites in Kosovo. *L. infantum* positive site is indicated as a black star with an orange frame.

Of the 3272 trapped specimens, 2930 (89.5%) were female, and 342 (10.5%) were male. Among all females, 497 (17%) were engorged. Additionally, 1149 specimens were individually identified (from all locations with less than 100 trapped specimens), representing seven species in two genera. These species include *Phlebotomus perfiliewi* (715, 62.2%), *Phlebotomus neglectus* (368, 32.0%), *Phlebotomus tobbi* (27, 2.3%), *Phlebotomus simici* (22, 1.9%), *Phlebotomus balcanicus* (8, 0.7%), *Phlebotomus papatasi* (2, 0.2%), and *Sergentomyia minuta* (7, 0.6%) (Table 5.4).

Table 5.4: Individually identified sand flies.

Species	Male	Female (Engorged)	Total	Percentage
Ph. perfiliewi	64	651 (110)	715	62.2%
Ph. neglectus	156	212 (61)	368	32.0%
Ph. tobbi	5	22 (2)	27	2.3%
Ph. simici	3	19 (4)	22	1.9%
Ph. balcanicus	4	4 (0)	8	0.7%
Ph. papatasi	1	1 (0)	2	0.2%
S. minuta	1	6 (2)	7	0.6%
Total	234	915 (179)	1149	100%

All other 2123 specimens originated from only five locations, namely 02/7 (45), 03/7 (146), 03/8 (925), 04/9 (715), and 07/8 (292). Among these, 372 specimens were individually identified as *Ph. perfiliewi* and *Ph. neglectus*, with *Ph. perfiliewi* being the predominant species (Table 5.5).

Table 5.5: Pooled sand flies at locations with more than 100 specimens.

Location ID	Total Number	Number Identified	Species		Pooled Specimens
			Ph. perfiliewi	***Ph. neglectus***	
02/7	133	88	74 (84.1%)	14 (15.9%)	45 (4 pools)
03/7	203	57	56 (98.3%)	1 (1.7%)	146 (9 pools)
03/8	1002	77	76 (98.7%)	1 (1.3%)	925 (32 pools)
04/9	828	113	87 (77.0%)	26 (23.0%)	715 (24 pools)
07/8	329	37	37 (100%)	0 (0.0%)	292 (11 pools)
total	2495	372	330 (88.7%)	42 (11.3%)	2123 (80 pools)

5.3.1. Distribution of sand flies

The number of caught sand flies per district varied between 34 (Ferizaj 05) and 1299 (Peja 03). Gjakova had the most sand fly species (5 species), followed by Gjilani and Ferizaj (4 species), Peja and Prizreni (3 species), and Prishtina and Mitrovica (2 species) (Table 5.6).

Table 5.6: Sand fly species per district.

Species	Prishtina	Mitrovica	Peja	Prizreni	Ferizaj	Gjilani	Gjakova	Total
Ph. perfiliewi	4	165	213	92	1	8	232	715
Ph. neglectus	54	61	12	107	24	53	57	368
Ph. tobbi	0	0	0	0	1	26	0	27
Ph. simici	0	0	0	0	8	13	1	22
Ph. balcanicus	0	0	0	0	0	0	8	8
Ph. papatasi	0	0	2	0	0	0	0	2
S. minuta	0	0	1	5	0	0	1	7
in mixed pools [a]	0	45	1071	715	0	0	292	2123
total	58	271	1299	919	34	100	591	3272

[a] only consisting of *Ph. perfiliewi* and *Ph. neglectus*.

Phlebotomus perfiliewi and *Ph. neglectus* were the only species found in all seven districts, whereas *Ph. balcanicus* was found only in Gjakova, and *Ph. papatasi* was found only in Peja (Table 5.6). Based on the living environment, different species are found in different habitats, as shown in Table 5.7 and Table 5.8.

Table 5.7: Distribution of species in different habitats.

Species	M.	F.	BF.	Cow in	Cow out	Ch. in	Ch. out	Dog in	Sh. in	G. in	P. in	Total
Ph. neglectus	156	151	61	128	41	56	61	37	7	5	33	368
Ph. perfiliewi	64	541	110	268	184	65	91	80	25	2	0	715
Ph. simici	3	15	4	18	2	0	1	1	0	0	0	22
Ph. tobbi	5	20	2	13	1	2	5	6	0	0	0	27
Ph. papatasi	1	1	0	2	0	0	0	0	0	0	0	2
Ph. balcanicus	4	4	0	3	0	2	0	3	0	0	0	8
Se. minuta	1	4	2	2	0	1	2	2	0	0	0	7
Total	**234**	**736**	**179**	**434**	**228**	**126**	**160**	**129**	**32**	**7**	**33**	**1149**

M=male, F=female, BF=blood fed, Ch=chicken, Sh=sheep, G=goat, P=pigeon.

Table 5.8: Distribution of species based on environment and districts.

Districts	Species (*Ph.*)	M.	F.	BF.	Cow in	Cow out	Ch. in	Ch. out	Dog out	Sh. in	G. in	P. in	Total
01	*neglectus*	32	19	3	7	3	1	3	9	0	2	29	54
	perifliewi	2	1	1	0	0	1	0	3	0	0	0	4
Total		**34**	**20**	**4**	**7**	**3**	**2**	**3**	**12**	**0**	**2**	**29**	**58**
02	*neglectus*	16	26	19	39	1	14	3	4	0	0	0	61
	perfiliewi	38	114	13	50	11	43	1	60	0	0	0	165
Total		**54**	**140**	**32**	**89**	**12**	**57**	**4**	**64**	**0**	**0**	**0**	**226**
03	*neglectus*	8	3	1	5	0	3	0	4	0	0	0	12
	perfiliewi	6	157	50	108	20	20	38	1	24	2	0	213
	papatasi	1	1	0	2	0	0	0	0	0	0	0	2
	S. minuta	0	1	0	1	0	0	0	0	0	0	0	1
Total		**15**	**162**	**51**	**116**	**20**	**23**	**38**	**5**	**24**	**2**	**0**	**228**
04	*neglectus*	50	37	20	19	17	31	22	13	2	0	3	107
	perfiliewi	6	59	27	13	43	0	31	5	0	0	0	92
	S. minuta	0	3	2	1	0	1	2	1	0	0	0	5
Total		**56**	**99**	**49**	**33**	**60**	**32**	**55**	**19**	**2**	**0**	**3**	**204**
05	*neglectus*	4	16	4	15	4	0	4	0	0	0	1	24
	perfiliewi	0	1	0	1	0	0	0	0	0	0	0	1
	simici	0	8	0	6	1	0	1	0	0	0	0	8
	tobbi	0	1	0	1	0	0	0	0	0	0	0	1
Total		**4**	**26**	**4**	**23**	**5**	**0**	**5**	**0**	**0**	**0**	**1**	**34**
06	*neglectus*	16	27	10	8	13	2	24	1	5	0	0	53
	perfiliewi	0	4	4	5	0	1	0	1	1	0	0	8
	simici	3	6	4	12	1	0	0	0	0	0	0	13
	tobbi	5	19	2	12	1	2	5	6	0	0	0	26
Total		**24**	**56**	**20**	**37**	**15**	**5**	**29**	**8**	**6**	**0**	**0**	**100**
07	*neglectus*	30	23	4	35	3	5	5	6	0	3	0	57
	perfiliewi	12	205	15	91	110	0	21	10	0	0	0	232
	simici	0	1	0	0	0	0	0	1	0	0	0	1
	S. minuta	1	0	0	0	0	0	0	1	0	0	0	1
	balcanicus	4	4	0	3	0	2	0	3	0	0	0	8
Total		**47**	**233**	**19**	**129**	**113**	**7**	**26**	**21**	**0**	**3**	**0**	**299**
		234	**736**	**179**	**434**	**228**	**126**	**160**	**129**	**32**	**7**	**33**	**1149**

Ph=*Phlebotomus*, S= *Sergentomyia*, M=male, F=female, BF=blood fed, Ch=chicken, Sh=sheep, G=goat, P=pigeon.

5.3.2. *Leishmania* DNA screening

Of the 2930 female specimens tested (915 individually and 2015 in 74 pools), three specimens (0.1%) were positive for *Leishmania* DNA: one *Ph. neglectus* and two *Ph. perfiliewi*. While the ssu rRNA (321 bp) and ITS1 (276 bp) PCRs gave positive results for all three samples, the cpb gene fragment (665 bp, 702 bp including primers) could only be amplified from the *Ph. neglectus* specimen. No nucleotide differences were observed between the respective ssu rRNA and cpb sequences of all three specimens. The kinetoplastid qPCR showed CT values of 24.9, 32.5, and 28.5 for the *Ph. neglectus* and the two *Ph. perfiliewi* specimens, respectively. Blast analysis results are given in Table 5.9. All three sand fly specimens positive for *Leishmania* DNA (3/828; 0.4%) originated from the same location (04/9): a cow farm in Semetisht village located in the Prizren district (Figure 5.7).

Table 5.9: Blast analysis results of generated sequences.

	Blast Identity Compared to Reference Sequences			
Gene	***L. infantum***	***L. donovani***	***L. tropica***	***L. major***
ssu rRNA [a]	100% (MN757921.1, MK495991.1)	100% (CP022642.1, ON934698.1)	99.69% (KF302745.1, GQ332363.1)	99.69% (MT560279.1) to 99.38% (MZ520154.1)
ITS1 [a]	100% (MZ362379.1, MN503527.1)	100% (MH202970.1, OQ184729.1)	92.39% (KC679052.1) to 90.78% (KY963131.1)	91.80% (MT423523.1) to 86.51% (ON243845.1)
Cpb [b]	100% (CP027841.1) to 97.89% (AY896778.1)	99.75% (EU637909.1) to 98.52% (AY896783.1)	90.86% (DQ286773.1) to 87.44% (JN400184.1)	90.15% (JN944175.1) to 88.92% (JN400175.1)

[a] same *Leishmania* DNA sequences were observed from all three sand fly specimens
[b] Blast algorithm neglects 39 bp deletion of *L. infantum*

5.3.3. Molecular characterization of *L. infantum* positive samples

Reference sequences were obtained from GenBank for further characterization of *Leishmania* DNA sequences. In total, 17 (ssu rRNA), 17 (ITS1), and 15 (cpb) sequences were downloaded, aligned, and subjected to pairwise distance (pd) analysis. Gaps in the alignment were excluded from the analysis. The overall mean distances were 0.2% (S.E. = 0.2), 6.6% (S.E. = 0.9), and 8.0% (S.E. = 0.8) for ssu rRNA, ITS1, and cpb genes, respectively. Pairwise distances were lowest between *L. infantum* and *L. donovani* for all three gene fragments (0.06%/0.3%/0.8%) (Table 5.10). For ssu rRNA, low pd was observed, ranging from 0.06 to 0.5%. For ITS1 and cpb, pd was low between *L. infantum* and *L. donovani* (0.3% and 0.8%), but comparably high between other species (4.7 to 22.0% for ITS1, 8.5 to 13.4% for cpb) (Table 5.10).

Table 5.10: Mean pairwise Kimura-2-parameter genetic distances (%) between analyzed Leishmania species based on three genes: ssu rRNA, ITS1, and cpb.

***Leishmania* spp.**	***L. infantum***	***L. donovani***	***L. tropica***	***L. major***	***L. mexicana***
L. infantum	-				
L. donovani	0.06/0.3/0.8	-			
L. tropica	0.3/4.7/9.3	0.4/5.0/9.9	-		
L. major	0.4/5.5/11.0	0.5/5.8/11.6	0.1/5.5/8.5	-	
L. mexicana	0.3/18.2/12.8	0.4/17.9/13.4	0.0/20.4/12.6	0.1/22.0/13.3	-

All sequences used for pd calculations were included in the ML analysis, and *L. mexicana* was used as an outgroup for all three genes. The low discriminatory power of the ssu rRNA gene was shown, as only two major clades were observed: clade I comprised *L. infantum* and *L. donovani*, whereas clade II comprised all other sequences of *L. tropica*, *L. major*, and *L. mexicana* (Figure 5.8a). ML analysis of ITS1 sequences revealed four clades, namely *L. donovani/infantum* (clade I), *L. tropica* (clade II), *L. major* (clade III), and *L. mexicana* (clade IV), thereby discriminating all species except *L. infantum* and *L. donovani* (Figure 5.8b).

The highest discriminatory power was shown for the cpb gene, which divided the included sequences into five clades, including the discrimination of *L. infantum* and *L. donovani*, supported by high bootstrap values (Figure 5.8c). The cpb sequence obtained in this study clustered with all

other *L. infantum* sequences (clade I), and a 39 bp deletion was observed for all *L. infantum* sequences in the alignment between positions 406 and 444, which is species-specific for *L. infantum* (Figure 5.8d). The complete alignment is provided in Appendix – Annex 3.

