University of Naples
"Federico II"

PhD THESIS

"*Canine Leishmaniasis: investigation on the possible role of dogs in the epidemiology of L. major infection*"

Coordinator	**Candidate**	**Supervisor**
Prof. Giuseppe CRINGOLI	Nour El Houda BEN FAYALA	Prof. Gaetano OLIVA

To the soul of my mom who would have been proud of this degree.

Table Of Contents

Table Of Contents

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List of Abbreviations

BCS	Body Condition Score
BM	Bone marrow
CanL	Canine leishmaniasis
CICs	Circulating Immune Complexes
CL	Cutaneous Leishmaniasis
ClinL	Clinical leishmaniasis
CVBDs	Canine Vector-Borne Diseases
CVL	Canine Visceral Leishmaniasis
DAT	Direct Agglutination Test
DNA	Deoxyribonucleic Acid
FNA	Fine Needle Aspiration
HL	Human Leishmaniasis
IL	Interleukin
L.	*Leishmania*
LNE	Lymph Node Enlargement
LPG	LipoPhosphoGlycan
MCL	mucocutaneous Leishmaniasis
P.	*Phlebotomus*
PCR	Polymerase Chain Reaction
PKDL	Post Kala Azar Leishmaniasis
PM	Peritrophic Matrix
PMNs	Polymorphonuclear Neutrophil Granulocytes
PSG	Promastigote Secretory Gel
TLRs	Receptors Toll-like
VL	Visceral leishmaniasis
ZCL	Zoonoti Cutaneous Leishmaniasis
ELISA	Enzyme Linked Immunosorbent Assay
IFAT	immunofluorescence antibody test
B.	*Babesia*
E.	*Ehrlichia*
µm	micrometre
RT-PCR	Reverse transcription polymerase chain reaction
ITS1	Internal Transcribed Spacer 1
MIP-1β	Macrophage inflammatory protein-1beta
PMNs	Polymorphonuclear leukocytes
LAMP.	Loop Mediated Isothermal Amplification
CD	Cluster of Differentiation
Th T	helper cells
DAT	Direct Agglutination Test

List of Figures

List of Figures

List of Tables

Abstract

Leishmaniasis is an infectious disease caused by protozoan parasites of over 20 species of *Leishmania* which occurs in three forms in humans: visceral (the most severe), cutaneous (the most common) and mucocutaneous. Almost all *Leishmania* species are pathogenic for mammals and many of them are zoonotic and have animal reservoirs, including the three species known to cause the disease in Tunisia: *Leishmania infantum*, *Leishmania major*, and *Leishmania killicki (syn. tropica)*. Domestic dogs are the main reservoir of *L. infantum* (the main aetiologic agent of canine leishmaniasis in Italy and in Tunisia) and can also host infections of *L. major and L. tropica*. Furthermore, CanL co-infection/co-exposure with other vector-borne pathogens plays a major role in clinical manifestations and influences the course of the infection and may consequently display important risk factors for its establishment, progression and further spread to other animals and humans. Such co-infections include Canine vector-borne diseases such as babesiosis, ehrlichiosis, anaplasmosis, borreliosis and heartworm disease.

In the present study, we will discuss *Leishmania infantum* and *Leishmania major*, given their major importance in humans and dogs respectively, through studies that provide a better understanding of CanL in all its aspects: epidemiological, clinical, and pathogenetic.

The thesis was divided into six chapters of equal importance: the introduction and the first chapter describe the general part while the other chapters discuss research works carried out during the three years of the PhD project.

Chapter 2-Examining the Relationship of Clinical and Laboratory Parameters with Infectiousness to *Phlebotomus perniciosus* and Its Potential Infectivity in Dogs with Overt Clinical Leishmaniasis, this study examines the relationship between different clinical parameters and the infectiousness to colonized *Phlebotomus perniciosus* sand flies having a blood meal on dogs. Data obtained in the present study come from an untreated group of *Leishmania* sick dogs submitted to xenodiagnosis for the evaluation of a spot-on insecticide solution. Seventeen dogs were diagnosed as affected by leishmaniasis through clinical examination, immunofluorescence antibody test (IFAT) serology, and loop-mediated isothermal amplification (LAMP). The disease severity (clinical score) was staged by using a numeric value derived from eight clinical and parasitological parameters. Xenodiagnosis was performed on caged dogs exposed for 1.5 h to sand-fly bites. The

following parameters related to sand flies were examined: blood feeding (% of blood engorged females), promastigote detection (% of promastigote-positive sand flies), promastigote burden, and the promastigote stage maturation (potential transmissibility rate). Statistical relationship between the clinical score and entomological parameters was investigated, as well as the possible correlation between each clinical and laboratory parameters and sand fly infection/infectivity. The severity of clinical score may influence the blood feeding by, and the probability of promastigote detection in, sand flies; skin lesions seem to be the main factor that influences the rate of blood feeding. Promastigote burden is related to IFAT titer, skin lesions, and clinical score. All entomological parameters are strongly related among them. This study confirms that both *P. perniciosus* infection and infectivity are influenced by a dog's clinical condition.

Chapter 3-Laboratory evidence that dinotefuran, pyriproxyfen and permethrin combination abrogates *Leishmania infantum* transmissibility by sick dogs, The effect of dinotefuran, pyriproxyfen and permethrin spot-on solution (Vectra®3D, Ceva Santé Animale, Libourne, France) on *Leishmania* transmissibility by naturally infected dogs via reared *Phlebotomus perniciosus*, was assessed. Dogs affected by leishmaniasis were submitted to xenodiagnosis and 6 infecting >10% of insects were treated topically on day 0. Antifeeding, insecticidal and anti-transmissibility effects were evaluated through xenodiagnoses performed on days 1, 7 and 28, using individual pre-treatment parameters as control. Feeding and mortality rates were assessed at 24 h, whereas promastigote infection, maturation and burden were assessed up to 96 h post blood meal (potentially infectious rate). On day 1, the anti-feeding efficacy was >95% in 4 dogs, insecticidal efficacy 100% in 4 dogs, and anti-transmissibility effect 100% in 6 dogs. Efficacy rates recorded on day 7 were very similar to day 1. On day 28, anti-feeding and insecticidal efficacy values were much broader, ranging 32.6-100% and 7.7-94.4%, respectively. Potentially infectious insects were recorded from two dogs, with sharp decrease in transmissibility rate as compared with pre-treatment condition. Altogether, Vectra®3D abrogated by >98% the potential *Leishmania* transmissibility by the examined pool of infected dogs over 1 month.

Chapter 4-Assessment of Circulating Immune Complexes During Natural and Experimental Canine Leishmaniasis, the aim of this study was to assess the serum level of CICs in dogs exposed to natural and experimental

infection. Fifty-two sera were examined, belonging to untreated groups of naïve beagles previously studied to assess the performance of anti-leishmanial vaccines under natural (no. 22 dogs) or experimental (no. 30 dogs) transmission. Sera were classified in five groups according to the dog's health condition, IFAT titer, and the BM nested (n)-PCR result. A: no.10 healthy dogs before the experimental infection; B: no.10 clinically healthy dogs infected experimentally, IFAT negative (= reciprocal titer <160) and n-PCR positive; C: no.10 clinically healthy dogs naturally infected, IFAT positive at titers 160–320 and n-PCR negative; D: no.10 sick dogs experimentally infected, IFAT positive at titer >320 and n-PCR positive; E: no.12 sick dogs naturally infected, IFAT positive at titer >320 and n-PCR positive. CICs levels were assessed by ELISA method (canine CIC assay—Cloude-Clone Corporation, USA). The two groups characterized by negative IFAT (A and B) had the lowest median level of CICs (16.09 and 12.78 μg/ml, respectively). CICs value increased progressively in the group C and reached the highest levels in the groups D and E, both characterized by high antibodies titer and severe disease, independently from the mode of infection. Significant differences in CICs concentration ($p < 0.0001$) were demonstrated between A, B, and C groups when compared with D or E groups of dogs. No differences were found inside the first three groups, while differences were recorded between the last two groups of sick dogs. CICs serum concentration increased with the progress of leishmaniasis, being significantly correlated with the increase of specific antibodies over time. High CICs levels detectable by commercial ELISA proved specific to an established *Leishmania* infection in dogs in the absence of other concomitant infections, as demonstrated by the similar trend assessed in experimentally and naturally infected dogs.

Chapter 5-Simultaneous detection of parasitic vector borne diseases: a robust cross-sectional survey in hunting, stray and sheep dogs in a mediterranean area, the aim of this study was to investigate leishmaniosis, babesiosis, and filarial infections in dogs with three different lifestyles (hunting, stray, and sheep dogs) in Molise, the smallest region of southern Italy, where data available about these parasitic infections are very scant. A cross-sectional survey was conducted on 318 hunting, 180 stray, and 218 sheep dogs. Immunofluorescence antibody test, blood smear, molecular techniques and Knott's test were performed to detect *Leishmania infantum*, *Babesia spp*. and filarial nematodes. Association between positivity to CVBDs, age, sex, and living conditions was evaluated. An overall

prevalence of 12.3% of CVBDs caused by *L. infantum* (10.2%), *B. canis canis* (0.3%) and filarial nematodes (2.1%) was detected. Three dogs showed co-infections of *L. infantum* and *B. c. canis* (0.1%) or *Acanthocheilonema reconditum* (0.3%). A significantly association was found only for filarial infection in hunting dogs. These parasites were reported also in dogs without clinical signs. It is very important to plan effective control programs for CVBDs to guarantee not only the health and welfare of pets, but also the public safety, because some of mentioned parasites are of zoonotic importance.

Chapter 6-Investigation on the possible role of dogs in the epidemiology of *L. major* infection, the aim of this study was to identify canine leishmaniasis due to *Leishmania major*, to compare two different populations of dogs living in distinct geographical areas, of VL and ZCL and to identify co-infections/co-exposures to others CVBDs. The sampling was carried out in pre-selected sites on 229 owned dogs which generally were in bad clinical conditions. Parasite culture, skin and LN smears, serology, molecular techniques, and Knott's test were carried out to identify *Leishmania* species, *Ehrlichia spp, Anaplasma spp., Babesia spp*., and *Dirofilaria spp.* Also, a statistical evaluation allowed to study the risk factors of infected dogs according to the region in which they live. Only *L. infantum* was detected in both groups with not statistically difference between them. The co-exposure to *Ehrlichia spp.* (36%) and *Anaplasma. spp.* (16%) and one case of *Dirofilaria repens* infection was identified. Isolation of *L. major* has not been verified this indicates that dogs do not play a role in the epidemiological cycle of ZCL in Tunisia.