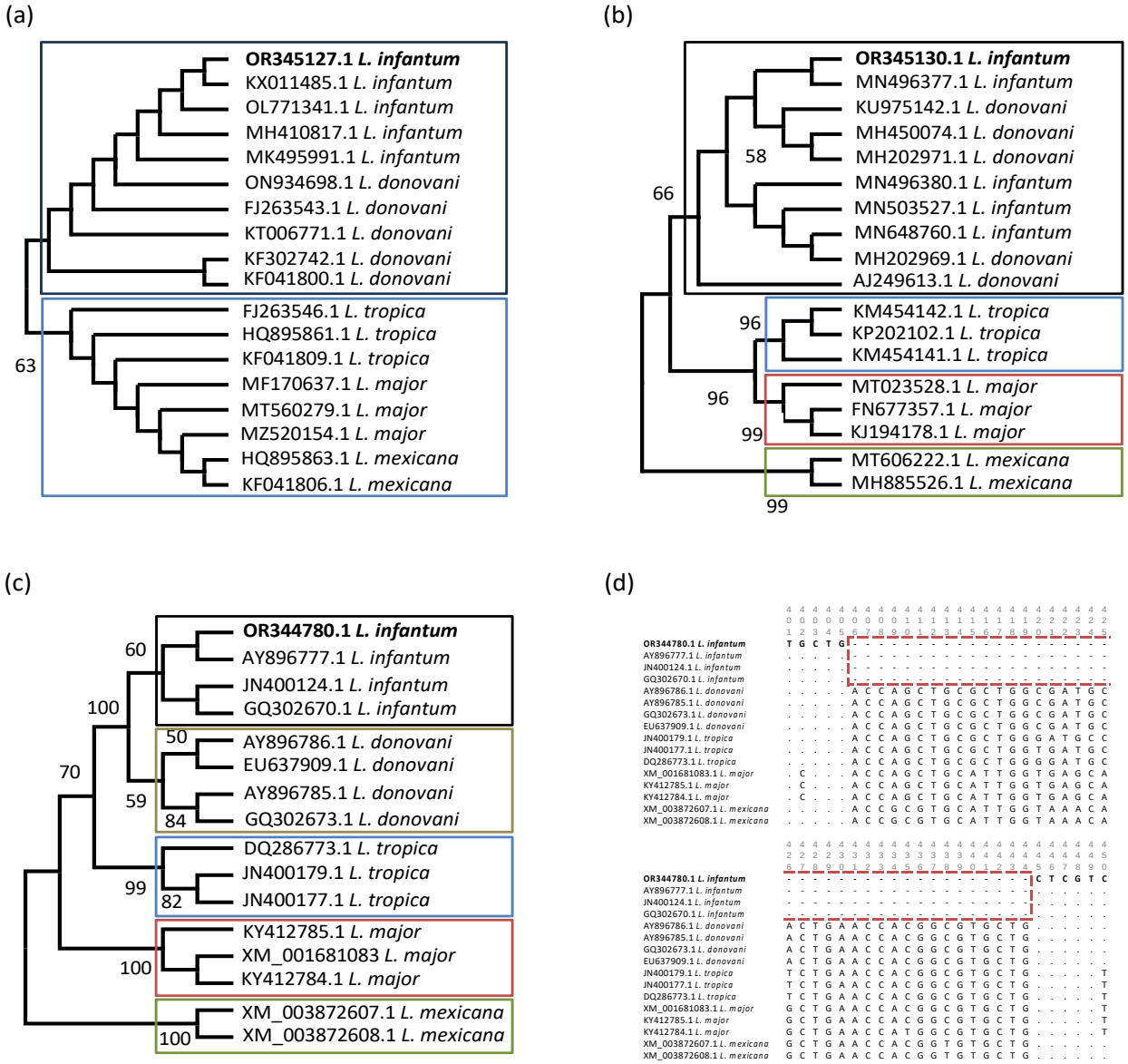


Figure 5.8: Discriminatory power of applied *Leishmania* PCRs. Maximum likelihood (ML) cladograms of *Leishmania* spp. based on three analyzed gene regions: ssu rRNA (a), ITS1 (b), and cpb (c). *L. mexicana* was used as an outgroup. Obtained sequences are shown, and they are marked with bold letters; colored squares indicate major clades observed. Bootstrap values over 50% are shown. Sequence alignment of *Leishmania spp*. showing the 39 bp deletion of *L. infantum* in the red dashed square (d).

5.4. Blood fed sand flies

Of all engorged females, 179 out of 497 were screened for host identification. The highest number of engorged females among the seven species found in the study were *Phlebotomus perfiliewi*, followed by *Phlebotomus neglectus*, *Phlebotomus simici*, *Phlebotomus tobbi*, and *Sergentomyia minuta*, whereas *Phlebotomus balcanicus* and *Phlebotomus papatasi* were not engorged.

The hosts of most species were *Bos taurus*, followed by *Ovis ammon*, *Homo sapiens*, *Canis lupus*, *Capra hircus*, *Gallus gallus*, and *Lepus europeanus* (Table 5.11).

Out of 179 tested sand flies, 13 samples could not be amplified. The *Leishmania* DNA screening results did not show any positive engorged female sand flies.

Table 5.11: Analysis for identification of the host in different engorged females.

Species	**Nr. of Blood fed**	***Bos taurus***	***Canis lupus***	***Capra hircus***	***Gallus gallus***	***Homo sapiens***	***Lepus europeanus***	***Ovis ammon***	**Not identified**
Ph. balcanicus	0	0	0	0	0	0	0	0	0
Ph. neglectus	62	51	2	0	0	3	0	2	4
Ph. papatasi	0	0	0	0	0	0	0	0	0
Ph. perfiliewi	109	87	3	3	1	2	1	4	8
Ph. simici	4	3	0	0	0	0	0	1	0
Ph. tobbi	2	2	0	0	0	0	0	0	0
Se. minuta	2	0	0	0	0	1	0	0	1
Total	**179**	**143**	**5**	**3**	**1**	**6**	**1**	**7**	**13**

6. DISCUSSION

This study provides a recent report of *Leishmania infantum* seropositivity in asymptomatic dogs in Kosovo, and the first detection of *Leishmania infantum* in sand flies. The wide distribution of anti-*Leishmania* antibodies in local dogs from six of the seven districts of Kosovo indicates an established leishmaniosis transmission cycles among asymptomatic dogs within the country. Meanwhile, the study on sand flies represents the most extensive sand fly sampling effort in Kosovo, covering all seven districts and including the first detection of *L. infantum* DNA in sand fly vectors. *Leishmania* DNA was detected in two sand fly species: *Phlebotomus neglectus* and *Phlebotomus perfiliewi*.

Overall, the seroprevalence rate of leishmaniosis in asymptomatic dogs was 4.21% in ELISA and 3.51% in IFAT, which complies with reports from previous studies in neighboring countries. Reported prevalence rates ranged from 5.1% to 10.6% in Albania, 2.5% to 20.3% in North Macedonia, 2.1% to 43.7% in Serbia, and 5.9% to 72.7% in Montenegro (for asymptomatic and symptomatic dogs, respectively) [17,32,96–100].

Historical data on the distribution of sand flies in Kosovo are limited to only a few studies reporting the presence of ten species, namely *Ph. major*, *Ph. neglectus*, *Ph. perfiliewi*, *Ph. perniciosus*, *Ph. tobbi*, *Ph. sergenti*, *Ph. papatasi*, *Ph. simici*, *Ph. balcanicus*, and *S. dentata* [101,102]. Several recent studies confirmed the presence of some of these species and added three new records to the species list, namely *Ph. mascittii*, *Ph. alexandri*, and *S. minuta* [103–105]. In our study, we trapped seven different species of two genera, namely *Phlebotomus neglectus, Ph. perfiliewi, Ph. tobbi, Ph. papatasi, Ph. simici, Ph. balcanicus and Sergentomyia minuta*, of which *Ph. perfiliewi* showed the highest trapping numbers, and *Ph. neglectus* was the most widespread sand fly species found. This was also observed by Dvorák et al. [105], but not by Vaselek et al. [103], who reported *Ph. neglectus* to be the most trapped and most widespread species in their survey. While this is the most extensive sand fly survey in Kosovo, we found fewer sand fly species than in the other two studies. However, some species, such as *Ph. mascittii* and *Ph. alexandri*, which were not detected in our study, were previously reported in very low numbers and at only a few locations. In this study, surveyed domestic and peridomestic sites as well as the sampling technique closely reflect

the sampling sites of previous surveys that report the presence of these two species in Balkan countries [103,105]. The absence of these species is attributed to their rarity in Kosovo and Balkan countries in general. Entomological sampling at or close to published locations of presence might further elucidate their actual distribution, particularly in southwestern parts of Kosovo.

The study on dog samples shows a non-significantly higher prevalence in male compared to female dogs. Similar findings were reported among *L. infantum*–infected dogs in Iran [106]. While we did not observe an age-dependent seroprevalence, dogs aged 8–10 years showed the highest seroprevalence, and dogs younger than 3 years did not have anti-*L. infantum* antibodies. In endemic areas, seroprevalence is often reported to increase with each passing year of life; thus, age is considered an important factor for the infection with *Leishmania* spp. [29,80]. On the contrary, other authors reported a bimodal age distribution of seroprevalence peaking at very young (1–2 years) and old age (7–8 years). This was explained by the breed composition of screened dogs, whereby some breeds (e.g. Boxer, German Shepherd) are more sensitive to the disease than others and have a higher chance of developing the disease at an early age. Latent infections may become active at a progressive age with immunosuppression or comorbidity [107]. The current study included only dogs without symptoms of CanL. There was no significant difference between the seropositive samples from dogs with normal and disrupted health status (cachexia, alopecia, lethargy, epistaxis, or skin lesions). Only one seropositive dog with disrupted health status showed moderate dermatitis with no other symptoms of the disease. Additionaly, there was no significant difference in seropositivity between the dogs with a mixed or specified breed status.

Of all reported sand fly species in Kosovo, *Ph. neglectus*, *Ph. perfliewi*, *Ph. tobbi*, and *Ph. balcanicus* are proven vectors of *L. infantum* [26,108,109]. In this study, *Leishmania* DNA was detected in *Ph. neglectus* and *Ph. perfiliewi*, both important vectors of *L. infantum* in Mediterranean countries. Based on the available data, *L. infantum* is the predominant *Leishmania* species circulating in the Balkan region. However, not all studies discriminate between species within the *L. donovani* complex. In this study, three different PCRs were performed for species confirmation and specification, and apparent differences in the specificity and sensitivity of the three applied PCRs were observed. A sensitive nested PCR approach targeting the SSU rRNA gene (Table 4.1) was used for the initial screening of all samples, with a significantly lower

discriminatory power [86,87]. A second PCR targeting the ITS1 region [88] confirmed all three positive samples; however, while discriminating all other *Leishmania* species as confirmed by ML analysis, the PCR lacks specificity to identify species within the *L. donovani* complex. A third species-specific PCR assay (cysteine protease B gene region) [89] for discriminating *L. infantum* and *L. donovani* gave a clear positive result only for the *Ph. neglectus* specimen, but only faint bands that could not be sequenced for the two *Ph. perfiliewi* specimens. It assumed that this is based on a lower sensitivity compared to the two other applied PCRs, and subsequent qPCR showed CT values of 24.9 for *Ph. neglectus* and higher values of 32.5 and 28.5 for the *Ph. perfiliewi* specimens. As stated by the Hide and Banuls [89], the cpb PCR can detect DNA yields of 50–100 pg. However, the sensitivity might be lower in mixed DNA samples. Therefore, establishing a nested PCR approach might be helpful for *L. donovani* complex typing from sand fly samples or other mixed samples with lower DNA yields compared to cultures or isolates. All positive specimens originated from the same cow farm in Semetisht village (Prizren district), representing an overall prevalence of 0.1% of sand flies positive for *Leishmania* DNA. Vaselek et al. [103], detected *L. tropica* DNA in one *Ph. neglectus* specimen (out of 118 tested) in Zhur (also in the Prizren district, approximately 35 km from Semetisht), close to the Albania border. Reports from neighboring countries such as Albania and Serbia have also reported the presence of *L. infantum* DNA in sand flies. In Serbia, *L. donovani/infantum* DNA was detected in three engorged females of *Ph. papatasi*, which is not regarded as its vector and thus probably only accidentally picking up parasites that would not establish an infection in the sand fly. In total, 80 specimens were tested, accounting for 4.0 % of total positives [110]. In Albania, 15% of pools made of 422 *Ph. neglectus* specimens were positive for *Leishmania* DNA [111], and in another survey, 3 of 55 mixed species pools including *Ph. neglectus* and/or *Ph. perfiliewi* were positive for DNA of the *L. donovani/infantum* complex [112]. However, these comparably higher prevalence rates compared to our study can be linked to specific sampling in areas with reported leishmaniosis cases. On the contrary, our survey represents a single-year, country-wide approach without targeted sampling. Interestingly, *Leishmania* spp. DNA has not yet been detected in sand flies from North Macedonia and Montenegro. However, leishmaniosis is circulating in dogs in all four neighboring countries of Kosovo [32].

The first report of CanL in Kosovo was published in 2008, reporting a seroprevalence of 1.7% [17]. Considering that samples analyzed by Lazri et al. [17] mainly originated from only three districts, namely Prishtina, Gjakova, and Ferizaj, our observed seroprevalence rates were more than four times higher in the Prishtina district and two times higher in the two other respective districts, clearly indicating increased transmission in the last decade. In another report from 2016, the prevalence rate of anti-*Leishmania* antibodies among stray dogs in the southwestern region of Kosovo was 18.4% [18]. The high discrepancies between these observed prevalence rates might result from a focally performed sample collection. While our seroprevalence results are represented by the seven districts of Kosovo, Xhekaj et al. [18] analyzed samples on a much smaller geographic scale, namely in four municipalities of Southwestern Kosovo, where subsequently high rates of neutralizing antibodies against *Toscana phlebovirus* (TOSV) and *Sicilian phlebovirus* (SFSV) have been detected in cattle [104]. Furthermore, *Ph. neglectus*, a proven vector for *L. infantum*, was observed to be the predominant sand fly species in these regions, which might contribute to locally established *L. infantum* transmission cycles [103]. Additionally, CanL caused by *L. infantum* is zoonotic and, thus, poses a significant medical risk for human infection. Although not significant, the highest seroprevalence was detected in the most populated Prishtina district, which also constitutes the homonymous capital city of the country (with more than 200,000 inhabitants). Considering the high number of dog shelters and stray dogs in this district, particularly in the capital city, as well as the potential zoonotic spillover of *L. infantum* to humans, this area might be of high public health relevance. However, there is little data on human leishmaniasis in the Prishtina district. A study on the detection of anti-*Leishmania* antibodies in Austrian Armed Forces returning from Kosovo showed that 55 out of 261 soldiers (21.1%) had anti-*Leishmania* antibodies, of these 13.8% showed positive and 7.3% showed borderline results. These results demonstrate a considerable risk of exposure to *Leishmania* spp. in Kosovo and emphasize the need for a one-health approach [113]. According to the World Health Organization (WHO), leishmaniosis is one of the most significant and neglected tropical diseases. The main etiologic agent of canine visceral leishmaniosis is *Leishmania infantum*, which is a major global zoonotic parasite potentially fatal to both dogs and humans [11].

Diagnosis of leishmaniosis is complex and is an example of an infection with overt clinical signs of disease due to the high prevalence of subclinical infection [13]. To diagnose CanL, serological tests are a useful tool to detect specific antibodies against *Leishmania* spp. and to determine the

spread of the disease, particularly among the high number of asymptomatic dogs. In our study, we applied two tests, ELISA and IFAT, considering that these are the most frequently used serological methods. ELISA is a highly sensitive method for screening large numbers of samples in epidemiological studies and is well-suited to be applied in field conditions [14]. Whereas the IFAT is considered the gold standard test. The assessment of agreement between the tests is indicative of test validity. With a kappa value of 0.81, signifying perfect agreement between the tests, the obtained results for determining the seroprevalence of canine leishmaniosis in dogs in Kosovo using the aforementioned commercial tests are deemed reliable and valid. The seroprevalence rates detected by ELISA were generally higher compared to IFAT results, with one exception of the Gjakova district. These results are in line with other studies, in which ELISA tests showed higher sensitivities compared to IFAT, particularly lacking sensitivity in samples of asymptomatic dogs [114,115]. Thus, as we screened dogs without apparent signs of CanL, better diagnostic performance by ELISA is not surprising. Furthermore, the results of IFAT vary based on the subjective interpretation of the operator's skills and different cut-of titers are used by laboratories [116]. Based on the exceptionally high diagnostic performance of our applied ELISA kit (ID Screen®) observed by Solano-Gallego et al. [65], ELISA-based methods should be favored over IFAT when screening asymptomatic dog populations. However, we need to highlight that the sensitivity of antibody detection is generally lower in early or in asymptomatic canine infections, and the possibility of false-negative samples should not be neglected [117].

The current study highlights the urgent need to enhance knowledge about the disease among health authorities in Kosovo. Dog owners should receive more information on how to protect their pets from infection and stop the transmission of the disease. Currently, four vaccines against leishmaniosis in dogs have been commercialized: Leishmune®, Leish-Tec®, CaniLeish®, and Letifend®. However, none of these is licensed in Kosovo. Baxarias et al. [73], reported that 39.8% of dog owners in Europe vaccinate their dogs against leishmaniosis, whereas 86.2% apply repellents as the first mode of action. Insecticide-impregnated dog collars (e.g. deltamethrin, imidacloprid, or permethrin) are highly effective in protecting dogs from sand fly bites. Thereby, uncollared dogs living with or close to collard dogs were shown to have a 3.5 times higher risk of being infected [118]. In Kosovo, pet dogs are mainly kept outside. Considering the high number of stray dogs as well as their frequent contact with pet dogs, the broad application of insecticide-

impregnated dog collars is highly recommended, particularly in regions with high *Leishmania* seroprevalences.

The present study underlines the necessity to increase the capacity of local authorities for better control of CanL in Kosovo. While research on sand fly distribution and CanL in Kosovo has advanced recently, published data on human leishmaniasis are scarce. In recent years, the number of human leishmaniasis cases in Kosovo has been increasing (unpublished data). The future control and prevention of leishmaniasis in Kosovo should be based on an integrated one-health approach involving surveillance of animals, sand flies, and humans.

7. CONCLUSIONS

This study presents the epidemiology of canine leishmaniosis in Kosovo and the role of *Phlebotomus* spp. in its spread. Samples were taken in all districts of Kosovo, and results were achieved using different diagnostic methods.

The main conclusions of the research presented in this thesis can be summarized as follows:

- Canine leishmaniosis was qualitatively confirmed in Kosovo. The prevalence rate of CanL antibodies among asymptomatic dogs in all seven districts of Kosovo was different in both ELISA and IFAT tests.
- It is indicative that *L. infantum* transmission cycles are active in different districts of Kosovo, particularly in the Prishtina district. The distribution of positive ELISA and IFAT samples by districts was different. The highest rate was found in Prishtina, followed by Gjakova, Prizreni, Ferizaj, Peja, and Gjilani. The Mitrovica district samples were negative.
- Quantitatively, there was no association found between seropositivity and asymptomatic dogs, nor with the regional distribution, sex, age, breed, and health status. The distribution of seropositive samples by gender was higher on male dogs compared to female. The distribution of seropositive samples by age on ELISA and IFAT was highest to dogs between 4–6 years, followed by dogs between 8–10 years, then dogs between 3–4 years, followed by dogs 6–8 years, and dogs older than 10 years, the dogs between 1–2 and 2–3 years old were all negative. The distribution of seropositive samples by health status on ELISA and IFAT was higher to normal dogs than for disrupted health status dogs, respectively. The distribution of seropositive samples by breed status on ELISA and IFAT was higher for the mix-breed, than for the specified-breed. There were no significant differences among the different districts, gender, ages, health status, and breed.
- Different species of *Phlebotomus* spp. were detected. Seven species of two genera were identified, namely *Ph. perfiliewi*, *Ph. neglectus*, *Ph. tobbi*, *Ph. simici*, *Ph. balcanicus*, *Ph. papatasi*, and *Sergentomyia minuta*.
- The detection of *L. infantum* DNA in *Ph. neglectus* and *Ph. perfiliewi*, along with previous seroprevalence data, suggests the existence of endemic leishmaniosis transmission cycles

in Kosovo. Out of all female specimens tested, three specimens were positive for *Leishmania* DNA: one *Ph. neglectus* and two *Ph. perfiliewi*. All three sand fly specimens positive for *Leishmania* DNA originated from the same location (04/9): a cow farm in Semetisht village located in the Prizren district (See supplementary table).

- The predominance of *Ph. neglectus* and *Ph. perfiliewi* and their universal occurrence in Kosovo corroborates their involvement in *L. infantum* transmission. *Phlebotomus perfiliewi* and *Ph. neglectus* were the only species found in all seven districts, whereas *Ph. balcanicus* was found only in Gjakova, and *Ph. papatasi* was found only in Peja.

Recommendations for future work related to this topic to fully elucidate *Leishmania* transmission cycles in Kosovo are as follows:

- Future studies should include domestic animals and wildlife in screening approaches.
- Future studies should include autochthonous human cases.
- Faunistic and seasonal studies are recommended, with targeted sand fly surveillance in the respective districts.

8. REFERENCES

1. Latrofaa, M.S.; Iattaa, R.; Dantas-Torresa, F.; Annoscia, G.; Gabrielli, S.; Pombi, M.; Gradoni, L.; Otranto, D. Detection of Leishmania Infantum DNA in Phlebotomine Sand Flies from an Area Where Canine Leishmaniosis Is Endemic in Southern Italy. *Vet. Parasitol.* **2018**, *253*, 39–42, doi:10.1016/j.vetpar.2018.02.006.

2. Bennai, K.; Tahir, D.; Lafri, I.; Bendjaballah-Laliam, A.; Bitam, I.; Parola, P. Molecular Detection of Leishmania Infantum DNA and Host Blood Meal Identification in Phlebotomus in a Hypoendemic Focus of Human Leishmaniasis in Northern Algeria. *PLoS Negl. Trop. Dis.* **2018**, *12*, doi:10.1371/journal.pntd.0006513.

3. Derghal, M.; Tebai, A.; Balti, G.; Souguir-Omrani, H.; Chemkhi, J.; Rhim, A.; Bouattour, A.; Guizani, I.; M'Ghirbi, Y.; Guerbouj, S. High-Resolution Melting Analysis Identifies Reservoir Hosts of Zoonotic Leishmania Parasites in Tunisia. *Parasit. Vectors* **2022**, *15*, 12, doi:10.1186/s13071-021-05138-x.

4. WHO; Fact Sheets Leishmaniasis in the WhO European Region. **2016**, (accessed on 09/02/2024).

5. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. *Parasit. Vectors* **2011**, *4*, 86, doi:10.1186/1756-3305-4-86.

6. El Goulli, A.F.; Zribi, L.; Sanhaji, R.; Chabchoub, A.; Bouratbine, A.; Gharbi, M.; Abdelmelek, H. Study of Ocular Manifestations and Humoral Immune Response in Eyes of Dogs with Leishmaniasis. *Vet. Med. Sci.* **2023**, *9*, 625–637, doi:10.1002/vms3.982.

7. Mendoza-Roldan, J.A.; Zatelli, A.; Latrofa, M.S.; Iatta, R.; Bezerra-Santos, M.A.; Annoscia, G.; Gernone, F.; Votypka, J.; Modry, D.; Volf, P.; et al. Leishmania (Sauroleishmania) Tarentolae Isolation and Sympatric Occurrence with Leishmania (Leishmania) Infantum in Geckoes, Dogs and Sand Flies. *PLoS Negl. Trop. Dis.* **2022**, *16*, 1–16, doi:10.1371/journal.pntd.0010650.

8. Colella, V.; Hodžić, A.; Iatta, R.; Baneth, G.; Alić, A.; Otranto, D. Zoonotic Leishmaniasis, Bosnia and Herzegovina. *Emerg. Infect. Dis.* **2019**, *25*, 385–386, doi:10.3201/eid2502.181481.

9. Jain, K.; Jain, N.K. Vaccines for Visceral Leishmaniasis: A Review. *J. Immunol. Methods* **2015**, *422*, 1–12, doi:10.1016/j.jim.2015.03.017.

10. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological Findings and Detection of Toll-like Receptor 2 in Cutaneous Lesions of Canine Leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163, doi:10.1016/j.vetpar.2015.03.004.

11. Peris, M.P.; Esteban-Gil, A.; Ortega-Hernández, P.; Morales, M.; Halaihel, N.; Castillo, J.A. Comparative Study of Real-Time PCR (TaqMan Probe and Sybr Green), Serological Techniques (ELISA, IFA and DAT) and Clinical Signs Evaluation, for the Diagnosis of Canine Leishmaniasis in Experimentally Infected Dogs. *Microorganisms* **2021**, *9*, doi:10.3390/microorganisms9122627.

12. Morea, A.; Stufano, A.; Sgroi, G.; Benedictis, L. De; Bezerra-Santos, M.A.; Mendoza-Roldan, J.A.; Veneziano, V.; Otranto, D.; Lovreglio, P.; Iatta, R. Seroprevalence of Leishmania Infantum in Outdoor Workers from Southern Italian Endemic Regions. *Zoonoses Public Health* **2023**, 459–464, doi:10.1111/zph.13041.

13. Strube, C.; Mehlhorn Heinz. *Dog Parasites Endangering Human Health*; 2021; Vol. 13; ISBN 978-3-030-53229-1.

14. Selim, A.; Shoulah, S.; Abdelhady, A.; Alouffi, A.; Alraey, Y.; Al-Salem, W.S. Seroprevalence and Risk Factors Associated with Canine Leishmaniasis in Egypt. *Vet. Sci.* **2021**, *8*, 236, doi:10.3390/vetsci8100236.

15. Morales-Yuste, M.; Martin-Sanchez, J.; Corpas-Lopez, V. Canine Leishmaniasis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. *Vet. Sci.* **2022**, *9*, 1–20, doi:10.3390/vetsci9080387.

16. Kaszak, I.; Planellas, M.; Dworecka-Kaszak, B. Canine Leishmaniosis – an Emerging Disease. *Ann. Parasitol.* **2015**, *61*, 69–76.

17. Lazri, T.; Duscher, G.; Edelhofer, R.; Bytyci, B.; Gjino, P.; Joachim, A. Infektionen Mit Arthropodenübertragenen Parasiten Bei Hunden Im Kosovo Und in Albanien Unter Besonderer Berücksichtigung Der Leishmanieninfektionen. *Wien. Klin. Wochenschr.* **2008**, *120*, 54–58, doi:10.1007/s00508-008-1076-4.

18. Xhekaj, B.; Alishani, M.; Rexhepi, A.; Jakupi, X.; Sherifi, K. Serological Survey of Canine Leishmaniasis in Southwestern Region of Kosovo. *Vet. Ital.* **2020**, *56*, 47–50, doi:10.12834/VetIt.1345.7407.5.

19. WHO. *Control of the Leishmaniasis: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010.*; 2010;

20. Sykes, J.E. *Canine and Feline Infectious Diseases*; 2014; ISBN 9781437707953.

21. Ayele, A.; Seyoum., Z. A Review on Canine Leishmaniasis; Etiology, Clinical Sign, Pathogenesis, Treatment and Control Methods. *Glob. Vet.* **2016**, *4*, 343–352, doi:10.5829/idosi.gv.2016.17.04.104151.