La leishmaniosi è una malattia infettiva ad andamento cronico causata da protozoi parassiti appartenenti ad oltre 20 specie di *Leishmania*, che si manifesta nell'uomo in tre forme: viscerale (la più grave), cutanea (la più comune) e mucocutanea. Nel cane, la malattia tipicamente evolve in due forme: viscerale e cutanea. Quasi tutte le specie di *Leishmania* sono patogene per i mammiferi, e molte di esse si rendono responsabili di zoonosi, comprese le tre specie note come agenti eziologici della malattia in Tunisia: *Leishmania infantum*, *Leishmania major* e *Leishmania killicki* (*syn. tropica*). I cani domestici sono il principale serbatoio di *L. infantum* (il principale agente eziologico della CanL in Italia e in Tunisia) e possono anche ospitare infezioni da *L. major* e *L. tropica*. Inoltre, stati di co-infezione/co-esposizione della leishmaniosi canina ad altre patologie a trasmissione vettoriale possono svolgere un ruolo importante nelle manifestazioni cliniche e influenzano il decorso dell'infezione, e può, di fatto, essere un importante fattore di rischio nell' insorgenza, progressione e ulteriore diffusione della stessa leishmaniosi, sia nell'ambito animale che umano. Tali co-infezioni includono più frequentemente: babesiosi, ehrlichiosi, anaplasmosi, borreliosi e filariosi cardiopolmonare.

Nel presente studio, discuteremo di *Leishmania infantum* e *Leishmania major*, data la loro maggiore importanza nell'uomo e nel cane, rispettivamente attraverso studi che forniscono una migliore comprensione della CanL in tutti i suoi aspetti: epidemiologici, clinici e patogenetici.

La tesi è stata suddivisa in sei capitoli di uguale importanza: l'introduzione e il primo capitolo descrivono la parte generale mentre le restanti parti trattano i lavori di ricerca svolti nel triennio del progetto di dottorato.

Capitolo 2-Valutando la relazione dei parametri clinici e di laboratorio con la potenziale infettività di *Phlebotomus perniciosus* in cani con patologia clinica conclamata, questo studio è volto ad esaminare il possibile rapporto tra le diverse alterazioni cliniche e l'infettività dei flebotomi che hanno effettuato un pasto di sangue sui cani .I dati ottenuti nel presente studio provengono da un gruppo non trattato di cani malati di leishmaniosi sottoposti a xenodiagnosi per la valutazione di una soluzione insetticida spot-on. Diciassette cani sono stati identificati come affetti da leishmaniosi attraverso l'esame clinico, la sierologia del test anticorpale di immunofluorescenza indiretta (IFAT) e l'amplificazione isotermica mediata dall'ansa (LAMP). La gravità della malattia (punteggio clinico) è stata

formulata utilizzando un valore numerico derivante da otto parametri clinici e parassitologici. La xenodiagnosi è stata eseguita su cani in gabbia esposti per 1,5 ore a punture di flebotomi. Sono stati esaminati i seguenti parametri relativi ai flebotomi: pasto di sangue (% di femmine con rigonfiamenti di sangue), rilevamento del promastigote (% di flebotomi positivi al promastigote), carica di promastigote e maturazione dello stadio del promastigote (potenziale tasso di trasmissibilità). È stata studiata la relazione statistica tra il punteggio clinico ei parametri entomologici, nonché la possibile correlazione tra ciascun parametro clinico e di laboratorio e l'infezione/infettività del flebotomo. La gravità del punteggio clinico può influenzare l'alimentazione del sangue e la probabilità di rilevamento del promastigote nei flebotomi; le lesioni cutanee sembrano essere il principale fattore che influenza la velocità di alimentazione del sangue. Il carico di promastigote è correlato al titolo IFAT, alle lesioni cutanee e al punteggio clinico. Tutti i parametri entomologici sono fortemente correlati tra loro. Questo studio conferma che sia l'infezione che l'infettività da *P. perniciosus* sono influenzate dalle condizioni cliniche del cane.

Capitolo 3- Prove di laboratorio evidenziano che la combinazione dinotefurano, piriproxifene e permetrina (Vectra®3D, Ceva Santé Animale, Libourne, Francia) abrogano la trasmissibilità della *Leishmania infantum* da parte di cani naturalmente infetti tramite flebotomi della specie *Phlebotomus perniciosus.* I cani affetti da leishmaniosi sono stati sottoposti a xenodiagnosi e 6 insetti infettanti >10% sono stati trattati localmente il giorno 0. Gli effetti insetticidi anti-alimentazione e anti-trasmissibilità sono stati valutati attraverso xenodiagnosi eseguite nei giorni 1, 7 e 28, utilizzando parametri individuali di pretrattamento come controllo. I tassi di alimentazione e mortalità sono stati valutati a 24 ore, mentre l'infezione, la maturazione e il carico del promastigote sono stati valutati fino a 96 ore dopo il pasto di sangue (tasso potenzialmente infettivo). Il giorno 1, l'efficacia anti-alimentazione era >95% in 4 cani, l'efficacia insetticida del 100% in 4 cani e l'effetto anti-trasmissibilità del 100% in 6 cani. I tassi di efficacia registrati il giorno 7 erano molto simili al giorno 1. Il giorno 28, i valori di efficacia anti-alimentazione e insetticida erano molto più ampi, rispettivamente del 32,6-100% e del 7,7-94,4%. In due cani sono stati registrati insetti potenzialmente infettivi, con una forte diminuzione del tasso di trasmissibilità rispetto alle condizioni di pretrattamento. Complessivamente, Vectra®3D ha abrogato di >98% la potenziale

trasmissibilità di *Leishmania infantum* da parte del pool esaminato di cani infetti in 1 mese.

Capitolo 4- Valutazione dei complessi immunitari circolanti (CICs) durante la leishmaniosi canina naturale e sperimentale: lo scopo di questo studio è stato di valutare il livello sierico di CICs nei cani esposti a infezioni naturali e sperimentali. Sono stati esaminati 52 sieri, appartenenti a gruppi non trattati di beagle naïve precedentemente studiati per valutare le prestazioni dei vaccini anti-*Leishmania spp.* in trasmissione naturale (n. 22 cani) o sperimentale (n. 30 cani). I sieri sono stati classificati in cinque gruppi in base alle condizioni di salute del cane, al titolo IFAT e al risultato di (n)-PCR del midollo osseo (BM): gruppo A: n.10 cani sani prima dell'infezione sperimentale; gruppo B: n.10 cani clinicamente sani infettati sperimentalmente, IFAT negativi (= titolo reciproco <160) e n-PCR positivi; gruppo C: n.10 cani clinicamente sani naturalmente infetti, IFAT positivi ai titoli 160–320 e n-PCR negativi; gruppo D: n.10 cani malati infettati sperimentalmente, IFAT positivi a titolo >320 e n-PCR positivi; gruppo E: n.12 cani malati naturalmente infetti, IFAT positivi a titolo >320 e n-PCR positivi. I livelli di CICs sono stati valutati con il metodo ELISA (canine CIC assay—Cloude-Clone Corporation, USA). I due gruppi caratterizzati da IFAT negativo (A e B) avevano il livello medio più basso di CICs (rispettivamente 16,09 e 12,78 μg/ml). Il valore di CICs è aumentato progressivamente nel gruppo C e ha raggiunto i livelli più alti nei gruppi D ed E, entrambi caratterizzati da alto titolo anticorpale e malattia grave, indipendentemente dalla modalità di infezione. Sono state dimostrate differenze statisticamente significative nella concentrazione di CICs ($p < 0,0001$) tra i gruppi A, B e C rispetto ai gruppi di cani D o E. Non sono state riscontrate differenze all'interno dei primi tre gruppi, mentre sono state registrate differenze tra gli ultimi due gruppi di cani malati. La concentrazione sierica di CICs è aumentata con il progredire della leishmaniosi, essendo significativamente correlata con l'aumento di anticorpi specifici nel tempo. I livelli elevati di CICs rilevabili mediante ELISA commerciale si sono rivelati specifici per un'infezione accertata da *Leishmania spp.* nei cani in assenza di altre infezioni concomitanti, come dimostrato dalla tendenza simile valutata nei cani infetti sperimentalmente e naturalmente.

Capitolo 5- Individuazione simultanea di malattie trasmesse da vettori parassiti: un'indagine trasversale in cani da caccia, randagi e da pastore in

un'area mediterranea. Lo scopo di questo studio era di indagare la presenza di patologie canine a trasmissione vettoriale (CVBDs), quali la leishmaniosi, la babesiosi e le infezioni da filaria, in cani con tre diversi stili di vita (cani da caccia, randagi e da pastore) in Molise, la più piccola regione del sud Italia, dove i dati disponibili su queste infezioni parassitarie sono molto scarsi. È stata condotta un'indagine trasversale su 318 cani da caccia, 180 randagi e 218 cani da pastore. Sono stati eseguiti test di immunofluorescenza per anticorpi, striscio di sangue, tecniche molecolari e test di Knott per rilevare *Leishmania infantum*, *Babesia spp*. e nematodi responsabili di filariosi. È stata valutata l'associazione tra positività a CVBDs, età, sesso e condizioni di vita. È stata rilevata una prevalenza complessiva del 12,3% dei CVBD causati da *L. infantum* (10,2%), *B. canis canis* (0,3%) e nematodi responsabili di filariosi (2,1%). Tre cani hanno mostrato coinfezioni di *L. infantum* e *B. c. canis* (0,1%) o *Acanthocheilonema reconditum* (0,3%). Un'associazione significativa è stata riscontrata solo per l'infezione da filaria nei cani da caccia. Questi parassiti sono stati segnalati anche in cani senza segni clinici. È molto importante pianificare programmi di controllo efficaci per le CVBDs per garantire non solo la salute e il benessere degli animali domestici, ma anche la sicurezza pubblica, poiché alcuni dei parassiti citati sono di importanza zoonotica.

Capitolo 6-. Indagine rivolta al possibile ruolo dei cani nell'epidemiologia dell'infezione da *L. major* in Tunisia. Lo scopo dello studio è stato di identificare casi di leishmaniosi canina sostenuti da *L. major*, e di comparare due popolazioni diverse di cani in differenti aree geografiche di VL e ZCL e di identificare le co-infezioni/co-esposizioni ad altre malattie a trasmissione vettoriale (CVBDs). I campioni sono stati ottenuti in aree geografiche pre-selezionate, da 229 cani padronali in scarse condizioni cliniche in generale. Sono stati messi a punto colture parassitarie, campionamenti di cute e linfonofi (LN), tecniche sierologiche e molecolari e test di Knott. per identificare le specie di *Leishmania spp*., *Ehrlichia spp*., *Anaplasma spp*., *Babesia spp*. e *Dirofilaria spp*. È stata inoltre eseguita una valutazione statistica per identificare fattori di rischio nei cani infetti a seconda della regione in cui essi vivono. In entrambi i gruppi è stata identificata soltanto *L. infantum*, senza differenze statisticamente significative tra i due; è stata riscontrata la co-esposizione a *Ehrlichia spp*.(36%) e *Anaplasma spp*. (16%), ed un solo caso a *Dirofilaria repens*. Di contro, non è stato individuato l'isolamento di *L. major*, e ciò potrebbe

indicare che i cani non svolgono un ruolo nel ciclo epidemiologico della ZCL in Tunisia.

Premise

This work is the result of researches made in different places and time periods. It revolves around canine leishmaniasis both in Italy and North Africa, aiming for a better understanding of the different aspects of transmissibility, pathogenesis, and distribution in the Campania and Molise region and in Tunisia.