22. Montaner-Angoiti, E.; Llobat, L. Is Leishmaniasis the New Emerging Zoonosis in the World? *Vet. Res. Commun.* **2023**, doi:10.1007/s11259-023-10171-5.

23. Mullen, G.R.; Durden, L.A. *Medical and Veterinary Entomology*; Elsevier, 2019; ISBN 978-0-12-814043-7.

24. Taylor, M.A.; Coop, R.L.; Wall, R.L. *Veterinary Parasitology*; 2016; ISBN 978-0-470-67162-7.

25. Service, M. *Medical Entomology for Students*; 2012; ISBN 978-1-107-66818-8.

26. Ready, P.D. Leishmaniasis Emergence in Europe. *Euro Surveill.* **2010**, *10*, 1–11.

27. Greene, C.E. *Infectious Diseases of the Dog and Cat*; 2012; ISBN 978-1-4160-6130-4.

28. Pal, M.; Ejeta, I.; Girma, A.; Dave, K.; Dave, P. Etiology, Clinical Spectrum, Epidemiology, Diagnosis, Public Health Significance and Control of Leishmaniasis: A Comprehensive Review. *Acta Sci. Microbiol.* **2022**, *5*, 140–151, doi:10.31080/ASMI.2022.05.1066.

29. Rombolà, P.; Barlozzari, G.; Carvelli, A.; Scarpulla, M.; Iacoponi, F.; Macrì, G. Seroprevalence and Risk Factors Associated with Exposure to Leishmania Infantum in Dogs, in an Endemic Mediterranean Region. *PLoS One* **2021**, *16*, 1–13, doi:10.1371/journal.pone.0244923.

30. Maia, C.; Conceicão, C.; Pereira, A.; Rocha, R.; Ortuño, M.; Muñoz, C.; Jumakanova, Z.; Perez-Cutillas, P.; Ozbel, Y.; Toz, S.; et al. The Estimated Distribution of Autochthonous Leishmaniasis by Leishmania Infantum in Europe in 2005-2020. *PLoS Negl. Trop. Dis.* **2023**, *17*, 1–25, doi:10.1371/journal.pntd.0011497.

31. ESCCAP Control of Vector-Borne Diseases in Dogs and Cats. In; 2019; pp. 1–42 ISBN 978-1-907259-69-2.

32. Vaselek, S. Canine Leishmaniasis in Balkan – A Review of Occurrence and Epidemiology. *Acta Trop.* **2021**, *224*, doi:10.1016/j.actatropica.2021.106110.

33. Dantas-Torres, F. The Role of Dogs as Reservoirs of Leishmania Parasites, with Emphasis on Leishmania (Leishmania) Infantum and Leishmania (Viannia) Braziliensis. *Vet. Parasitol.* **2007**, *149*, 139–146, doi:10.1016/j.vetpar.2007.07.007.

34. Ribeiro, R.R.; Michalick, M.S.M.; da Silva, M.E.; Santos, C.C.P. dos; Frézard, F.J.G.; da Silva, S.M. Canine Leishmaniasis: An Overview of the Current Status and Strategies for Control. *Biomed Res. Int.* **2018**, *2018*, doi:10.1155/2018/3296893.

35. Dennis, J.; Fox, M.; Gibbons, L.; Hermosilla, C. *Principles of Veterinary Parasitology*; 2016;

36. Bowman, D.D. *Georgis' Parasitology for Veterinarians*; 2021; ISBN 978-0-323-54396-5.

37. Cecílio, P.; Cordeiro-da-Silva, A.; Oliveira, F. Sand Flies: Basic Information on the Vectors of Leishmaniasis and Their Interactions with Leishmania Parasites. *Commun. Biol.* **2022**, *5*, 305, doi:10.1038/s42003-022-03240-z.

38. González, M.A.; Ruiz-Arrondo, I.; Gutiérrez-López, R.; Barceló, C.; Miranda, M.Á. First Record of Phlebotomus (Larroussius) Perfiliewi (Diptera: Psychodidae), Vector of Leishmania Infantum and Phleboviruses, in Spain. *Diversity* **2023**, *15*, doi:10.3390/d15030400.

39. Zé-Zé, L.; Amaro, F.; Osório, H.C.; Giovanetti, M.; Lourenço, J.; Alves, M.J. Molecular Identification and Ecology of Portuguese Wild-Caught Phlebotomine Sandfly Specimens. *Zoonotic Dis.* **2022**, *2*, 19–31, doi:10.3390/zoonoticdis2010003.

40. Tsirigotakis, N.; Pavlou, C.; Christodoulou, V.; Dokianakis, E.; Kourouniotis, C.; Alten, B.; Antoniou, M. Phlebotomine Sand Flies (Diptera: Psychodidae) in the Greek Aegean Islands: Ecological Approaches. *Parasit. Vectors* **2018**, *11*, 1–14, doi:10.1186/s13071-018-2680-4.

41. Elhadi, F.; Babeker, A.; Elhaj, H.; Alahmri, F.; Almuflh, L.; Alkibbashi, R. Entomological Survey of Sandflies Vector (Diptera: Psychodidae: Phlebotominae) in Asser Region, Saudi Arabia. *Eur. J. Biol. Biotechnol.* **2022**, *3*, 47–50, doi:10.24018/ejbio.2022.3.1.317.

42. Michael, L. *The Biology of Blood-Sucking in Insects.*; 2005; ISBN 978-0-521-83608-1.

43. Munstermann, L.E. *Phlebotomine Sand Flies and Moth Flies (Psychodidae)*; Elsevier Inc., 2018;

44. Wall, R.; Shearer, D. *Veterinary Entomology.*; Springer Netherlands: Dordrecht, 1997; ISBN 978-0-412-61510-8.

45. Eldridge, B.F.; Edman, J.D. *Medical Entomology.*; Eldridge, B.F., Edman, J.D., Eds.; Springer Netherlands: Dordrecht, 2004; ISBN 978-1-4020-1794-0.

46. Alexander, B. Sampling Methods for Phlebotomine Sandfies. *Med. Vet. Entomol.* **2000**, *14*, 109–122.

47. Huemer, H.; Prudhomme, J.; Amaro, F.; Baklouti, A.; Walder, G.; Alten, B.; Moutailler, S.; Ergunay, K.; Charrel, R.N.; Ayhan, N. Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part II: Important Points to Consider for Fieldwork and and Subsequent Virological Screening. *Vector-Borne Zoonotic Dis.* **2017**, *17*, 81–90, doi:10.1089/vbz.2016.1965.

48. Alten, B.; Ozbel, Y.; Ergunay, K.; Kasap, O.E.; Cull, B.; Antoniou, M.; Velo, E.; Prudhomme, J.; Molina, R.; Bañuls, A.-L.; et al. Sampling Strategies for Phlebotomine Sand Flies (Diptera: Psychodidae) in Europe. *Bull. Entomol. Res.* **2015**, *105*, 664–678, doi:10.1017/S0007485315000127.

49. ECDC and EFSA; *Field Sampling Methods for Mosquitoes, Sandflies, Biting Midges and Ticks.*; 2018;

50. Baxarias, M.; Jornet-rius, O.; Donato, G.; Mateu, C.; Alcover, M.M.; Pennisi, M.G.; Solano-Gallego, L. Signalment, Immunological and Parasitological Status and Clinicopathological Findings of Leishmania-Seropositive Apparently Healthy Dogs. *Animals* **2023**, *13*, doi:10.3390/ani13101649.

51. Saridomichelakis, M.N. Advances in the Pathogenesis of Canine Leishmaniosis: Epidemiologic and Diagnostic Implications. *Vet. Dermatol.* **2009**, *20*, 471–489, doi:10.1111/j.1365-3164.2009.00823.x.

52. Molina, C.C.; Dias, M.J.; Domingues, T.D.; Englar, R.E.; Leal, R.O. Clinical Findings and Prognostic Factors for Mortality in Hospitalized Dogs with Leishmaniosis: Aretrospective Study. *Comp. Immunol. Microbiol. Infect. Dis.* **2023**, *101*, doi:10.1016/j.cimid.2023.102041.

53. Symeonidou, I.; Angelou, A.; Theodoridis, A.; Sioutas, G.; Papadopoulos, E. Canine Leishmaniosis in Greece: An Updated Countrywide Serological Study and Associated Risk Factors. *Pathogens* **2021**, *10*, doi:10.3390/pathogens10091129.

54. Koutinas, A.F.; Koutinas, C.K. Pathologic Mechanisms Underlying the Clinical Findings in Canine Leishmaniosis Due to Leishmania Infantum/Chagasi. *Vet. Pathol.* **2014**, *51*, 527–538, doi:10.1177/0300985814521248.

55. Veras, P.S.T.; Fraga, D.B.M.; Solcà, M. da S.; Guedes, C.E.S. New Advances in the Diagnosis of Canine Visceral Leishmaniasis. *Intech open Sci.* **2014**, 239–273, doi:10.5772/57573.

56. Macia, M.; Marín-García, P.-J.; Ahuir-Baraja, A.-E.; Llobat, L. Immunological Profile of Two Canine Breeds in an Endemic Region of Leishmania Infantum. *Vet. Parasitol. Reg. Stud. Reports* **2023**, *40*, doi:10.1016/j.vprsr.2023.100861.

57. Rigo, R.S.; Carvalho, C.M.E.; Honer, M.R.; Andrade, G.B. de; Silva, I.S.; Rigo, L.; Figueiredo, H.R.; Barreto, W.T.G. Renal Histopathological Findings in Dogs with Visceral Leishmaniasis. *Rev. Inst. Med. Trop. Sao Paulo* **2013**, *55*, 113–116, doi:10.1590/S0036-46652013000200008.

58. Baneth, G.; Koutinas, A.F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends Parasitol.* **2008**, *24*, doi:10.1016/j.pt.2008.04.001.

59. Alvar, J.; Canavate, C.; Molina, R.; Moreno, J.; Nieto, J. Canine Leishmaniasis. *Elsevier* **2004**, *57*, doi:10.1016/S0065-308X(04)57001-X.

60. Kniha, E.; Aspöck, H.; Auer, H.; Walochnik, J. Leishmania Infections and Leishmania Species in Central Europe. *Wien Tierarztl Monat – Vet Med Austria* **2023**, *110*, doi:10.5680/wtm000015.

61. Takamiya, N.T.; Rogerio, L.A.; Torres, C.; Leonel, J.A.F.; Vioti, G.; Oliveira, T.M.F. de S.; Valeriano, K.C.; Porcino, G.N.; Santos, I.K.F. de M.; Costa, C.H.N.; et al. Parasite Detection in Visceral Leishmaniasis Samples by Dye-Based QPCR Using New Gene Targets of Leishmania Infantum and Crithidia. *Trop. Med. Infect. Dis.* **2023**, *8*, doi:10.3390/ tropicalmed8080405.

62. Beugnet, F.; Halos, L.; Guillot, J. *Textbook of Clinical Parasitology in Dogs and Cats*; 2018; ISBN 978-2-9550805-2-8.

63. Siqueira, W.F.; Cardoso, M.S.; Fraga, V.G.; Ottino, J.; Ribeiro, V.M.; Gondim, C.N.; Barcante, J.M. de P.; Gomes, A.C.A.; Galdino, A.S.; Eersels, K.; et al. Immunogenic Mapping of RDyn-1 and RKDDR- plus Proteins and Selection of Oligopeptides by Immunoblotting for the Diagnosis of Leishmania Infantum-Infected Dogs. *PLoS Negl. Trop. Dis.* **2023**, *17*, doi:10.1371/journal. pntd.0011535.

64. Cavalera, M.A.; Zatelli, A.; Donghia, R.; Mendoza-Roldan, J.A.; Gernone, F.; Otranto, D.; Iatta, R. Conjunctival Swab Real Time-PCR in Leishmania Infantum Seropositive Dogs: Diagnostic and Prognostic Values. *Biology (Basel).* **2022**, *11*, doi:10.3390/biology11020184.

65. Solano-Gallego, L.; Villanueva-Saz, S.; Carbonell, M.; Trotta, M.; Furlanello, T.; Natale, A. Serological Diagnosis of Canine Leishmaniosis: Comparison of Three Commercial ELISA Tests (Leiscan ®, ID Screen ® and Leishmania 96 ®), a Rapid Test (Speed Leish K ®) and an in-House IFAT. *Parasit. Vectors* **2014**, *7*, doi:10.1186/1756-3305-7-111.

66. Florin-Christensen, M.; Schnittger, L. *Parasitic Protozoa of Farm Animals and Pets.*; 2018; ISBN 978-3-319-70131-8.

67. Paiva-Cavalcanti, M. de; Morais, R.C.S. de; Pessoa-e-Silva, R.; Trajano-Silva, L.A.M.; Gonçalves-de-Albuquerque, S. da C.; Tavares, D. de H.C.; Brelaz-de-Castro, M.C.A.; Silva, R. de F. e; Pereira, V.R.A. Leishmaniases Diagnosis: An Update on the Use of Immunological and Molecular Tools. *Cell Biosci.* **2015**, *5*, doi:10.1186/s13578-015-0021-2.

68. Paltrinieri, S.; Solano-Gallego, L.; Fondati, A.; Lubas, G.; Gradoni, L.; Castagnaro, M.; Crotti, A.; Maroli, M.; Oliva, G.; Roura, X.; et al. Guidelines for Diagnosis and Clinical Classification of Leishmaniasis in Dogs. *J Am Vet Med Assoc* **2010**, *236*, 1184–1191.

69. WOAH. *Leishmaniosis*; 2021;

70. Gizzarelli, M.; Manzillo, V.F.; Inglese, A.; Montagnaro, S.; Oliva, G. Retrospective Long-Term Evaluation of Miltefosine-Allopurinol Treatment in Canine Leishmaniosis. *Pathogens* **2023**, *12*, doi:10.3390/pathogens12070864.

71. Oliva, G.; Roura, X.; Crotti, A.; Maroli, M.; Castagnaro, M.; Gradoni, L.; Lubas, G.; Paltrinieri, S.; Zatelli, A.; Zini, E. Guidelines for Treatment of Leishmaniasis in Dogs. *J Am Vet Med Assoc* **2010**, *236*, 1192–1198.

72. Solano-Gallego, L.; Koutinas, A.; Miro, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G.. Directions for the Diagnosis, Clinical Staging, Treatment and Prevention of Canine Leishmaniosis. *Vet. Parasitol. J.* **2009**, *165*, 1–18, doi:10.1016/j.vetpar.2009.05.022.

73. Baxarias, M.; Homedes, J.; Mateu, C.; Attipa, C.; Solano-Gallego, L. Use of Preventive Measures and Serological Screening Tools for Leishmania Infantum Infection in Dogs from Europe. *Parasit. Vectors* **2022**, *15*, 1–11, doi:10.1186/s13071-022-05251-5.

74. Otranto, D.; Dantas-Torres, F. The Prevention of Canine Leishmaniasis and Its Impact on Public Health. *Trends Parasitol.* **2013**, *29*, 339–345, doi:10.1016/j.pt.2013.05.003.

75. Velez, R.; Gállego, M. Commercially Approved Vaccines for Canine Leishmaniosis: A Review of Available Data on Their Safety and Efficacy. *Trop. Med. Int. Heal.* **2020**, *25*, 540–557, doi:10.1111/tmi.13382.

76. Miró, G.; Petersen, C.; Cardoso, L.; Bourdeau, P.; Baneth, G.; Solano-Gallego, L.; Pennisi, M.G.; Ferrer, L.; Oliva, G. Novel Areas for Prevention and Control of Canine Leishmaniosis. *Trends Parasitol.* **2017**, *33*, 718–730, doi:10.1016/j.pt.2017.05.005.

77. Yadav, P.; Azam, M.; Ramesh, V.; Singh, R. Unusual Observations in Leishmaniasis — An Overview. *Pathogens* **2023**, *12*, doi:10.3390/ pathogens12020297.

78. Bharati, K. Human Genetic Polymorphism and Leishmaniasis. *Infect. Genet. Evol.* **2022**, *98*, doi:10.1016/j.meegid.2021.105203.

79. Andric, B.; Terzic, D.; Dupanovic, B.; Andric, A. Public Health Aspects of Visceral Leishmaniasis in Montenegro. *Open J. Clin. Diagnostics* **2013**, *3*, 195–201, doi:10.4236/ojcd.2013.34035.

80. Tamponi, C.; Scarpa, F.; Carta, S.; Knoll, S.; Sanna, D.; Gai, C.; Pipia, A.P.; Dessì, G.; Casu, M.; Varcasia, A.; et al. Seroprevalence and Risk Factors Associated with Leishmania Infantum in Dogs in Sardinia (Italy), an Endemic Island for Leishmaniasis. *Parasitol. Res.* **2021**, 289–300, doi:10.1007/s00436-020-06973-0.

81. Dantas-Torres, F.; Miró, G.; Baneth, G.; Bourdeau, P.; Breitschwerdt, E.; Capelli, G.; Cardoso, L.; Day, M.J.; Dobler, G.; Ferrer, L.; et al. Canine Leishmaniasis Control in the Context of One Health. *Emerg. Infect. Dis.* **2019**, *25*, doi:10.3201/eid2512.190164.

82. Buleshkaj, S.H.; Rugova, N. Challenges in Treating Cancer in Post-Conflict Kosovo, a Systematic Review. *Res. Sq.* **2022**, 1–20, doi:10.21203/rs.3.rs-1233024/v1.

83. USAID. *Country Profile Property Right and Resource Governance. Kosovo.*; 2015;

84. Lewis, D.J. A Taxonomic Review of the Genus Phlebotomus (Diptera: Psychodidae). *Bull. Br. Museum* **1982**, *45*, 121–209.

85. Dantas-Torres, F.; Tarallo, V.D.; Otranto, D. Morphological Keys for the Identification of Italian Phlebotomine Sand Flies (Diptera: Psychodidae: Phlebotominae). *Parasites and Vectors* **2014**, *7*, doi:10.1186/s13071-014-0479-5.

86. van Eys, G.J.J.M.; Schoone, G.J.; Kroon, N.C.M.; Ebeling, S.B. Sequence Analysis of Small Subunit Ribosomal RNA Genes and Its Use for Detection and Identification of Leishmania Parasites. *Mol. Biochem. Parasitol.* **1992**, *51*, 133–142.

87. Cruz, I.; Canavate, C.; Rubio, J.M.; Morales, M.A.; Chicharro, C.; F.Laguna; M.Jimenez-Majias; Sirera, G.; Videla, S.; Alvar, J. A Nested Polymerase Chain Reaction (Ln-PCR) for Diagnosing and Monitoring Leishmania Infantum Infection in Patients Co-Infected with Human Immunodeficiency Virus. *Trans. R. Soc. Trop. Med. Hyg.* **2002**, *96*, 185–189.

88. El Tai, N.O.; Osman, O.F.; El Fari, M.; Presber, W.; Schonian, G. Genetic Heterogeneity of Ribosomal Internal Transcribed Spacer in Clinical Samples of Leishmania Donovani Spotted on Filter Paper as Revealed by Single-Strand Conformation Polymorphisms and Sequencing. *Trans. R. Soc. Trop. Med. Hyg.* **2000**, *94*, 575–579.

89. Hide, M.; Banuls, A.-L. Species-Specific PCR Assay for L. Infantum/L. Donovani Discrimination. *Acta Trop.* **2006**, *100*, 241–245, doi:10.1016/j.actatropica.2006.10.012.

90. Mary, C.; Faraut, F.; Lascombe, L.; Dumon, H. Quantification of Leishmania Infantum DNA by a Real-Time PCR Assay with High Sensitivity. *J. Clin. Microbiol.* **2004**, *42*, 5249–5255, doi:10.1128/JCM.42.11.5249.

91. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299.

92. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; Mcgettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X Version 2.0. *Bioinformatics.* **2007**, *23*, 2947–2948, doi:10.1093/bioinformatics/btm404.

93. Nicholas, K.B. Genedoc: A Tool for Editing and Annotating Multiple Sequence Alignments. **1997**.

94. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549, doi:10.1093/molbev/msy096.

95. QGIS Development Team. QGIS Geographic Information System. Open Source Geospatial Foundation Project. 2019. Available Online: Http://Qgis.Osgeo.Org (Accessed on 26 January 2024).

96. Bizhga, B.; Laci, D.; Dhamo, G.; Keci, R.; Belegu, K.; Bakiasi, I.; Turmalaj, L. Survey for Canine Leishmaniosis. *J. Anim. Vet. Adv.* **2013**, *12*, 442–446.

97. Pavicevic, Z.; Lausevic, D.; Pejovic, N. Seroprevalence of Dogs to Leishmania Infantum Antigens in the Territory of Crna Gora. *Vet. Glas.* **2014**, *68*, 301–309, doi:10.2298/VETGL1406301P.

98. Hamel, D.; Shukullari, E.; Rapti, D.; Silaghi, C.; Pfister, K.; Rehbein, S. Parasites and Vector-Borne Pathogens in Client-Owned Dogs in Albania. Blood Pathogens and Seroprevalences of Parasitic and Other Infectious Agents. *Parasitol. Res.* **2016**, *115*, 489–499, doi:10.1007/s00436-015-4765-8.

99. Marcic, D.; Zekic Stosic, M.; Milosevic, S.; Pusic, I.; Potkonjak, A.; Tasic Otasevic, S.; Savic, S. Seroprevalence of Leishmania Spp. in Dogs in Northern Serbia. *Vet. Glas.* **2020**, *74*, 187–193, doi:10.2298/vetgl201030015m.

100. Tasic-Otasevic, S.; Savic, S.; Jurhar-Pavlova, M.; Stefanovska, J.; Stalevic, M.; Ignjatovic, A.; Randelovic, M.; Gajic, B.; Cvetkovikj, A.; Gabrielli, S. Molecular Survey of Dirofilaria and Leishmania Species in Dogs from Central Balkan. *Animals* **2022**, *12*, doi:10.3390/ani12070911.

101. Simic, C.; Zivkovic, V. Prilog Poznavanju Faune Nasih Flebotomina I Deo—Makedonija, Juzni Deo Srbije i Kosmet [Contribution to the Knowledge of Sandflies in Yugoslavia. Part I—Macedonia, Southern Serbia and Kosovo]. *Glas. Srp. Akad. Nauk. Med.* **1949**, *1*, 151–181.

102. Zivkovic, V. Repartition de Phlebotomus Chinensis Balcanicus Theodor, 1958 (Diptera, Psychodidae) En Yougoslavie. *Acta Parasitol. Jugoslavica* **1974**, *5*, 3–9.

103. Vaselek, V.; Oguz, G.; Ayhan, N.; Ozbel, Y.; Kadriaj, P.; Cupina, A.I.; Velo, E.; Muja, N.; Baymak, D.; Alishani, M.; et al. Sandfly Surveillance and Investigation of Leishmania Spp. DNA in Sandflies in Kosovo. *Med. Vet. Entomol.* **2020**, *34*, 394–401, doi:10.1111/mve.12451.

104. Ayhan, N.; Sherifi, K.; Taraku, A.; Bërxholi, K.; Charrel, R.N. High Rates of Neutralizing Antibodies to Toscana and Sandfly Fever Sicilian Viruses in Livestock, Kosovo. *Emerg. Infect. Dis.* **2017**, *23*, 989–992, doi:10.3201/eid2306.161929.

105. Dvorak, V.; Kasap, O.E.; Ivovic, V.; Mikov, O.; Stefanovska, J.; Martinkovic, F.; Omeragic, J.; Pajovic, I.; Baymak, D.; Oguz, G.; et al. Sand Flies (Diptera: Psychodidae) in Eight Balkan Countries: Historical Review and Region - Wide Entomological Survey. *Parasit. Vectors* **2020**, *13*, doi:10.1186/s13071-020-04448-w.

106. Fakhar, M.; Derakhshani-nia, M.; Gohardehi, S.; Karamian, M.; Hezarjaribi, H.Z.; Mohebali, M.; Akhoundi, B.; Sharbatkhori, M. Domestic Dogs Carriers of Leishmania Infantum, Leishmania Tropica and Crithidia Fasciculata as Potential Reservoirs for Human Visceral Leishmaniasis in Northeastern Iran. *Vet Med Sci.* **2022**, *8*, 2329–2336, doi:10.1002/vms3.929.

107. Galvez, R.; Miro, G.; Descalzo, M.A.; Nieto, J.; Dado, D.; Martın, O.; Cubero, E.; Molina, R. Emerging Trends in the Seroprevalence of Canine Leishmaniosis in the Madrid Region (Central Spain). *Vet. Parasitol.* **2010**, *169*, 327–334, doi:10.1016/j.vetpar.2009.11.025.

108. Svobodova, M.; Alten, B.; Zidkova, L.; Dvorak, V.; Hlavackova, J.; Myskova, J.; Seblova, V.; Kasap, O.E.; Belen, A.; Votypka, J.; et al. Cutaneous Leishmaniasis Caused by Leishmania Infantum Transmitted by Phlebotomus Tobbi. *Int. J. Parasitol.* **2009**, *39*, 251–256, doi:10.1016/j.ijpara.2008.06.016.

109. Giorgobiani, E.; Lawyer, P.G.; Babuadze, G.; Dolidze, N.; Jochim, R.C.; Tskhvaradze, L.; Kikaleishvili, K.; Kamhawi, S. Incrimination of Phlebotomus Kandelakii and Phlebotomus Balcanicus as Vectors of Leishmania Infantum in Tbilisi, Georgia. *PLoS Negl Trop Dis.* **2012**, *6*, doi:10.1371/journal.pntd.0001609.

110. Vaselek, S.; Ayhan, N.; Oguz, G.; Kasap, O.E.; Savic, S.; Muccio, T. Di; Gradoni, L.; Ozbel, Y.; Alten, B.; Petric, D. Sand Fly and Leishmania Spp. Survey in Vojvodina (Serbia): First Detection of Leishmania Infantum DNA in Sand Flies and the First Record of Phlebotomus (Transphlebotomus) Mascittii Grassi, 1908. *Parasit. Vectors* **2017**, *10*, doi:10.1186/s13071-017-2386-z.

111. Velo, E.; Bongiorno, G.; Kadriaj, P.; Myrseli, T.; Crilly, J.; Lika, A.; Mersini, K.; Muccio, T. Di; Bino, S.; Gramiccia, M.; et al. The Current Status of Phlebotomine Sand Flies in Albania and Incrimination of Phlebotomus Neglectus (Diptera, Psychodidae) as the Main Vector of Leishmania Infantum. *PLoS One* **2017**, *12*, doi:10.1371/journal.pone.0179118.

112. Ayhan, N.; Velo, E.; Lamballerie, X. de; Kota, M.; Kadriaj, P.; Ozbel, Y.; Charrel, R.N.; Bino, S. Detection of Leishmania Infantum and a Novel Phlebovirus (Balkan Virus) from Sand Flies in Albania. *Vector-Borne Zoonotic Dis.* **2016**, doi:10.1089/vbz.2016.2002.