It should be noted that the work carried out in Tunisia has several limitations that should be highlighted first, to explain its progress: the most important is the limited number of samples taken due to COVID-19-related travel and movement restrictions and relatively to the short period of the anti-rabies vaccination campaigns that we had to accompany in order to be able to access certain regions due to the lack of confidence and collaboration of the owners living in rural areas

The second limitation encountered was the difficulty of taking the samples of the different biological materials and especially the skin samples which required making biopsy punches mainly in lesions localized on the muzzle and ears of agitated and aggressive poorly contained dogs, therefore the other matrices were less invasive and easily performed, such as the blood sampling and lymph node aspiration, were favoured.

At the time of the submission of the thesis, the study is in progress to continue research in this subject

Leishmaniasis is a neglected disease caused by a flagellated protozoan parasite of over 20 species belonging to the Leishmania genus. It affects humans and many domestic and wild animals with a worldwide distribution and an endemic presence in more than 90 countries.
The World Health Organization (WHO) reports that more than 1 billion people live in areas endemic for leishmaniasis and are at risk of infection and more than one million new cases occur annually around the world, making it one of the most important parasitic diseases.
Most people and animals infected by the parasite have truly silent infection, and do not develop any signs or symptoms. Others develop mild to moderate disease, but some develop severe infection which can lead to permanent damage and potentially life-threatening complications.
In humans, the disease can be displayed in three basic clinical: visceral, cutaneous and mucocutaneous, while in animals usually cutaneous and /or visceral forms are observed. It can manifest with a variety of clinical signs, depending on the species involved, the geographic region and the host response.
As it involves several overlapping species and different vectors, the disease has a complex ecology and epidemiology.
In Tunisia, visceral leishmaniasis (VL) caused by *Leishmania infantum* and cutaneous zoonotic leishmaniasis (ZCL) caused by *Leishmania major* are endemic diseases. Visceral leishmaniasis is observed in the northern part of the country while ZCL is frequent in the center and in the south; respective outbreaks of the two diseases remain geographically separate.
As elsewhere in the Mediterranean basin and the Middle East, the dog is the main reservoir of *L. infantum* while wild rodents of the genera *Psammomys spp.* and *Meriones spp*. are the main reservoir hosts of *L. major.*
Previous reports described cases of canine leishmaniasis due to *Leishmania major* like in Saudi Arabia (1984) then some cases of cutaneous infection in dogs were reported in Egypt (1987) and in Israel (2016) identified with appropriate molecular enzymatic and biochemical techniques.

I. Taxonomy

Fossil sand flies, *Paleomyia burmitis,* from Burma's *Cretaceous amber* have been found to contain blood believed to be dinosaur blood. The whole blood of 21 fossil sand flies was examined under a microscope, the developmental stages of a fossil form of *ProtoLeishmania, Paleoleishmania proterus,* were

identified in 10 of them, which explains the capacity of adaptation and resistance of this parasite.
Leishmania parasites belong to the Kingdom *Protista*, the Sub-kingdom *Protozoa*, the Phylum *Sarcomastigophora*, the Subphylum *Mastigophora*, the *Zoomastigophora* Class, the *Kinetoplastid*a Order, the *Trypanosomatinae* Suborder, the *Trypanosomatidae* Family, the *Salivaria* Section, and the *Leishmania* Genus.

Leishmania species are a dixenous parasite, with two hosts in their lifecycle: They live in the phagocytes of the reticuloendothelial system of mammals and in the phlebotomine sandflies gut in addition to the *Forcipomyia spp.* (*Diptera*: *Ceratopogonidae*) and ticks which have been reported as potential vectors.

The use of different laboratory techniques, such as electrophoretic analysis of isoenzymes, DNA analysis, evaluation of species-specific monoclonal antibodies, with the evaluation of the behavior of the parasite in the definitive host and in the vector, its geographic distribution and its clinical implications, have allowed the description of about 53 species of *Leishmania* (without considering the synonyms and including all five subgenera and complexes: *Leishmania, Viannia, Sauroleishmania, L. Enriettii complex*, and *Paraleishmania*); of these, 31 species are known as mammalian parasites and 21 species are pathogenic for human beings.

Parasites cause three main clinical syndromes, depending on the location in mammalian tissues: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL).
The genus *Leishmania spp.*is divided into two subgenera: *Leishmania leishmania* parasites of the Old and New World, and *Leishmania viannia*, parasites of the New World, in which we find many other classified species.
The main species of *Leishmania* in the Old World are *L. donovani, L. infantum, L. major* and *L. tropica.* However, *L. infantum* is the only protozoan reported in both the Old and the New Worlds.

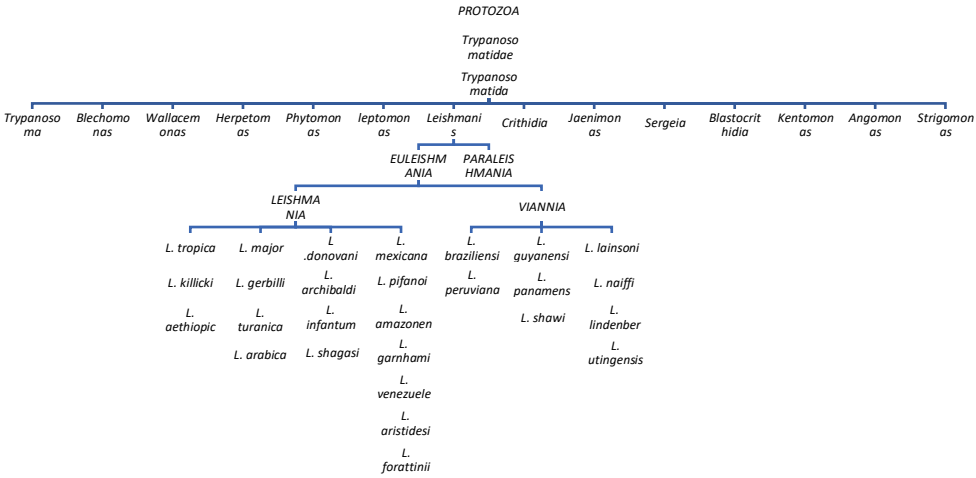

Fig. 1 Classification of Leishmania species

Currently, the taxonomy of *Leishmania spp.* has undergone profound modifications using new technologies, through which the intrinsic characteristics of the parasite (isoenzymes, DNA, proteins, antigens) have been defined. Among these, the standard method is the electrophoretic analysis of the isoenzymes which allows to group *Leishmania spp.* in zymodemes, which represent a set of strains having identical electrophoretic mobility for the enzymes examined (usually a minimum of 12).

There are two main coding systems of zymodemes:

-The MON code is attributed by the Medical Ecology Laboratory of Montpellier-France and used in all the Mediterranean countries.

-The LON code by the London School of Hygiene and Tropical Medicine in London-Great Britain, is no longer used.

In Tunisia, isoenzymatic typing allowed to establish that the most frequent parasite, responsible for the most common human and canine visceral forms is represented by *L. infantum.*In dogs, the disease is mainly caused by the Montpellier 1 zymodeme (*MON-1)* and secondly by *L. infantum MON-80.* while *L. infantum MON-24* has only been identified in humans

and the other forms of HL are represented by *Leishmania major MON-25* and *Leishmania killicki MON-317* and *MON-80*, both were not found in dogs

II. Morphology

Leishmania species are unicellular eukaryotes with clonal reproduction. Like the other members of the *Kinetoplastida* Order, they are characterized by the presence of a large mitochondrion, in which there is an accumulation of extranuclear DNA, called kinetoplast. Depending on the stage of their life cycle, there are two structural variants:

- The amastigote form is an intracellular and immotile form, found in the reticuloendothelial system of the vertebrate host.
- The promastigote form is an extracellular and motile form found in the digestive tract of sand flies.

The amastigote morphology is characterized by a spherical cell body with a short immotile flagellum that barely emerges from the flagellar pocket and is potentially more focused on sensory functions. It is of great importance in these parasites as it is the only site of endocytosis and exocytosis and is therefore a critical interface between the parasite and its host.

The Parasites multiply by binary fission within the macrophage, producing 50-200 new parasites.

The promastigote morphology is defined by an elongated ovoid cell body (15-30 µm) with a long motile flagellum extending out of the flagellar pocket that provides propulsive force likely responsible for facilitating the traverse through the sand fly digestive tract. These two morphologies retain the same basic intracellular organelles.

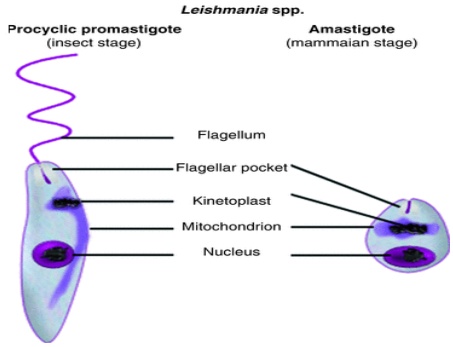

Fig. 2 Schematic representation of a promastigote (left) and an amastigote (right) of Leishmania spp.

III. Vector

This term is universally applied to hematophagous arthropods that can be both biological, where the parasite completes part of its life cycle, and mechanical, where the vector simply carries and transmits the parasite without undergoing any development.
Typically, the vector of *Leishmania spp.* is a biological vector and could be considered as just another host in the parasite's life cycle. Sandflies are the most important vectors of *Leishmania*. However, the secondary modes of transmission have been extensively discussed and hypothesized in particular, the hypothesis of ticks as vectors of *L. infantum*

Sandflies belong to:

- *Kingdom: Animalia*
- *Phylum: Arthropoda*
- *Class: Insecta*
- *Order: Diptera*
- *Family: Psychodidae*
- *Subfamily: Phlebotominae*
- *Genus: Phlebotomus*

The sandflies of the genus *Phlebotomus* are very numerous; about 10% of the approximately 600 known species of sandflies are vectors of *Protozoa*, and only 30 of these are important. *Leishmania spp.* is transmitted by the infected female sand fly's bites: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Central and South America).

Currently, the most used classification according to a conservative approach based on practical criteria, claims the subdivision of *Phlebotominae* into six genera: three Old World genera (*Phlebotomus, Sergentomyia and Chinius*) and three from the New World (*Lutzomyia, Brumptomyia and Warileya*).
The phlebotomine is a small insect, haematophagous diptera, its adults are usually less than 5 mm long, its scales are erect but sparser and can be grayish, brownish, or yellowish. The head is small and hypognathous, with dark, conspicuous eyes and no ocelli. Legs are long and thin; this allows them to typically perform small "hops" before preparing to settle down to eat the blood meal. Lanceolate wings are maintained erect over the body, when landing, and head and thorax are at 90° angle. The latter is covered with long and thin hair, which allows the sandflies to have a particularly

silent flight. In the daytime, they rest in dark and sheltered places while they are active at dusk and during the night.