113. Kniha, E.; Walochnik, J.; Poeppl, W.; Mooseder, G.; Obwaller, A.G. Leishmania Spp. Seropositivity in Austrian Soldiers Returning from the Kosovo. *Wien. Klin. Wochenschr.* **2020**, *132*, 47–49, doi:10.1007/s00508-019-01598-5.

114. Mettler, M.; Grimm, F.; Capelli, G.; Camp, H.; Deplazes, P. Evaluation of Enzyme-Linked Immunosorbent Assays, an Immunofluorescent-Antibody Test, and Two Rapid Tests (Immunochromatographic-Dipstick and Gel Tests) for Serological Diagnosis of Symptomatic and Asymptomatic Leishmania Infections in Dogs. *J. Clin. Microbiol.* **2005**, *43*, 5515–5519, doi:10.1128/JCM.43.11.5515.

115. Rodriguez-Cortes, A.; Ojeda, A.; Todoli, F.; Alberola, J. Performance of Commercially Available Serological Diagnostic Tests to Detect Leishmania Infantum Infection on Experimentally Infected Dogs. *Vet. Parasitol.* **2013**, *191*, 363–366, doi:10.1016/j.vetpar.2012.09.009.

116. Ferroglio, E.; Vitale, F. Diagnosis of Leishmaniosis: Between Old Doubts and New Uncertainties. *Vet. Res. Commun.* **2006**, *30*, 35–38, doi:10.1007/s11259-006-0008-x.

117. Leontides, L.S.; Saridomichelakis, M.N.; Billinis, C.; Kontos, V.; Koutinas, A.F.; Galatos, A.D.; Mylonakis, M.E. A Cross-Sectional Study of Leishmania Spp. Infection in Clinically Healthy Dogs with Polymerase Chain Reaction and Serology in Greece. *Vet. Parasitol.* **2002**, *109*, 19–27.

118. Matsumoto, P.S.S.; Taniguchi, H.H.; Pereira, V.B.R.; Hiramoto, R.M.; Rampazzi, K.L.S.; Barbosa, J.E. de R.; Neto, R.A.P.; Campriger, V.M.; Cortez, L.R.P. de B.; Rahaman, K.R.; et al. Efficacies of Insecticide Dog Collars against Visceral Leishmaniasis in Low and High-Income Areas and the Effects for Non-Collared Neighbor Dogs. *Acta Trop.* **2022**, *235*, doi:10.1016/j.actatropica.2022.106626.

9. APPENDIX

9.1. Annex 1. Dog samples included in the study.

Nr.	Age	Breed	Gender	Clinical	District	ELISA	IFAT
1	1	MIX	F	S	01	-	-
2	3	MIX	F	H	01	-	-
3	4	MIX	M	H	01	-	-
4	6	MIX	M	H	01	-	-
5	3	MIX	F	H	01	-	-
6	3	MIX	F	H	01	-	-
7	5	B	F	H	01	-	-
8	2	MIX	F	H	01	-	-
9	3	MIX	M	H	01	-	-
10	7	B	F	H	01	-	-
11	6	MIX	M	S	01	-	-
12	4	MIX	M	H	01	-	-
13	2	MIX	F	H	01	-	-
14	2	MIX	F	H	01	-	-
15	1	MIX	M	H	01	-	-
16	3	MIX	F	H	01	-	-
17	3	MIX	F	H	01	-	-
18	5	MIX	M	H	01	-	-
19	3	MIX	M	H	01	-	-
20	7	MIX	F	H	01	-	-
21	8	MIX	F	S	01	-	-
22	10	MIX	M	S	01	-	-
23	12	B	M	H	01	+	-
24	5	MIX	M	H	01	-	-
25	3	B	M	H	01	-	-
26	3	MIX	M	H	01	-	-
27	2	MIX	M	H	01	-	-
28	1	MIX	M	H	01	-	-
29	3	MIX	M	H	01	-	-
30	8	MIX	F	H	01	-	-
31	2	MIX	F	H	01	-	-
32	8	B	M	H	01	+	+

33	2	B	F	H	01	-	-
34	8	MIX	M	H	01	-	-
35	10	B	M	H	01	-	-
36	8	MIX	M	H	01	-	-
37	4	MIX	F	H	01	-	-
38	2	MIX	F	H	01	-	-
39	3	MIX	F	H	01	-	-
40	3	B	M	H	01	-	-
41	3	MIX	F	H	01	-	-
42	8	MIX	F	S	01	+	+
43	4	MIX	F	S	01	-	-
44	3	B	F	H	01	-	-
45	7	MIX	F	H	01	+	+
46	2	MIX	M	H	01	-	-
47	5	MIX	M	H	01	-	-
48	3	MIX	F	H	01	-	-
49	3	B	M	H	01	-	-
50	4	MIX	F	H	01	-	-
51	13	MIX	F	S	02	-	-
52	1	MIX	F	H	02	-	-
53	1	MIX	M	H	02	-	-
54	1	MIX	F	H	02	-	-
55	8	MIX	F	S	02	-	-
56	12	MIX	F	S	02	-	-
57	4	B	M	H	02	-	-
58	15	MIX	M	S	02	-	-
59	4	MIX	M	H	02	-	-
60	1	MIX	M	H	02	-	-
61	2	B	M	H	02	-	-
62	6	B	F	H	02	-	-
63	2	B	F	H	02	-	-
64	1	B	F	H	02	-	-
65	5	B	F	H	02	-	-
66	4	B	F	H	02	-	-
67	2	B	M	H	02	-	-
68	1	B	M	H	02	-	-
69	3	B	M	H	02	-	-
70	4	B	M	H	02	-	-

71	3	MIX	F	H	02	-	-
72	2	MIX	F	H	02	-	-
73	1	B	F	H	02	-	-
74	1	MIX	F	H	02	-	-
75	3	MIX	M	H	02	-	-
76	1	MIX	F	H	02	-	-
77	1	MIX	M	H	02	-	-
78	2	MIX	M	H	02	-	-
79	5	B	M	H	02	-	-
80	3	MIX	F	H	02	-	-
81	5	MIX	M	S	02	-	-
82	4	B	F	H	02	-	-
83	12	MIX	M	S	02	-	-
84	7	B	M	S	02	-	-
85	2	MIX	M	S	02	-	-
86	3	MIX	M	S	02	-	-
87	4	MIX	F	S	02	-	-
88	1	MIX	F	H	02	-	-
89	1	MIX	F	H	02	-	-
90	3	MIX	F	S	02	-	-
91	4	B	M	H	04	-	-
92	2	B	M	H	04	-	-
93	1	B	F	H	04	-	-
94	2	B	M	H	04	-	-
95	2	B	F	H	04	-	-
96	10	B	M	H	04	-	-
97	5	B	M	H	04	-	-
98	1	B	M	H	04	-	-
99	2	B	F	H	04	-	-
100	1	B	F	H	04	-	-
101	3	B	F	H	04	-	-
102	3	B	M	H	04	-	-
103	2	B	F	H	04	-	-
104	2	B	M	H	04	-	-
105	5	B	M	H	04	+	-
106	2	B	M	H	04	-	-
107	7	B	F	H	04	-	-
108	2	B	F	H	04	-	-

109	4	B	F	H	04	-	-
110	6	B	M	H	04	-	-
111	3	B	F	H	04	-	-
112	1	B	M	H	04	-	-
113	1	B	M	H	04	-	-
114	2	B	F	H	04	-	-
115	2	B	M	H	04	-	-
116	4	B	F	H	04	-	-
117	3	B	M	H	04	-	-
118	6	B	F	H	04	-	-
119	4	B	M	H	04	-	-
120	11	B	F	H	04	-	-
121	3	MIX	F	S	05	-	-
122	2	MIX	F	H	05	-	-
123	3	MIX	M	S	05	-	-
124	1	MIX	M	H	05	-	-
125	7	MIX	M	H	05	-	-
126	3	MIX	M	H	05	-	-
127	5	MIX	F	H	05	-	-
128	7	MIX	M	H	05	-	-
129	3	MIX	F	H	05	-	-
130	1	MIX	M	H	05	-	-
131	5	MIX	F	H	05	-	-
132	3	MIX	F	H	05	-	-
133	1	MIX	M	H	05	-	-
134	7	MIX	F	H	05	-	-
135	3	MIX	M	S	05	-	-
136	3	MIX	F	H	05	-	-
137	4	MIX	M	H	05	+	+
138	8	MIX	F	H	05	-	-
139	6	MIX	M	H	05	-	-
140	9	MIX	M	H	05	-	-
141	4	B	F	H	05	-	-
142	5	MIX	F	H	05	-	-
143	5	MIX	M	H	05	-	-
144	2	B	M	H	05	-	-
145	3	MIX	F	H	05	-	-
146	4	MIX	F	H	05	-	-

147	1	B	M	H	05	-	-
148	2	B	M	H	05	-	-
149	2	B	M	H	05	-	-
150	1	B	M	H	05	-	-
151	8	MIX	M	H	07	-	-
152	6	MIX	F	H	07	-	-
153	2	MIX	M	H	07	-	-
154	2	MIX	M	H	07	-	-
155	2	MIX	F	H	07	-	-
156	1	B	F	H	07	-	-
157	1	B	F	H	07	-	-
158	1	MIX	F	H	07	-	-
159	3	MIX	F	H	07	-	-
160	4	B	F	H	07	-	-
161	4	B	M	H	07	-	+
162	3	B	F	H	07	-	-
163	3	B	M	H	07	-	-
164	4	B	F	H	07	-	-
165	4	B	M	H	07	+	+
166	1	MIX	M	H	07	-	-
167	1	MIX	F	H	07	-	-
168	5	MIX	M	H	07	+	+
169	2	MIX	F	H	07	-	-
170	7	B	F	H	07	-	-
171	2	MIX	F	H	07	-	-
172	2	MIX	M	H	07	-	-
173	1	B	M	H	07	-	-
174	1	MIX	F	H	07	-	-
175	2	MIX	F	H	07	-	-
176	4	MIX	F	H	07	-	-
177	5	B	F	H	07	-	-
178	4	MIX	F	H	07	-	-
179	4	B	F	H	07	-	-
180	3	B	M	H	07	-	-
181	1	MIX	F	H	03	-	-
182	6	MIX	F	S	03	-	-
183	1	MIX	F	S	03	-	-
184	5	MIX	M	H	03	-	-

185	1	MIX	F	H	03	-	-
186	3	MIX	F	S	03	-	-
187	1	MIX	M	H	03	-	-
188	3	B	M	H	03	-	-
189	1	MIX	F	H	03	-	-
190	9	MIX	M	H	03	-	-
191	1	MIX	M	H	03	-	-
192	2	MIX	M	H	03	-	-
193	3	B	F	H	03	-	-
194	2	MIX	M	H	03	-	-
195	4	MIX	M	S	03	-	-
196	2	MIX	M	S	03	-	-
197	10	B	M	S	03	-	-
198	10	MIX	F	S	03	-	-
199	10	MIX	M	S	03	-	-
200	2	MIX	F	H	03	-	-
201	2	B	F	H	06	-	-
202	2	MIX	M	S	06	-	-
203	6	B	F	S	06	-	-
204	1	B	F	H	06	-	-
205	9	B	M	H	06	-	-
206	2	B	M	H	06	-	-
207	3	MIX	M	H	06	-	-
208	2	B	F	H	06	-	-
209	4	MIX	M	H	06	-	-
210	4	B	M	S	06	-	-
211	2	MIX	M	H	06	-	-
212	1	MIX	M	H	06	-	-
213	3	B	M	H	06	-	-
214	2	MIX	F	H	06	-	-
215	2	B	M	H	06	-	-
216	2	B	F	H	06	-	-
217	5	B	M	H	06	-	-
218	4	MIX	M	H	06	-	-
219	1	MIX	F	H	06	-	-
220	2	MIX	M	H	06	-	-
221	4	MIX	M	H	06	+	+
222	3	MIX	M	H	06	-	-

223	3	MIX	M	H	06	-	-
224	3	B	F	H	06	-	-
225	2	B	M	H	06	-	-
226	2	B	F	H	06	-	-
227	2	MIX	M	H	06	-	-
228	4	MIX	M	H	06	-	-
229	2	MIX	M	H	06	-	-
230	3	MIX	M	H	06	-	-
231	2	MIX	M	H	06	-	-
232	4	MIX	M	H	06	-	-
233	3	B	F	H	06	-	-
234	4	B	M	H	06	-	-
235	3	MIX	M	H	06	-	-
236	2	MIX	F	H	06	-	-
237	2	MIX	F	H	06	-	-
238	2	MIX	M	H	06	-	-
239	4	MIX	M	H	06	-	-
240	7	B	M	H	06	-	-
241	8	B	F	H	03	-	-
242	4	MIX	F	H	03	-	-
243	3	MIX	F	H	03	-	-
244	10	MIX	F	S	03	-	-
245	3	MIX	F	H	03	+	+
246	2	MIX	F	H	03	-	-
247	4	MIX	F	H	03	-	-
248	4	MIX	M	H	03	-	-
249	4	B	F	H	03	-	-
250	3	MIX	F	H	03	-	-
251	4	B	M	S	03	-	-
252	2	B	F	S	03	-	-
253	5	MIX	F	H	03	-	-
254	3	MIX	M	H	03	-	-
255	5	MIX	M	H	03	-	-
256	4	MIX	F	H	05	-	-
257	5	MIX	F	H	05	-	-
258	2	MIX	F	S	05	-	-
259	3	MIX	F	H	05	+	-
260	3	MIX	F	S	05	-	-

261	7	B	M	H	04	-	-
262	2	B	F	H	04	-	-
263	7	MIX	M	H	04	-	-
264	4	MIX	M	H	04	-	-
265	3	MIX	F	H	04	-	-
266	5	MIX	F	H	04	-	-
267	16	B	F	H	04	-	-
268	8	B	M	H	04	-	-
269	3	B	F	H	07	-	-
270	2	MIX	F	H	07	-	-
271	2	MIX	F	H	07	-	-
272	3	MIX	F	S	07	-	-
273	2	MIX	F	S	07	-	-
274	6	B	M	H	07	-	-
275	4	B	M	H	07	-	-
276	13	B	M	S	07	-	-
277	10	B	F	H	07	-	-
278	5	MIX	M	H	04	+	+
279	5	B	M	S	04	-	-
280	7	MIX	M	H	05	-	-
281	3	MIX	F	H	05	-	-
282	3	MIX	F	H	05	-	-
283	4	MIX	F	H	05	-	-
284	2	B	M	H	07	-	-
285	4	MIX	F	H	05	-	-

Mix=Mix breed, B=Pure breed; M=Male, F=Female; H=Healthy (no clinical signs), S=Sick (disrupted health); - & + = Results on tests.