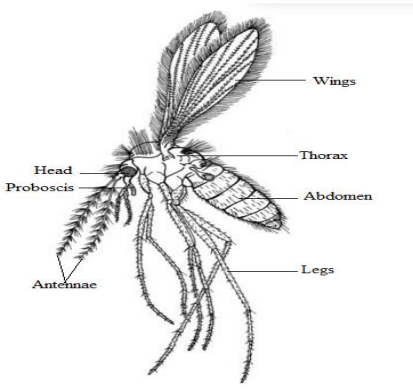

Fig. 3 Sand fly morphology

Only the females are hematophagous, while the males feed on sugary vegetable juices. Sandflies can be autogenous or anautogenic. Females of the autogenous species complete the first egg development cycle without a blood meal but require one or more blood meals to complete each subsequent cycle, whereas females of autogenic species require one or more blood meals to complete each cycle, including the first one.

Autogenesis has been observed under laboratory conditions in only a few species (e.g., *Lutzomyia lichyi*, *Phlebotomus bergeroti* and *P. kazeruni*). In a study, 8% of *P. papatasi females* produced autogenous eggs with a mean brood size of 12, compared with a brood size of 60 to 70 from anautogenic females. Multiple blood meals in a single egg cycle have been demonstrated in several species. Females of most species can complete multiple cycles.

Fig 4 Male and female Phlebotomus papatasi. The female (upper figure) has a fully engorged midgut. (x 9)

In Tunisia, *Phlebotomus perniciosus* has been reported to be the vector of *L. infantum MON-1* and the full life cycle has been demonstrated.
However, the vectors of the other two zymodemes *L. infantum MON-24* and *MON-80* are still unknown but recently Remadi *et al.*, reported through experimental study that colonized *P. perniciosus* is able to acquire, retain, and develop in its midgut the zymodemes MON-24 and MON-80 and also to transmit such zymodemes through bites.
Other studies demonstrated the high involvement of *P. longicuspis*, *P. perfiliewi*, and *P. perniciosus* in *L. infantum* transmission and the presence of the DNA of *L. infantum* in the midgut of sand flies of the *Larroussius* subgenera (*P. langeroni*, *P. longicuspis*, *P. perfiliewi*), in *Phlebotomus papatasi*, as well as in the genus *Sergentomyia* (*S. minuta*). The role of *P. perfiliewi* and *P. longicuspis* has been confirmed as vectors whereas the presence of DNA of *L. infantum* in *S. minuta* and *P. papatasi*, is not sufficient to definitively incriminate these species of sandflies as vectors but could be explained by recent feedings on infected animals resulting in parasite DNA residues after blood digestion.

For the transmission of *L. major* in Tunisia, other than *P. papatasi* as a vector, *Sergentomyia minuta and Sergentomyia clydei* sand flies are also confirmed vectors, whereas for *L. killicki* (belonging to *the L. tropica complex*) it is generally recognized as an anthroponotic parasite, and it is transmitted by *Phlebotomus sergenti.*

IV. Reservoir

The reservoir is defined as the ecological system in which the infectious agent finds the appropriate conditions that allow it to survive and multiply and from where it can be transmitted to another sensitive host.
A vertebrate host responsible for the long-term maintenance of a population of infectious agents is called a “reservoir host” which can be wild or domestic, capable of hosting an infectious or parasitic agent in nature (often asymptomatically), and a potential source of infection for humans or domestic animals, so as to cause the endemic reappearance of the infection in a given geographic area.

Therefore, "maintenance hosts" are those mammals that can be infected and maintain the infection whereas "amplifying hosts" are those mammals that, in addition to maintaining the infection, show a characteristic that favours

transmission (more parasites in the blood and in the skin for longer periods). These conditions are interchangeable according to the health conditions of the host, such as immune suppression and concomitant parasitic infections that can lead to their shift *e.g.* A significant association has been found between CanL and canine ehrlichiosis caused by *Ehrlichia canis*, as co-infected dogs can develop a higher parasitic load of the skin and consequently increase the possibility of contracting an infection through vectors.

There are two main sources of leishmaniasis; zoonotic leishmaniasis, in which the reservoir hosts are wild animals, commensals, or domestic animals, and anthroponotic leishmaniasis, in which the reservoir host is human. Although each *Leishmania* species generally falls into one or another of these categories, there are exceptions where anthroponotic species cause zoonotic transmissions (*e.g.*, *L. tropica*).

In Tunisia, dogs represent the main reservoir for the spread of human visceral leishmaniasis caused by *L. infantum*, while several wild animals are hosts of *L. major* and *L. killicki (syn. tropica)*, but the most common are rodents and more particularly *Psammomys obesus*, *Ctenodactylus gundi*, the gerbils *Meriones shawi* and *Meriones libycus.*

Other small mammals have been identified as reservoir hosts of *L. major*, *L. infantum* and *L. tropica* like hedgehogs of the *Atelerix algirus* species and of the *Paraechinus aethiopicus* species, whereas *Mustela nivalis* was also considered as a possible reservoir host for ZCL.

V. Biological cycle

Leishmania are dixenous parasites, i.e., they need two hosts to complete their life cycle:

- A vector, represented by a hematophagous dipteran insect belonging to the *Psychodidae* family (genera *Phlebotomus* in the Old World and *Lutzomya* in the New World)
- A mammal, which plays the role of host/reservoir of infection.

Consequently, there are 2 cycles in which the parasite develops, one cycle in the vector and the next in the host:

- *Leishmania* life cycle in the vector:

During its life within the phlebotomine vector, *Leishmania* undergoes a complex development process, mainly limited to the midgut, in which

parasites have to overcome various difficulties. Both the parasite and the vector reach an equilibrium that implies a significant interaction.

For its survival and the successful completion of its biological cycle in the sand fly, *Leishmania* must resist digestive enzymes (in particular proteases), it must avoid expulsion from the gut (for this purpose it produces a myo-inhibitory peptide that determines discrete gut distension, also preventing the digestion of the peritrophic matrix) and in the last stage, it must migrate anteriorly, detaching itself from the epithelium gut for transmission to the mammalian host through the meal.

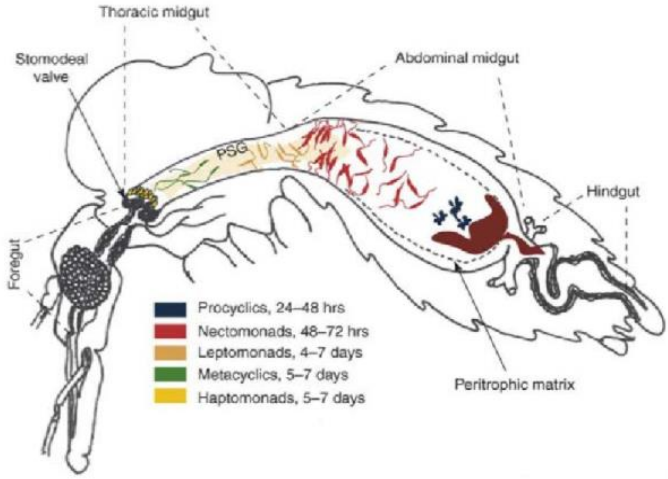

Fig. 5 Main developmental forms of Leishmania promastigotes in the sand fly vector

Digestive proteases represent the first and one of the most significant barriers to parasite survival. Within the first 6-12 hours after infection, most *Leishmania* are killed, probably by the action of these proteases. Parasites in the transition phase between amastigotes and promastigotes (procyclic) are the most vulnerable to this proteolytic attack and become less sensitive as they fully mature in the following stage of development.

Released amastigotes transform into procyclics and undergo rapid multiplication for the next 24-48 hours. The procyclic forms subsequently develop into nectomonads, detached forms, of large dimensions, whose function is to: escape the confinement of PM (through the synthesis of chitinolytic enzymes useful for freeing themselves from the peritrophic matrix), anchor themselves to the epithelial cells that line the midgut (thanks

to the residues of β-galactose-binding lectin, of LPG molecules, present at the level of the flagellum, which bind to the lectin of the digestive tract) and migrate forward towards the thoracic midgut.
Three days post-blood meal, the PM of sandflies constitutes the second barrier to the development of *Leishmania* but also serves to protect parasites from an onslaught of digestive proteases. It starts to destroy itself under the action of chitinase secreted by the sand fly but also by a parasitic chitinase that accelerates the process of exiting the nectomonads in the intestinal lumen.
These nectomonad promastigotes adhere firmly to the surface of the midgut epithelium which protects them from excretion together with the undigested blood material as the last development phase and before the sand fly moves to feed on another host.

Once they reach the stomodeal valve, the promastigote nectomonads transform into leptomonads, shorter forms that resume replication. These are responsible for the secretion of promastigote secretory gel (PSG) which plays a key role in transmission. Some of the nectomonads / leptomonads promastigotes also attach to the cuticle-lined surface of the valve and differentiate into haptomonad promastigotes.
This form of attachment is mechanically different from that seen in the midgut epithelium and is mediated by the expansion of the flagellar tip into hemidesmosome-like structures.

Finally, some leptomonads differentiate into metacyclic promastigotes, which are the infectious phases of mammals. In this case, the chitinases secreted by *Leishmania* also attack the lining of this valve, which contains chitin, irreparably damaging the valve, preventing it from functioning correctly. In addition, the parasite secretion gel (PSG) forms a plug that prevents or significantly reduces the blood supply to the midgut. Combined, the PSG plug and the lack of a functioning stomodeal valve create a mechanism that facilitates expulsion (or regurgitation) of metacyclic promastigotes.

The infectious form, being mobile and free, accumulates just behind the stomodeal valve, in an ideal position to be regurgitated by the buccal apparatus of the phlebotomine, during the next blood meal. It is likely that the infecting promastigotes are not only those located at the level of the proboscis, but also others, more posterior, located at the level of the thoracic

midgut, from which they are regurgitated directly and therefore more rapidly at the puncture site.

The entire development cycle from amastigotes to metacyclic promastigotes takes 1-2 weeks depending on the *Leishmania* species.

- *Leishmania life cycle in the host:*

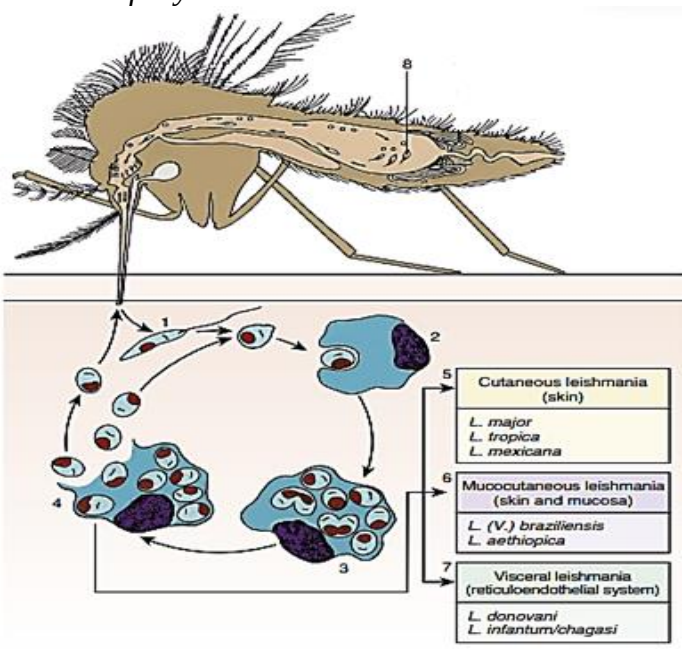

Fig. 6 Life cycle of Leishmania spp.

During its blood meal, the infected vector regurgitates the parasites in their form of flagellated promastigotes in the dermis of mammals (1) these latter will be phagocytosed by neutrophil granulocytes; forming the promastigote-neutrophil complex, which, in turn, is phagocytosed by macrophages.
In the macrophages, parasites lose their flagella to become rounded amastigotes known as "Leishman-Donovan bodies" (2) and they divide by asexual binary fission to form "cell nests" (3) which lead to the rupture of the host cell (4). The progeny is phagocytosed by other local or circulating macrophages. Depending on the parasite species, amastigotes can remain in the superficial tissues and continue the reproductive cycle (5). Others can be transported to the mucosa (6) or to the reticuloendothelial system including

the bone marrow, spleen, and liver (7). From there, they spread to the skin and become located at lesional and even healthy-looking dermal tissue.
As soon as the vector sucks amastigote-laden blood (in the skin or in the peripheral circulation) from an infected host, a new life cycle in the vector will start. These amastigotes will transform into promastigotes and divide by binary fission (8).
The incubation period of the disease before the onset of clinical signs can last for months or years, during which the parasite spreads throughout the host's body.

Although the natural transmission of *Leishmania spp.* occurs via a vector, other modes of transmission have also been reported such as through blood, products during blood transfusions from infected donors, vertical transplacental transmission and venereal transmission.
Direct dog-to-dog transmission by contact or bite without involvement of a haematophagous vector has been suspected in some cases of infection in areas where disease vectors are apparently absent.

VI. Pathogenesis and immune response

The immunopathogenetic aspect of vector-borne diseases and especially of leishmaniasis is of great importance for understanding their pathological evolution and consequently their treatment and adequate prevention. The immune response profile can trigger a pattern of resistance or susceptibility during parasitic infection, resulting in different clinical forms of the disease. To date, this aspect is not fully understood yet, neither in medicine in general nor in veterinary medicine in particular.

The clinical development of the disease after infection depends on:

- The parasitic species and the inoculum
- The genetic susceptibility and the host immunity
- Previous exposure to the vector and especially to its salivary components

Regarding the primary contact of the vector with the host, in addition to the formation of local lesions induced by the feeding of the vector, deposition of promastigotes occurs in the dermis along with large amounts of saliva at the bite site. Thirty distinct proteins were characterized in the saliva of sand flies. Some are anticoagulants to stimulate blood flow and prevent formation

of blood clots; well-researched protein, Maxadilan, is a powerful vasodilator for increasing blood flow, but many are immunogenic, meaning human and animal hosts develop antibodies to salivary proteins and appear to provide protection against *Leishmania.*
This process recruits phagocytic cells to the site, such as neutrophils, macrophages, and dendritic cells, creating a pro-inflammatory environment. The parasite's first contact with the immune system was demonstrated in an in vitro study showing that neutrophils are effector cells with the ability to control initial infection, resulting in reduced parasite viability. Furthermore, neutrophils have been observed to have the ability to produce high levels of IFN-γ when stimulated with soluble *L. infantum* antigen; and other innate immunity molecules have been correlated with ongoing CVL, such as TLRs (receptors Toll-like) and chemokines.

In cutaneous leishmaniasis due *to L. major,* it has been shown that PMNs are the first leukocytes to migrate to the site of infection and meet parasites, which engulf the microorganism without killing it. Furthermore, their physiological apoptosis is delayed and takes 2 days. The infected neutrophil secretes high levels of the chemokine MIP-1β, which attracts macrophages, which in turn engulf the PMNs and therefore the parasites, *Leishmania* internalized by this indirect way survives and multiplies in macrophages. In this way it can abuse granulocytes like a "Trojan horse" to enter their "silent" and unrecognized final host cells.

Another escape method has experimentally showed, in vitro and in vivo, that the virulent inoculum of promastigotes contains a high ratio of apoptotic parasites, and this parasite subpopulation is characterized by a rounded shape, a swollen kinetoplast, a condensed nucleus and a lack of multiplication representing dying or already dead parasites. These apoptotic promastigotes, altruistically, allow the intracellular survival of viable parasites; confirming previous observations on *Leishmania* apoptosis, which advanced the hypothesis of an evolutionary adaptation under the pressure of the host.
From the cutaneous site of infection, the parasite can be disseminated through the blood and lymphatic pathways, infecting macrophages in the bone marrow, lymph nodes, liver, spleen, kidneys, and gastrointestinal tract. Despite this, the pattern of immune responses in infected dogs is a mixture of Th1 and Th2 responses with no clear delineation between both, but the

imbalance between these responses is what prompts the infected host towards resistance or predisposition to clinical disease.
In fact, the CD4 + and CD8 + T helper lymphocytes can direct the immune system towards a humoral response (Th2), or towards a cell-mediated response (Th1).

- The main immune response against the parasite is induced by the adaptive response, in particular the type 1 immune response, characterized by the production of IFN-γ, TNF-α and IL-2 related to the resistance profile. This type of immune response is related to the upregulation of anti-leishmaniasis activity in macrophages, as the main effector mechanism of intracellular death of *Leishmania* amastigotes. In this sense, the type 1 immune response induces cytokines, such as IFN-γ and TNF-α, predominant in asymptomatic dogs, demonstrating their protective potential against the disease.
It has been shown that infected dogs with elevated IFN-γ levels had lower parasite loads than infected dogs that did not produce this cytokine and therefore dogs lacking this cytokine have more severe clinical symptoms, with increased parasitemia.

-On the contrary, the type 2 immune response, characterized by the cytokines IL-4, IL-5, IL-10 and TGF-β, is related to the susceptibility in CVL. These susceptible dogs exhibit a common pattern in the progression of clinical signs, with the severity and variety of signs increasing with disease progression, in which most clinical pathological changes become evident after 12 months of infection. Type 2 immune response provides an anti-inflammatory cytokine microenvironment that disactivates cellular immune response against *L. infantum* infection. Furthermore, a pronounced anti-*Leishmania* humoral response leads to the production of high levels of non-immunoprotective antibodies by highlighting the polyclonal B-cell response characteristic of CVL susceptibility. B lymphocytes are activated via Th2-dominated responses to produce more antibodies as plasma cells, and these antibodies, in addition to causing hyperglobulinemia, also bind to parasite antigen and complement C3 to form circulating immune complexes. Consequently, the formation of these latter increases with the progress of the disease.
Immune complexes that deposit in various tissues cause various manifestations like polyarthritis, glomerulonephritis, vasculitis, and uveitis.

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Chapter 1

Leishmaniasis

1.1 Human leishmaniasis

Human leishmaniasis (HL) is manifested in three main clinical forms: cutaneous, visceral and mucocutaneous. It is one of the oldest vector-borne tropical diseases that has remained endemic for decades due to many favouring factors such as climate change, migration, and urbanization. Unlike dogs, humans are much more resistant to *Leishmania* infection or its clinical manifestation.
Therefore, the most predisposed individuals to visceral leishmaniasis are children (whose immune system is still immature), immunosuppressed subjects (HIV positive, organ-transplanted) or those who, in any case, have predisposing pathophysiological conditions.

1.1.1 Visceral leishmaniasis

Annually, Visceral leishmaniasis causes about 200,000-400,000 new cases worldwide. Also called kala-azar (translation of "black fever", into Hindi, indicating that the skin can become dark in color), it represents the most serious and lethal disease, if left untreated.

After an incubation period of 2 weeks to 18 months, it appears with a systemic reticuloendotheliosis, characterized by irregular and progressive fever, weight loss, anemia, and hepatosplenomegaly of a considerable degree with the possible lethal outcome from terminal hemorrhages or intercurrent infections.

Fig. 1.1 Hepatosplenomegaly due to visceral leishmaniasis (VL).

Nearly 90% of VL is found in just three regions: the Indian subcontinent, East Africa, and Brazil. In the Indian subcontinent and East Africa, *L.*

donovani occurs in discrete outbreaks mainly in poor rural areas causing recurring cycles of epidemics. It is predominantly anthroponotic, although dogs and rodents can serve as reservoirs in East Africa. In contrast, *L. infantum*, which occurs sporadically in the Mediterranean, the Middle East and South America, is a zoonosis with dogs as the main reservoir.

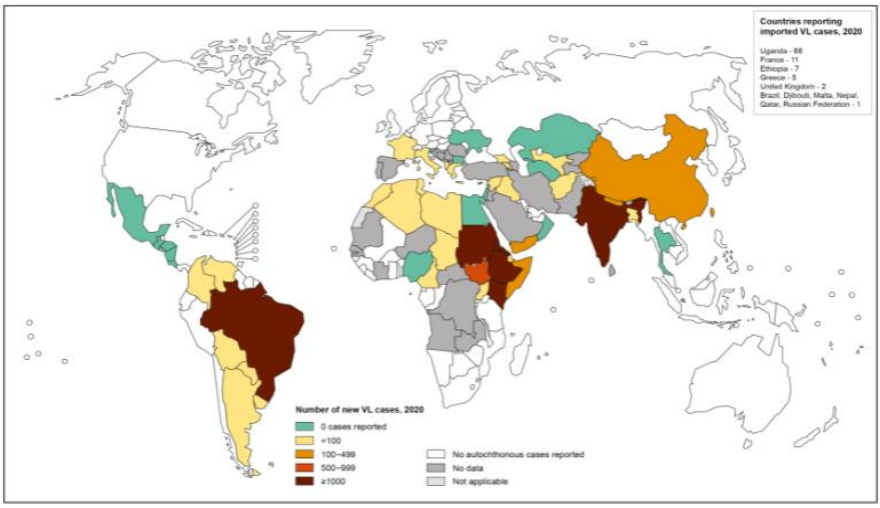

Fig. 1.2 Number of VL cases reported in 2020, but they likely underestimate the true extent of the disease.

In non-indigenous individuals and in the early stages of its outbreaks, the onset is sudden, with recurrent and irregular nightly fever sweats. It then evolves with weight loss, hepatosplenomegaly, pancytopenia and hyper-γ-globulinemia. In patients living in endemic areas, the onset of the disease is very insidious and can take place in an inconspicuous way for a long time. It is one of the most important opportunistic infections among HIV-infected individuals in geographic areas where the two infections are endemic.

In healthy persons, human-parasite contact can be evaluated with the use of specific intradermal reactions, which generally reveals a high percentage of asymptomatic infections in endemic areas.
Their frequency increases with age, therefore, in the most active outbreaks, over 40% of the adult population is positive. In addition to natural transmission via infected sand flies, artificial transmission by syringe exchange has been demonstrated among drug addicts.
Immunocompromised people are more susceptible to develop the disease, such as patients with HIV and anti-rejection immunosuppressive therapies.

Other predisposing pathophysiological factors seem to be the state of pregnancy, malnutrition, and concomitant diseases such as diabetes mellitus and chronic hepatitis. They often develop disseminated forms in different usual and unusual organs like digestive tract, lungs, cutaneous and mucosal membranes but unlike the dog's case, in humans, Glomerulonephritis is an unusual complication. Only a small percentage of adults display no apparent risk factors.