9.2. Annex 2. Sand fly samples included in the study.

Nr.	Location	District	Latitude	Longitude	Traps	Results
1	01_1	01	42.668764	21.146394	4	negative
2	01_2	01	42.642658	21.122259	4	negative
3	01_3	01	42.679402	21.019922	3	negative
4	01_4	01	42.67886	21.020865	3	negative
5	01_5	01	42.678809	21.012387	3	positive
6	01_6	01	42.594575	21.156799	4	negative
7	01_7	01	42.689994	21.172159	3	positive
8	01_8	01	42.684549	21.173746	3	positive
9	01_9	01	42.684283	21.172569	3	positive
10	01_10	01	42.626261	20.892525	2	negative
11	01_11	01	42.634392	21.014673	4	positive
12	01_12	01	42.539502	21.009598	3	positive
13	01_13	01	42.522345	20.924403	3	positive
14	01_14	01	42.536376	20.909426	2	negative
15	01_15	01	42.536627	20.909491	2	negative
16	01_16	01	42.932162	21.182975	3	negative
17	01_17	01	42.95431	21.184873	3	positive
18	01_18	01	42.969911	21.160249	3	positive
19	01_19	01	42.993166	21.154464	2	positive
20	01_20	01	42.92723	21.154539	3	positive
21	02_1	02	42.835659	20.958662	3	negative
22	02_2	02	42.840227	20.962818	3	positive
23	02_3	02	42.775186	20.902402	3	positive
24	02_4	02	42.799151	20.949435	3	positive
25	02_5	02	42.757957	21.052255	3	negative
26	02_6	02	42.736546	20.821495	3	positive
27	02_7	02	42.721264	20.810887	3	positive
28	02_8	02	42.806402	20.743851	2	positive
29	02_9	02	42.808698	20.738821	2	positive
30	02_10	02	42.754549	20.768225	2	positive
31	02_11	02	42.760194	20.792985	2	positive
32	02_12	02	42.876729	20.889338	3	negative
33	02_13	02	42.866804	20.853528	3	positive
34	02_14	02	42.857093	20.84189	3	negative
35	02_15	02	42.841512	20.846446	3	positive
36	02_16	02	42.850608	20.884741	3	positive

37	03_1	03	42.659835	20.51891	3	positive
38	03_2	03	42.719678	20.471844	2	positive
39	03_3	03	42.747889	20.460078	2	negative
40	03_4	03	42.735167	20.469194	3	positive
41	03_5	03	42.750309	20.554989	4	positive
42	03_6	03	42.802207	20.561346	2	positive
43	03_7	03	42.646088	20.590062	3	positive
44	03_8	03	42.658518	20.59838	3	positive
45	03_9	03	42.608569	20.56501	2	positive
46	03_10	03	42.611035	20.518651	3	positive
47	03_11	03	42.588664	20.559713	2	positive
48	03_12	03	42.633389	20.436331	3	negative
49	03_13	03	42.634622	20.433919	3	positive
50	03_14	03	42.654823	20.324136	3	negative
51	03_15	03	42.637022	20.318137	3	negative
52	03_16	03	42.630479	20.317202	3	positive
53	04_1	04	42.245912	20.735907	3	positive
54	04_2	04	42.251841	20.736529	3	positive
55	04_3	04	42.251726	20.743639	4	positive
56	04_4	04	42.252987	20.745992	2	positive
57	04_5	04	42.244203	20.757265	3	negative
58	04_6	04	42.324103	20.879075	2	negative
59	04_7	04	42.296707	20.878172	3	positive
60	04_8	04	42.33377	20.910125	3	positive
61	04_9	04	42.395806	20.804161	3	positive
62	04_10	04	42.355312	20.759284	3	positive
63	04_11	04	42.29388	20.686654	3	negative
64	04_12	04	42.293537	20.684464	3	negative
65	04_13	04	42.291271	20.864112	3	positive
66	04_14	04	42.304757	20.706458	3	negative
67	04_15	04	42.292863	20.886809	3	positive
68	05_1	05	42.458127	21.198266	3	negative
69	05_2	05	42.457838	21.201952	3	negative
70	05_3	05	42.444747	21.155297	3	negative
71	05_4	05	42.442015	21.175357	3	positive
72	05_5	05	42.440223	21.180144	3	negative
73	05_6	05	42.374909	21.181727	3	negative
74	05_7	05	42.356196	21.223439	3	negative
75	05_8	05	42.323345	21.198911	3	positive

76	05_9	05	42.317885	21.198926	3	negative
77	05_10	05	42.305965	21.198321	3	negative
78	05_11	05	42.275778	21.240491	3	positive
79	05_12	05	42.242806	21.231225	3	positive
80	05_13	05	42.252157	21.251885	3	positive
81	05_14	05	42.14429	21.289385	2	positive
82	05_15	05	42.141137	21.232028	3	positive
83	06_1	06	42.521638	21.375534	2	positive
84	06_2	06	42.505099	21.378929	3	positive
85	06_3	06	42.494595	21.389822	2	negative
86	06_4	06	42.471952	21.40529	2	negative
87	06_5	06	42.480567	21.379723	3	positive
88	06_6	06	42.482104	21.378179	3	positive
89	06_7	06	42.363773	21.349704	3	negative
90	06_8	06	42.377014	21.3465	2	negative
91	06_9	06	42.370242	21.419351	4	positive
92	06_10	06	42.29957	21.38915	3	positive
93	06_11	06	42.31382	21.366361	2	negative
94	06_12	06	42.568911	21.507358	3	positive
95	06_13	06	42.567564	21.526801	3	positive
96	06_14	06	42.626706	21.598017	3	positive
97	06_15	06	42.621446	21.601966	3	positive
98	06_16	06	42.573108	21.553697	3	positive
99	07_1	07	42.477431	20.304914	3	positive
100	07_2	07	42.480186	20.291309	3	positive
101	07_3	07	42.479126	20.289872	3	positive
102	07_4	07	42.444579	20.320648	3	positive
103	07_5	07	42.444868	20.321997	3	positive
104	07_6	07	42.438939	20.607013	3	positive
105	07_7	07	42.446807	20.601024	2	positive
106	07_8	07	42.415831	20.577364	2	positive
107	07_9	07	42.427216	20.536459	2	positive
108	07_10	07	42.436514	20.53552	3	positive
109	07_11	07	42.456674	20.536838	2	positive
110	07_12	07	42.330631	20.647703	3	positive
111	07_13	07	42.329927	20.576112	3	negative
112	07_14	07	42.316858	20.586673	3	positive
113	07_15	07	42.355969	20.522267	3	positive
114	07_16	07	42.366092	20.437181	2	negative

Continues: Result of the sand fly species found in the study.

Nr.	*Ph. perfiliewi*	*Ph. neglectus*	*Ph. tobbi*	*Ph. simici*	*Ph. balcanicus*	*Ph. papatasi*	*Se. minuta*	Pooled specimens	Total
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	7	0	0	0	0	0	0	7
6	0	0	0	0	0	0	0	0	0
7	0	3	0	0	0	0	0	0	3
8	0	2	0	0	0	0	0	0	2
9	0	29	0	0	0	0	0	0	29
10	0	0	0	0	0	0	0	0	0
11	3	2	0	0	0	0	0	0	5
12	0	4	0	0	0	0	0	0	4
13	0	1	0	0	0	0	0	0	1
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	2	0	0	0	0	0	0	2
18	0	3	0	0	0	0	0	0	3
19	0	1	0	0	0	0	0	0	1
20	1	0	0	0	0	0	0	0	1
21	0	0	0	0	0	0	0	0	0
22	12	0	0	0	0	0	0	0	12
23	0	1	0	0	0	0	0	0	1
24	4	0	0	0	0	0	0	0	4
25	0	0	0	0	0	0	0	0	0
26	2	4	0	0	0	0	0	0	6
27	74	14	0	0	0	0	0	45	133
28	1	19	0	0	0	0	0	0	20
29	0	6	0	0	0	0	0	0	6
30	6	13	0	0	0	0	0	0	19
31	5	1	0	0	0	0	0	0	6
32	0	0	0	0	0	0	0	0	0
33	0	1	0	0	0	0	0	0	1
34	0	0	0	0	0	0	0	0	0
35	11	0	0	0	0	0	0	0	11
36	50	2	0	0	0	0	0	0	52

37	5	0	0	0	0	0	0	0	5
38	0	2	0	0	0	0	0	0	2
39	0	0	0	0	0	0	0	0	0
40	1	0	0	0	0	0	0	0	1
41	13	0	0	0	0	0	0	0	13
42	0	1	0	0	0	0	0	0	1
43	56	1	0	0	0	0	0	146	203
44	76	1	0	0	0	0	0	925	1002
45	13	1	0	0	0	0	0	0	14
46	24	5	0	0	0	2	0	0	31
47	25	0	0	0	0	0	0	0	25
48	0	0	0	0	0	0	0	0	0
49	0	1	0	0	0	0	0	0	1
50	0	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	1	0	1
53	0	2	0	0	0	0	0	0	2
54	0	4	0	0	0	0	0	0	4
55	0	2	0	0	0	0	0	0	2
56	0	3	0	0	0	0	0	0	3
57	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0
59	0	45	0	0	0	0	3	0	48
60	0	10	0	0	0	0	0	0	10
61	87	26	0	0	0	0	0	715	828
62	5	3	0	0	0	0	1	0	9
63	0	0	0	0	0	0	0	0	0
64	0	0	0	0	0	0	0	0	0
65	0	2	0	0	0	0	0	0	2
66	0	0	0	0	0	0	0	0	0
67	0	10	0	0	0	0	1	0	11
68	0	0	0	0	0	0	0	0	0
69	0	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0
71	0	1	0	0	0	0	0	0	1
72	0	0	0	0	0	0	0	0	0
73	0	0	0	0	0	0	0	0	0
74	0	0	0	0	0	0	0	0	0
75	0	1	0	0	0	0	0	0	1

76	0	0	0	0	0	0	0	0	0
77	0	0	0	0	0	0	0	0	0
78	1	0	0	0	0	0	0	0	1
79	0	1	0	0	0	0	0	0	1
80	0	5	0	0	0	0	0	0	5
81	0	10	1	7	0	0	0	0	18
82	0	6	0	1	0	0	0	0	7
83	0	1	0	0	0	0	0	0	1
84	0	1	0	0	0	0	0	0	1
85	0	0	0	0	0	0	0	0	0
86	0	0	0	0	0	0	0	0	0
87	0	10	0	0	0	0	0	0	10
88	0	31	0	0	0	0	0	0	31
89	0	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0	0
91	1	1	1	0	0	0	0	0	3
92	3	3	1	0	0	0	0	0	7
93	0	0	0	0	0	0	0	0	0
94	0	5	0	0	0	0	0	0	5
95	2	0	13	11	0	0	0	0	26
96	1	0	0	0	0	0	0	0	1
97	1	1	8	0	0	0	0	0	10
98	0	0	3	2	0	0	0	0	5
99	0	12	0	0	0	0	0	0	12
100	0	27	0	0	7	0	0	0	34
101	0	3	0	0	1	0	0	0	4
102	0	1	0	0	0	0	0	0	1
103	0	1	0	0	0	0	0	0	1
104	7	5	0	0	0	0	0	0	12
105	27	4	0	0	0	0	0	0	31
106	37	0	0	0	0	0	0	292	329
107	53	0	0	0	0	0	0	0	53
108	58	1	0	0	0	0	0	0	59
109	45	0	0	0	0	0	0	0	45
110	1	1	0	0	0	0	1	0	3
111	0	0	0	0	0	0	0	0	0
112	0	1	0	0	0	0	0	0	1
113	4	1	0	1	0	0	0	0	6
114	0	0	0	0	0	0	0	0	0