Some persons develop complication called post-kala-azar dermal leishmaniasis PKDL. This syndrome is characterized by a cutaneous manifestation (patches macular, maculo-papular and nodular rash) mainly located on the face. Usually, the rash starts around the mouth from where it spreads to other parts of the body depending on the immune response of the person. These lesions can develop during treatment, a few months after the start of therapy (as in East Africa), or years after the end of therapy (as in India).
PKDL plays an important role in the inter-epidemic periods of VL, because the patient acts as a reservoir for parasites. Their pathogenesis is largely immunologically mediated; thus, elevated concentrations of IL-10 in the blood of patients with VL can predict the development of PKD.

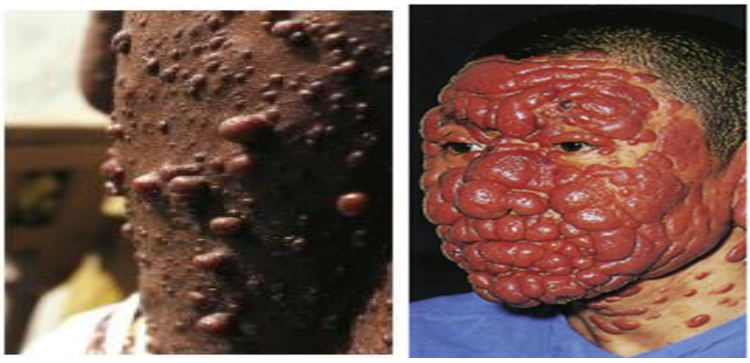

Fig 1.3 Post kala-azar dermal leishmaniasis

There are several markers of infection in VL, determined by antibody tests such as the DAT is a semi-quantitative method. The antigen is prepared from formalin-killed promastigote stages of parasite cultures and stained blue for visibility. Stained dead *L. donovani* promastigotes are mixed with patient serum in increasing dilutions on a microtiter plate. It detects antibodies *against L. donovani.*

Besides, the rK39-ELISA, is a rapid diagnostic test K39. This rapid diagnostic test detects rK39, a protein found in *L. infantum* and *L. donovani*. It is highly sensitive, inexpensive and takes less than 20 minutes to perform. Other tools to evaluate the exposure to *Leishmania* infection is found in cellular immunity markers such as the Quantiferon assay and molecular markers such as quantitative PCR.
SNP / HLA genotyping (Human Leukocyte Antigen Alleles (HLA) and Single Nucleotide Polymorphisms (SNPs) found in the HLA region) has also been demonstrated to be associated with VL.
The result should always be interpreted in conjunction with the clinical syndrome since many healthy individuals in endemic areas have asymptomatic VL infection or previous VL exposure.

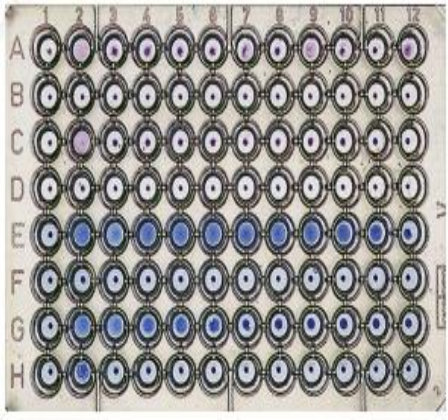

Fig 1.4 DAT for visceral leishmaniasis, samples E and G on this plate, are strongly positive

Fig 1.5 K39 rapid diagnostic test for visceral leishmaniasis. A negative test is seen on the left and a positive test on the right

1.1.2 Cutaneous leishmaniasis

CL is the most common form of leishmaniasis and is characterized by a dermatological lesion. About 95% of CL cases occur in the Mediterranean basin, the Middle East, the Americas, and Central Asia.

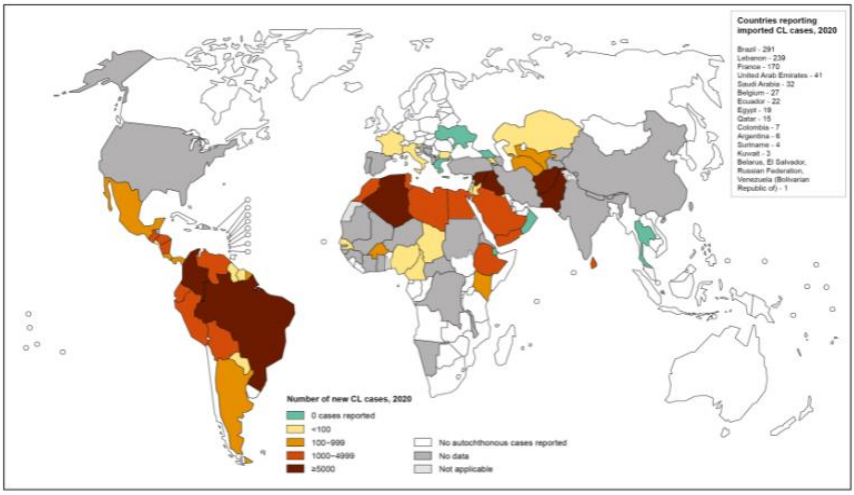

Fig 1.6 Number of CL cases reported in 2020

In the Old World, it is a zoonotic infection caused by *L. major, L. tropica and L. aethiopica. L. donovani* and *L. infantum*, which mainly cause visceral leishmaniasis and may occasionally cause localized cutaneous lesions.
CL local names are Oriental sore, Baghdad boil, Chiclero ulcer, and Aleppo boil.

This form is often associated with migratory movements, armed conflicts, when people are displaced and sand flies' habitats are destroyed, as has been seen the case in Syria where the number of reported cases has more than doubled since the start of the civil war as the result of the introduction of non-immune people into an endemic area. In fact, poor socio-economic conditions (such as poorly maintained anarchist homes, poor waste, and water management, as well as overcrowding) favour the proliferation of sand flies and their access to humans. Indeed, ecologically understudied urban area development and deforestation can lead to naive populations in areas with a high density of rodents and a very active transmission cycle. As a result, Leishmaniasis is a disease closely linked to environmental and climatic factors.

Cutaneous leishmaniasis can be classified as benign, represented by localized cutaneous leishmaniasis (LCL) which is the most prevalent clinical manifestation worldwide, characterized by the formation of slow-healing skin lesions at or near the site of the bite of the infected sand fly. Lesions begin as small red papules and develop into painless nodules, which

normally ulcerate to form circumscribed ulcers, corresponding to a Th1-type cellular immune response of the host, usually with Bacterial-leishmanial co-infection and especially with *Staphylococcus aureus*.

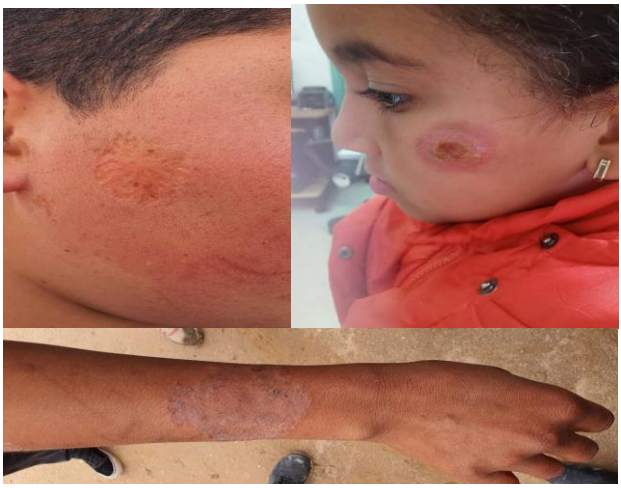

Fig. 1.7 Inhabitants from Kairouan demonstrating LCL lesions due to L. major

While diffuse cutaneous leishmaniasis (DCL) represents the severe form, with nodules disseminated on the body, with incomplete response to treatment and frequent relapses, it is associated with a Th2-type cellular response.

Between these two forms, mucocutaneous leishmaniasis corresponds, in its initial phase to an LCL, from which it appropriates the biological attributes, whereas the secondary involvement of the mucosa coincides with cellular hyperreactivity and a granuloma with mixed Th1 /Th2 cytokine profiles.

There are other minor forms of LCL described in the literature: disseminated cutaneous leishmaniasis, which is defined by a number of lesions greater than ten, and recurrent cutaneous leishmaniasis which is characterized by the reappearance of lesions at the periphery of an already healed scar tissue.

In the LCL, a multitude of morphological forms has been reported in the literature, sometimes with different names for the same clinical aspect, which displays a great source of error and confusion.

The incubation period is usually 2-4 months, but exceptionally it could last up to 3 years.

1.1.3 Mucocutaneous leishmaniasis

Leishmania infection of the mucosa of the nasopharynx is relatively rare. It is believed to be usually caused by hematogenic dissemination of the leishmaniasis inoculated in the skin for nasal mucosa, oropharynx, palates, lips, tongue, larynx and, exceptionally, trachea and upper respiratory tract. More rarely, the ocular conjunctiva and mucosa of genital organs and anus can be attacked.

If the disease is quickly diagnosed, the therapy is very effective and does not lead to relapses. In the clinical phase, human infection is of the "closed" type, meaning that the presence of parasites in the peripheral blood and in the dermis is so rare that the patient cannot act as a reservoir in turn, as is the case for the dog.

1.2 Canine Leishmaniasis

1.2.1 Symptomatology

Leishmania infantum has a dermotropic and viscerotropic tropism. It is among the most proteiform parasitic infections in dogs, which tend to become chronic and after a long debilitating phase and in the absence of therapeutic intervention, leads to the animal's death.

CanL is characterized by a large variety of clinical signs and clinicopathological alterations, the majority of which result from immune-mediated mechanisms and are attributed to CICs formation and deposition in specific tissues, causing general, cutaneous, ocular, and many other variable and nonspecific clinical spectrum, because CanL is a chronic and multisystemic disease that may potentially involve any organ and presents different symptoms as indicated in the table here below.

Tab. 1.1 Clinical signs of canine leishmaniasis.

General	**Cutaneous**
○ Generalized lymphadenomegaly ○ Loss of body weight ○ Decreased or increased appetite ○ Lethargy ○ Mucous membranes pallor ○ Splenomegaly ○ Polyuria and polydipsia ○ Fever ○ Vomiting ○ Diarrhea (including chronic colitis)	○ Non-pruritic exfoliative dermatitis with or without alopecia ○ Erosive-ulcerative dermatitis ○ Nodular dermatitis ○ Papular dermatitis ○ Pustular dermatitis ○ Onychogryphosis

Ocular	**Other signs**
○ Blepharitis (exfoliative, ulcerative, or nodular) and conjunctivitis (nodular) ○ Keratoconjunctivitis, either common or sicca ○ Anterior uveitis/Endophtalmitis	○ Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital and nasal) ○ Epistaxis ○ Lameness (erosive or non-erosive polyarthritis, osteomyelitis, polymyositis) ○ Atrophic masticatory myositis ○ Vascular disorders (systemic vasculitis, arterial thromboembolism) ○ Neurological disorders

In dogs; clinical signs may be present within three months to several years of infection. The most frequent clinical signs of the disease are the enlargement of lymph nodes that mainly affects the popliteal, prescapular, and submaxillary lymph nodes caused by the increasing of lymphoid follicles, the marked hypertrophy and hyperplasia of medullary macrophages in the cords and sinuses, and weight loss as a frequent systemic sign involving up to 50% of sick cases but cutaneous signs are typically late. The classic patterns include nonpruritic exfoliative dermatitis with or without alopecia, which can be localized or disseminated. Other manifestations often reported are splenomegaly, hepatomegaly, ophthalmopathy, chronic enteritis, and muscle atrophy (e. g. chronic myositis), as well as unusual or atypical signs such as arthritis and neurological manifestations.

In the largest study conducted in six European countries, including more than 2000 veterinary clinics, the frequency of clinical signs in descending order comprises weight loss, alopecia, lymph adenomegaly, lethargy, pale mucous membranes, exfoliative dermatitis, onychogryphosis. Atypical forms have also been described, including cardiorespiratory disorders (myocarditis, syncope, chronic cough) or neurological signs.
In endemic areas, only one of the signs described above is sufficient to suspect canine leishmaniasis.
In the early stages of the disease, renal failures may be clinically reversible though they are considered of poor prognosis as they are usually the main cause of death in the leishmaniasis patient.

1.2.2 Clinical Staging

There are two proposed classification systems:

Solano-Gallego (LEISHVET SYSTEM) and Canine Leishmaniasis Working Group (CLWG)

There is a good agreement between the two classification systems with a significant level of association.

According to the International Renal Interest Society (IRIS), the LeishVet system has a more clinical approach, dividing patients into four clinical stages and two substages with detailed definition of creatinine serum concentration and proteinuria.

Tab. 1.2 LeishVet staging system

Clinical stage	Serology	Clinical signs	Laboratory findings
I, mild disease	Negative to low positive antibody levels	Mild clinical signs such as peripheral lymphadenopathy, or papular dermatitis	No clinicopathological abnormalities
II, moderate disease	Low to high positive antibody levels	Dogs with signs of stage I may present: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/ onicogryphosis, ulcerations, anorexia, weight loss, fever, epistaxis	Clinicopathological abnormalities such as: mild non-regenerative anemia, hypergammoglobuminemia, hypoalbuminemia, serum hyperviscosity syndrome. Substage A: creatinine
III, severe disease	Medium to high positive antibody levels	Dogs with signs of stage II may present signs originating from immune-complex lesions: vasculitis, arthritis, uveitis and glomerulonephritis	Clinicopathological abnormalities listed in stage II, chronic renal disease IRIS stage I with UP/UC >1 or IRIS stage II (creatinine 1.4-2 mg/ dl)
IV, very severe disease	Medium to high positive	Dogs with signs of stage III may present with	Clinicopathological abnormalities listed in stage II, chronic renal disease IRIS

	antibody levels	Pulmonary thromboembolism, or nephrotic syndrome and end stage renal disease	stage III (creatinine 2-5 mg/dl) or IRIS stage IV (creatinine >5 mg/dl); marked proteinuria UP/UC >5

Whereas the Canine Leishmaniasis Working Group (CLWG) proposed the classification of infected dogs with positive serological tests, or those in which the parasite has been identified via direct diagnostic methods, into 5 stages (A to E), including, asymptomatic dogs as well as those with clinical signs unlike the previous system.

Tab.1.3 Canine Leishmaniasis Working Group staging system.

Stage and definition	Diagnostic methods results	Clinical signs	Laboratory findings
A, exposed	Low positive antibody levels and negative cytology, histology, or PCR	Clinically normal dogs or with signs associated with other diseases	No clinicopathological abnormalities
B, infected	Low positive antibody levels with positive cytology, histology or PCR	Clinically normal dogs or with signs associated with other diseases	No clinicopathological abnormalities
C, sick	High positive antibody levels or low level with positive cytology, histology or PCR	Clinical signs associated with leishmaniasis	Clinicopathological abnormalities suggestive of leishmaniasis
D, severely sick	-	Signs of chronic renal failure (IRIS stage III or IV) or proteinuric	Clinicopathological abnormalities

		nephropathy; severe ocular disease or severe joint disease and/ or require immunosuppressive therapy; important concomitant conditions, including neoplastic, endocrine or metabolic diseases	suggestive of leishmaniasis
E, unresponsive to treatment or early relapse	-	Clinically unresponsive to recommended treatment – clinical relapse soon after cessation of recommended treatment	-

1.2.3 Diagnosis

Diagnosis of CanL can often be complex due to the variety of clinical signs and laboratory alterations. In addition, most of the signs are also common to other pathologies; therefore, it must be suitably differentiated from other diseases such as: erlichiosis, anaplasmosis, rickettsiosis, babesiosis, hepatozoonosis, lymphoma, sarcoptic and demodectic mange, dermatitis of different etiology.

For this reason, the diagnosis must be based on an integrated approach that considers: signalment, history, laboratory tests and findings, and etiologic diagnosis (direct and indirect methods).

These tests can be divided into two groups:

- Non-specific tests: the results can be directly or indirectly correlated with leishmaniasis. They allow the determination of haematological and biochemical parameters that permit to complete the clinical diagnostic and to stage the patient
- Specific tests: allow to confirm the diagnosis of leishmaniasis

Non-specific tests:
The minimum database should include a complete blood count (CBC), serum biochemical panel with serum protein electrophoresis, and urinalysis.

Tab. 1.4 Non-specific tests for the diagnosis of CanL.

Basic tests	Findings consistent with leishmaniasis
Complete Blood Count (CBC)	Anaemia normocytic normochromic form Poorly regenerative or non-regenerative type (medullary hypoplasia) Thrombocytopenia Leukocytosis or leukopenia
Basic coagulation profile	Hyperfibrinogenemia, possible increase of PT and aPTT
Serum biochemical panel	Hyperproteinemia: hypoalbuminemia/ hyperglobulinemia: decresed A/G ratio Increased α2- globulins and poly/oligoclonal gammopathy Renal azotemia and creatinine (high values) Elevated hepatic enzyme activities
Urinalysis	Isosthenuric or poorly concentrated urine Proteinuria Increased UP/UC

Further tests can be elaborated such as Electrolytes, minerals, blood gas analysis, acute phase protein, Cytofluorimetry to detect antierythrocytes antibodies.

- Specific tests:

They allow to identify the parasite directly or indirectly (the body response: antibodies).

Diagnostic methods are cyto-histologic, parasitologic, molecular, and serologic:

Cyto-histologic methods: permit the identification of amastigotes inside intralesional macrophages or extracellularly but are not very sensitive. The adequate organs or tissues chosen are the bone marrow, the lymph nodes, the spleen, and the last choice is blood. Histologic examination is always advisable when a strong suspect remains despite negative cytological examination especially in presence of dermatitis and in cutaneous forms characterized by focal lesions. Another method that can be used with the biopsy samples is immunohistochemical stains using antibodies against Leishmania antigens.

Parasitological methods:

- Culture test: This is certainly the most specific test because the development in the culture of vital promastigotes can be attributed exclusively to genus *Leishmania* if the samples were collected in endemic areas of the Old World. It is performed by planting the sample (bone marrow, lymph node ...) on a suitable culture medium such as Novy-MacNeal-Nicolle. It also allows iso-enzymatic identification. However, it has the disadvantage of requiring long labour times.

- Xenodiagnosis: This method consists in feeding on the suspected dog some *Phlebotomus* bred in the laboratory and then examining them a few days later to identify for the presence of promastigotes in the gut. Although highly sensitive, this method is primarily used for research.

Molecular methods:

Different molecular methods have successively been evaluated for leishmaniasis diagnosis, but the PCR is considered to be the most sensitive and specific technique. It allows the detection of extremely few numbers of microorganisms or fragments of their genetic material. It could be done on several biologic samples. For leishmaniasis diagnosis, the highest probability to identify by PCR the DNA includes, in decreasing order of sensitivity: bone marrow, lymph nodes, skin, conjunctive, buffy coat and peripheral blood.

The three most commonly used techniques are:

Conventional or traditional PCR: *Leishmania* DNA is amplified using a couple of primers (base sequences complementary to the target sequence contained in *Leishmania* DNA).

“Nested” PCR: more sensitive but less specific, usually involves two sequential amplification reactions, each of which uses a different pair of primers, the increased number of passages tends to raise the risk of contamination by foreign DNA and, thus, of finding false positive results.

Quantitative PCR (“real-time”): using fluorescent probes, it is possible to quantify the number of parasite’s DNA copies present in the biological sample. Its sensitivity is similar to PCR “nested”, but if it is performed with

"closed systems" it is more specific because the sample is undergoing a lesser number of handlings and therefore is less exposed to contaminations.

Serological methods:
Serologic diagnosis in laboratories is traditionally carried out by rapid immunomigration test, immunofluorescent antibody test (IFAT), and enzyme-linked immunosorbent assays (ELISA).

Rapid immunomigration test: It is one of the fastest and most practical techniques for detecting antibody-antigen interactions and can be performed in-house too, but its diagnostic efficiency is lower than ELISA and IFAT. In the case of positive results, the limit is that the test doesn't allow to evaluate antibody titer.

Immunofluorescent antibody test: is one of the most commonly used techniques for detection of anti-*Leishmania* antibodies and is recommended by World Organization for Animal Health (OIE) as the reference of the serological method. It is performed placing the serum to evaluate on slides with *Leishmania* promastigotes. Any present antibody bind to promastigotes and positivity is shown using fluorescent anti-antibodies. In this case, it is also possible to determine antibody titer using a serial dilution of serum for evaluation. Sensitivity and specificity are near 100%. High antibody levels are associated with high levels of parasitism and provide a definitive diagnosis of CanL.

Enzyme-linked immunosorbent assays: In an ELISA assay, the antigen is immobilized to a solid surface. This is done either directly or via the use of a capture of antibody itself immobilized on the surface. The antigen is then complexed to a detection of antibody conjugated with a molecule amenable for detection such as an enzyme or a fluorophore. In positive cases, colorimetric reaction appears and can be quantified by spectrophotometry and therefore isn't dependent on operator-related variables.
The sensitivity and specificity of the ELISA technique varies depending on which antigen is used. The use of amastigotes as antigen appears to be more sensitive than promastigote antigen for detection of antibodies in both sick and subclinical dogs.

1.2.4 Therapy

The combination of meglumine antimoniate and allopurinol is the treatment of choice for canine leishmaniasis. This combined therapy is much more effective than that obtained by each of the two drugs when used alone because a high percentage of sick dogs show a very fast and tangible clinical improvement.

- Pentavalent antimonials may inhibit protozoal enzymes and damage protozoal DNA. The most common protocol for therapy is 75-100mg/kg/day SC for 4 weeks.
- Allopurinol is an analogue of hypoxanthine. The product interferes with protein synthesis by Leishmania spp. It is used at the dosage of 10 mg/kg twice a day-per oral administration for at least 6-12 months.
- Miltefosine has proved to be a powerful leishmanicidal drug and is recommended as an alternative to meglumine antimoniate. It is used as follows: 2 mg/kg/once a day for 28 days per oral administration.