9.3. Annex 3. Supplementary figure of sequence alignment of *Leishmania* spp.

OR344780.1 *L. infantum*	**A**	**G**	**G**	**G**	**C**	**G**	**C**	**G**	**T**	**G**	**C**	**G**	**G**	**G**	**T**	**C**	**G**	**T**	**G**	**C**	**T**	**G**	**G**	**G**	**C**	**G**	**T**	**T**	**C**	**T**	**C**	**G**	**G**	**C**	**G**	**G**	**T**	**C**	**G**	**G**	**C**	**A**	**A**	**C**	**A**	**T**	**C**	**G**	**A**	**G**	**[50]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
AY896786.1 *L. donovani*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
AY896785.1 *L. donovani*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
GQ302673.1 *L. donovani*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
EU637909.1 *L. donovani*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
JN400179.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	[50]
JN400177.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	[50]
DQ286773.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	[50]
XM_001681083.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
KY412785.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
KY412784.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
XM_003872607.1 *L. mexicanc*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]
XM_003872608.1 *L. mexicanc*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[50]

OR344780.1 *L. infantum*	**T**	**C**	**G**	**C**	**A**	**G**	**T**	**G**	**G**	**G**	**C**	**C**	**C**	**G**	**T**	**G**	**C**	**C**	**G**	**G**	**C**	**C**	**A**	**C**	**G**	**G**	**C**	**T**	**T**	**G**	**G**	**T**	**G**	**A**	**G**	**C**	**C**	**T**	**G**	**T**	**C**	**G**	**G**	**A**	**G**	**C**	**A**	**G**	**C**	**A**	**[100]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
JN400179.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	G	C	.	.	A	C	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	G	C	.	.	A	C	.	G	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	[100]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	A	C	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	A	A	G	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	A	A	G	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	A	A	G	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
XM_003872607.1 *L. mexicanc*	G	G	.	.	.	.	.	.	.	T	A	.	.	T	.	.	.	.	.	.	.	.	.	.	A	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]
XM_003872608.1 *L. mexicanc*	G	G	.	.	.	.	.	.	.	T	A	.	.	T	.	.	.	.	.	.	.	.	.	.	A	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[100]

OR344780.1 *L. infantum*	**G**	**C**	**T**	**G**	**G**	**T**	**G**	**A**	**G**	**C**	**T**	**G**	**C**	**G**	**A**	**T**	**G**	**A**	**C**	**A**	**A**	**A**	**G**	**A**	**C**	**A**	**A**	**T**	**G**	**G**	**C**	**T**	**G**	**C**	**A**	**A**	**C**	**G**	**G**	**C**	**G**	**G**	**G**	**C**	**T**	**G**	**A**	**T**	**G**	**C**	**[150]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
JN400179.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	G	.	.	.	.	.	.	.	.	T	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	G	.	.	.	.	.	.	.	.	T	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	G	.	.	.	.	.	.	.	.	T	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	[150]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	A	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	A	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[150]

OR344780.1 *L. infantum*	**T**	**G**	**C**	**A**	**G**	**G**	**C**	**G**	**T**	**T**	**C**	**G**	**A**	**G**	**T**	**G**	**G**	**C**	**T**	**G**	**C**	**T**	**G**	**C**	**G**	**A**	**C**	**A**	**C**	**A**	**T**	**G**	**T**	**A**	**C**	**G**	**G**	**G**	**A**	**T**	**C**	**G**	**T**	**G**	**T**	**T**	**C**	**A**	**C**	**G**	**[200]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	[200]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[200]
JN400179.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	[200]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	[200]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	[200]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	[200]
KY412785.1 *L. major*	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	[200]
KY412784.1 *L. major*	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	[200]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	C	.	A	.	.	.	.	.	C	A	.	C	.	.	.	A	.	.	.	.	.	.	[200]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	C	.	A	.	.	.	.	.	C	A	.	C	.	.	C	A	.	.	.	.	.	.	[200]

OR344780.1 *L. infantum*	**G**	**A**	**G**	**A**	**A**	**G**	**A**	**G**	**C**	**T**	**A**	**C**	**C**	**C**	**C**	**-**	**T**	**A**	**C**	**A**	**C**	**G**	**T**	**C**	**C**	**G**	**G**	**C**	**A**	**A**	**C**	**G**	**G**	**T**	**G**	**A**	**T**	**G**	**T**	**G**	**G**	**C**	**C**	**G**	**A**	**G**	**T**	**G**	**C**	**T**	**[250]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
AY896786.1 *L. donovani*	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[250]
JN400179.1 *L. tropica*	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	.	.	.	.	A	.	.	.	C	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
JN400177.1 *L. tropica*	.	.	A	G	.	C	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	.	.	.	.	A	.	.	.	C	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
DQ286773.1 *L. tropica*	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	.	.	.	.	A	.	.	.	G	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	[250]
XM_003872607.1 *L. mexicanc*	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	[250]
XM_003872608.1 *L. mexicanc*	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	-	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	[250]

OR344780.1 *L. infantum*	**T**	**G**	**A**	**A**	**C**	**A**	**G**	**C**	**A**	**G**	**T**	**A**	**A**	**A**	**C**	**T**	**C**	**G**	**T**	**T**	**C**	**C**	**C**	**G**	**G**	**C**	**G**	**C**	**G**	**C**	**A**	**A**	**A**	**T**	**C**	**G**	**A**	**C**	**G**	**G**	**C**	**T**	**A**	**C**	**G**	**T**	**G**	**A**	**T**	**G**	**[300]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[300]
JN400179.1 *L. tropica*	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C	.	[300]
JN400177.1 *L. tropica*	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	[300]
DQ286773.1 *L. tropica*	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C	.	[300]
XM_001681083.1 *L. major*	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	C	.	[300]
KY412785.1 *L. major*	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	C	.	[300]
KY412784.1 *L. major*	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	C	.	[300]
XM_003872607.1 *L. mexicanc*	C	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	G	T	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	[300]
XM_003872608.1 *L. mexicanc*	C	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	G	T	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	[300]

OR344780.1 *L. infantum*	**A**	**T**	**C**	**C**	**C**	**G**	**A**	**G**	**C**	**A**	**A**	**C**	**G**	**A**	**A**	**A**	**C**	**G**	**G**	**T**	**T**	**A**	**T**	**G**	**G**	**C**	**T**	**G**	**C**	**G**	**T**	**G**	**G**	**C**	**T**	**T**	**G**	**C**	**G**	**G**	**A**	**G**	**A**	**A**	**T**	**G**	**G**	**C**	**C**	**C**	**[350]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[350]
JN400179.1 *L. tropica*	.	.	.	G	A	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
JN400177.1 *L. tropica*	.	.	.	G	A	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
DQ286773.1 *L. tropica*	.	.	.	G	A	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
XM_001681083.1 *L. major*	.	.	G	G	A	A	.	.	.	.	G	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
KY412785.1 *L. major*	.	.	G	G	A	A	.	.	.	.	G	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
KY412784.1 *L. major*	.	.	G	G	A	A	.	.	.	.	G	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	[350]
XM_003872607.1 *L. mexicanc*	.	.	.	G	G	A	.	.	.	.	G	.	.	.	.	.	A	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	[350]
XM_003872608.1 *L. mexicanc*	.	.	.	G	G	A	.	.	.	.	G	.	.	.	.	.	A	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	[350]

OR344780.1 *L. infantum*	**C**	**A**	**T**	**C**	**G**	**C**	**G**	**A**	**T**	**T**	**G**	**C**	**G**	**G**	**T**	**C**	**G**	**A**	**C**	**G**	**C**	**C**	**A**	**G**	**C**	**T**	**C**	**C**	**T**	**T**	**C**	**A**	**T**	**G**	**T**	**C**	**T**	**T**	**A**	**C**	**C**	**A**	**G**	**A**	**G**	**C**	**G**	**G**	**C**	**G**	**[400]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
JN400179.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
JN400177.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
DQ286773.1 *L. tropica*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[400]
XM_001681083.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	[400]
KY412785.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	[400]
KY412784.1 *L. major*	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	[400]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	[400]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	[400]

OR344780.1 *L. infantum*	**T**	**G**	**C**	**T**	**G**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**C**	**T**	**C**	**G**	**T**	**C**	**[450]**
AY896777.1 *L. infantum*	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	[450]
JN400124.1 *L. infantum*	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	[450]
GQ302670.1 *L. infantum*	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	[450]
AY896786.1 *L. donovani*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	C	G	A	T	G	C	A	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	.	[450]
AY896785.1 *L. donovani*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	C	G	A	T	G	C	A	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	.	[450]
GQ302673.1 *L. donovani*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	C	G	A	T	G	C	A	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	.	[450]
EU637909.1 *L. donovani*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	C	G	A	T	G	C	A	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	.	[450]
JN400179.1 *L. tropica*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	G	A	T	G	C	C	T	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
JN400177.1 *L. tropica*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	T	G	A	T	G	C	T	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
DQ286773.1 *L. tropica*	.	.	.	.	.	A	C	C	A	G	C	T	G	C	G	C	T	G	G	G	G	A	T	G	C	T	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
XM_001681083.1 *L. major*	.	C	.	.	.	A	C	C	A	G	C	T	G	C	A	T	T	G	G	T	G	A	G	C	A	G	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
KY412785.1 *L. major*	.	C	.	.	.	A	C	C	A	G	C	T	G	C	A	T	T	G	G	T	G	A	G	C	A	G	C	T	G	A	A	C	C	A	C	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
KY412784.1 *L. major*	.	C	.	.	.	A	C	C	A	G	C	T	G	C	A	T	T	G	G	T	G	A	G	C	A	G	C	T	G	A	A	C	C	A	T	G	G	C	G	T	G	C	T	G	.	.	.	.	.	T	[450]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	A	C	C	G	C	G	T	G	C	A	T	T	G	G	T	A	A	A	C	A	G	C	T	G	A	A	C	C	A	C	G	G	T	G	T	G	C	T	G	.	.	.	.	.	.	[450]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	A	C	C	G	C	G	T	G	C	A	T	T	G	G	T	A	A	A	C	A	G	C	T	G	A	A	C	C	A	C	G	G	T	G	T	G	C	T	G	.	.	.	.	.	.	[450]

OR344780.1 *L. infantum*	**G**	**G**	**G**	**T**	**A**	**C**	**A**	**A**	**C**	**A**	**A**	**G**	**A**	**C**	**C**	**G**	**G**	**T**	**G**	**G**	**G**	**G**	**T**	**T**	**C**	**C**	**G**	**T**	**A**	**C**	**T**	**G**	**G**	**G**	**T**	**G**	**A**	**T**	**C**	**A**	**A**	**G**	**A**	**A**	**C**	**T**	**C**	**G**	**T**	**G**	**[500]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
JN400179.1 *L. tropica*	T	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	.	G	.	.	.	T	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	.	G	.	.	.	T	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[500]

OR344780.1 *L. infantum*	**G**	**G**	**G**	**T**	**G**	**A**	**G**	**G**	**A**	**C**	**T**	**G**	**G**	**G**	**G**	**C**	**G**	**A**	**G**	**A**	**A**	**G**	**G**	**G**	**C**	**T**	**A**	**C**	**G**	**T**	**G**	**C**	**G**	**C**	**G**	**T**	**G**	**G**	**C**	**C**	**A**	**T**	**G**	**G**	**G**	**G**	**C**	**T**	**G**	**A**	**[550]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[550]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[550]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[550]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[550]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	[550]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	[550]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[550]
JN400179.1 *L. tropica*	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	[550]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	G	.	.	[550]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	G	.	.	[550]

OR344780.1 *L. infantum*	**A**	**C**	**G**	**C**	**G**	**T**	**G**	**C**	**C**	**T**	**G**	**C**	**T**	**C**	**A**	**G**	**T**	**G**	**A**	**A**	**T**	**A**	**C**	**C**	**C**	**C**	**G**	**T**	**G**	**T**	**C**	**C**	**G**	**C**	**G**	**C**	**A**	**T**	**G**	**T**	**G**	**C**	**C**	**G**	**C**	**A**	**G**	**A**	**G**	**T**	**[600]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	[600]
JN400179.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
JN400177.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
DQ286773.1 *L. tropica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[600]
XM_001681083.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	[600]
KY412785.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	C	[600]
KY412784.1 *L. major*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	[600]
XM_003872607.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	[600]
XM_003872608.1 *L. mexicanc*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	[600]

OR344780.1 *L. infantum*	**C**	**T**	**C**	**A**	**C**	**C**	**C**	**C**	**T**	**[609]**
AY896777.1 *L. infantum*	.	.	.	.	.	.	.	.	.	[609]
JN400124.1 *L. infantum*	.	.	.	.	.	.	.	.	.	[609]
GQ302670.1 *L. infantum*	.	.	.	.	.	.	.	.	.	[609]
AY896786.1 *L. donovani*	.	.	.	.	.	.	.	.	.	[609]
AY896785.1 *L. donovani*	.	.	.	.	.	.	.	.	.	[609]
GQ302673.1 *L. donovani*	.	.	.	.	.	.	.	.	.	[609]
EU637909.1 *L. donovani*	.	.	.	.	.	.	.	.	.	[609]
JN400179.1 *L. tropica*	.	.	T	C	.	.	.	.	.	[609]
JN400177.1 *L. tropica*	.	C	.	.	.	.	.	.	.	[609]
DQ286773.1 *L. tropica*	.	C	.	.	.	.	.	.	.	[609]
XM_001681083.1 *L. major*	.	C	.	.	.	.	.	.	.	[609]
KY412785.1 *L. major*	.	C	.	.	.	.	.	.	.	[609]
KY412784.1 *L. major*	.	C	.	.	.	.	.	.	.	[609]
XM_003872607.1 *L. mexicanc*	G	C	.	G	.	.	.	.	.	[609]
XM_003872608.1 *L. mexicanc*	G	C	.	G	.	.	.	.	.	[609]