The clinical response to treatments can vary from poor to good depending on their overall initial clinicopathological status and their specific response to the therapy.

It should be emphasized that infected but clinically healthy dogs do not require immediate treatment against the parasite, as the use of unnecessary treatments could upset the balance of immunocompetence that these dogs have. These animals should be monitored for early detection of the possible appearance of clinical signs and/or laboratory abnormalities suggesting the presence of disease.

1.3 Canine Leishmaniasis and Co-Infections/co-exposure

CanL co-infections reported in published literature are bacterial, protozoal helminthic, and fungal. The most common ones are Canine vector-borne diseases (CVBDs) that comprise a group of globally distributed and rapidly spreading illnesses caused by a range of pathogens transmitted by arthropods including ticks, fleas, mosquitoes, and phlebotomine sandflies. In general, these co-infections have a role in host immune response and in the development of clinical signs.

Dogs, not regularly treated and living outdoors in highly endemic areas of various vector-borne pathogens, are more exposed to co-infections. This co-

infection is not entirely benign. Studies confirmed that clinical signs were more frequent in dogs with more than one infection, and the severity is increased when co-infection with other vector-borne pathogens is present.

The most common co-infection is between *L. infantum* and *Ehrlichia spp.* In fact, recent epidemiological studies have found this strong association and the aggravation of clinical progression. In north-eastern Brazil and Spain, the odds of co-infection found are 41% and 56% while it is lower in Germany. In Cyprus, the rate was estimated at 12 times the probability of clinical leishmaniasis dogs being co-infected with *E. canis* than clinically healthy dogs.
Another important parasite that can co-infect dogs and be zoonotic *is Anaplasma spp.* This co-infection *leishmania/Anaplasma spp.* increases the risk of leishmaniasis by 79%.
In Spain, *Borrelia/ Leishmania* co-infection was reported in a recent study. However, the prevalence rate was low, not exceeding 12% in the North-eastern U.S. and being lower in Europe. Dogs are probably incidental hosts and not part of the enzootic cycle and are mostly subclinical (up to 95%).
Babesia spp. co-infection is very common in an endemic area, but seropositive dogs are often subclinically infected. The co-infection does not influence the hematocrit values compared to dogs singly infected by *Babesia.*
Trypanosoma co-infection is possible, especially in an endemic area where both pathogens coexist, but the cross-reactivity between the two parasites complicates its diagnosis.
Toxoplasma co-infection has been reported in several studies, but this parasite of worldwide distribution is frequently diagnosed in dogs. For this reason, finding seropositivity of both species is very frequent. In Brazil, between 57 % and 59% of dogs are seropositive for both and probably *L. infantum*-exposed dogs are mainly predisposed to *T. gondii* but no association exists between anti-*Leishmania* or anti-Toxoplasma antibody titer.

Helminthic co-Infection: no significant differences in the amount of adult worm recovery were observed between *L. infantum*-seropositive or seronegative dogs. Even among 265 dogs in Italy, Guardone *et al* found no statistical association between helminth infection and *L. infantum* serology. However, the presence of the gastrointestinal cestode *Dipylidium caninum* was significantly correlated with *L. infantum* seroreactivity.

Dirofilaria co-infection: infections are subclinical, or the clinical signs are non-specific, and most infections go undiagnosed until the microfilaria burden rises, and most clinical signs appear. In a study of 118 samples of dogs from South-eastern Spain, 29 microfilaria-infected dogs had significantly increased severity of clinical signs when co-infected with *L. infantum*. However, dogs infected with *L. infantum* did not have more severe signs of CanL if they were also co-infected.
Leishmania co-infections with other canine endemic pathogens potentially affect the dog's immune response. They would be more susceptible *to L. infantum* infection thus favouring higher parasite burdens and more severe clinical signs. Thus, further investigations should attempt to identify adapted tests for the simultaneous diagnoses of co-infections of canine leishmaniasis in order to know the exact role of the dog in the spread and transmission of these diseases. This would promote better control and prevention measures to achieve the principles of One Health.

In Tunisia, there are no studies that describe the co-infection of CanL with other pathogens but there are a few other studies that described CVBDs.

1.4 Epidemiology of Leishmaniasis in Tunisia

Geographically, Tunisia is located in Northern Africa. Its east coast is only 140 km away from the Italian shores, situated between Algeria and Libya. It is influenced in the North by the Mediterranean, the South being under the influence of the Sahara. The Center is under the joint effect of these two elements. It directly influences the climate of the country which varies from sub-humid in the North-eastern region to a desert climate in the South of the country. This difference is due to the Tunisian dorsal mountains, which separate the region with a Mediterranean climate from the arid region influenced by the desert.

Today, Tunisia is afflicted by growing economic disparities that favour the coastal regions to the detriment of the Central and Southern regions. Tunisia's economy was already fragile and facing many structural obstacles exacerbated by the political instability following the 2011 Revolution, which also led to reducing epidemic surveillance protocols, public health, and animal diseases controls like zoonotic diseases including leishmaniasis, rabies, brucellosis…

Since it was found in Tunisia in 1908 by Nicolle and Comte, *Leishmania infantum* agent of visceral leishmaniasis, sporadic cutaneous leishmaniasis and CanL is endemic especially in Northern Tunisia with *P. perniciosus* as a proven vector and dogs as a domestic reservoir. While the most common form is represented by cutaneous leishmaniasis due to *Leishmania major*, transmitted by one of the most common sand flies in Tunisia, *Phlebotomus papatasi*, the disease was considered endemic in 15 of 24 governorates. It is present particularly in the central and southern parts. In these same parts of the country, for the first time, Rioux et al. identified *L. killicki* (*syn. tropica)* in the Tataouine province (Southeastern Tunisia). Then other sporadic cases were reported in the Center, South Western, and Northern Tunisia, mostly transmitted by *P. sergenti.*

In Tunisia, Leishmaniasis is clinically divided into three major categories: visceral, cutaneous, and mucocutaneous. Every one of them has its own epidemiological profile including different vectors and hosts. In fact, 17 species of sand flies have been identified in the country: they belong to the genera *Phlebotomus* and *Sergentomyia*, and many wild reservoirs hosts have been confirmed or suspected as part of the leishmaniasis cycle.

The distribution of these forms is due to the ecologic and socio-economic conditions which favour the survival of the reservoir and the vector, thereby maintaining the epidemiological cycle of the disease.

1.5 Canine leishmaniasis caused by *Leishmania major*

Leishmania major is a parasite of great importance because it is one of the parasites that cause the most diffuse form of human leishmaniasis. This dermotropic parasite is responsible for pure cutaneous forms. However, non-specific clinical forms have been described in immunodeficient people, such as diffuse cutaneous leishmaniasis and visceral leishmaniasis.
Cutaneous leishmaniasis is not a lethal disease but it can lead to severe social stigma caused by life-long scars and in some cases severe disabilities.
The predominant reservoirs of *L. major* are rodents, frequently from the subfamily *Gerbillinae*, but also other small mammalian reservoir hosts have been identified.
In the 80s, *Leishmania major* was found in domestic dogs from Alexandria, Egypt, and later a few other studies in Saudi Arabia, Iraq and Israel reported some cases of canine leishmaniasis due to *L. major*. Recently both molecular

and phylogenetic studies showed that the dog *L. major* strain is closely related to that isolated in humans in the same area.
These indications cast doubt on the role of dogs in *Leishmania's major* life cycle in endemic areas and suggest that the dog is a potential reservoir.

Alanazi *et al.*, reported that the prevalence of *L. major* in dogs living in the most endemic areas of Saudi Arabia oscillated between 5.2- 6.3%, male dogs (5.2%), and dogs over one-year-old were most infected with *L. major*, contrary to what was reported by Baneth *et al.*, who found the parasite in young dogs, which means dog's gender and age had significant effects on parasite prevalence in Saudi Arabia.
Al-Bajalan *et al*. reported that antibodies in response to the *Leishmania* infection were present in the infected dogs blood with *L. major* by molecular analysis, even if the kit used was designed for diagnosing *L. donovani* because serological cross-reactivities among *Leishmania species* are not uncommon and this was also reported by Baneth *et al.*

A clinical case well described by Baneth *et al.*of a 6-month dog with *L. major* demonstrated on the physical examination that the prescapular lymph node was moderately enlarged with ulcerative skin lesions on the muzzle and footpads. A complete blood count (CBC), serum biochemistry panel and urinalysis were within normal limits.
The use of blood for leishmaniasis PCR is advantageous, as samples can be obtained less invasively from dogs and are easy to process but blood as a clinical specimen yielded low sensitivity to detect cutaneous leishmaniasis. All previous studies on L*eishmania major* in dogs reported that blood was negative by PCR.
In the study of Baneth *et al.*, only both LNA and skin biopsy were positive and yielded a DNA sequence that was 100 % identical to *Leishmania major*. Dog treated for *Leishmania major* infection with allopurinol at 10mg/kg every 12h, as recommended for dogs infected with *L. infantum*, responded to the therapy after a period in which the lymph node remained mildly enlarged with mild leukocytosis, neutrophilia, hypoalbuminemia, also serology and culture still positive for a while then a general improvement was described, and all values were normalized.

Although *Leishmania major* has been found in dogs, its epidemio-clinical role remains uncertain because it is not yet clear whether the dog is an

accidental host or a reservoir. Further research is necessary for a better understanding of this pathology, which is extremely widespread in the world and which could present a risk for Europe, since the recent discovery of voles of the species *Microtus guentheri*, (a common rodent in the Balkan countries which were infected by *L. major* in Israel) which indicates that its vector is present in Europe as well.

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Chapter 2

Examining the Relationship of Clinical and Laboratory Parameters with Infectiousness to *Phlebotomus perniciosus* and Its Potential Infectivity in Dogs with Overt Clinical Leishmaniasis

Manuela Gizzarelli, Antonio Bosco, Valentina Foglia Manzillo, Gioia Bongiorno, Riccardo Bianchi, Daniela Giaquinto, ***Nour El Houda Ben Fayala****, Marie Varloud, Alessia Crippa, Luigi Gradoni, Giuseppe Cringoli, Maria Paola Maurelli, Laura Rinaldi and Gaetano Oliva*

2.1 Introduction

Phlebotomine sand flies (*Diptera: Psychodidae*) are the biological vectors of *Leishmania* parasites (*Kinetoplastida: Trypanosomatidae*), which are transmitted to vertebrate hosts by the bite of blood-sucking females. Phlebotomines become infected when they ingest amastigote forms from peripheral blood or resident skin macrophages of an infected reservoir host. In the gut of competent vectors, amastigotes transform into flagellate forms (promastigotes) which undergo multiplication, migration to the foregut, and maturation to non-dividing metacyclic promastigotes within 7–10 days, ready to be transmitted into the skin of a new host during a subsequent blood meal. Several *Phlebotomine* species of the *Phlebotomus* (*Larroussius*) subgenus in the Old World, and some species of the *Lutzomyia* genus in the New World, are proven vectors of *Leishmania infantum*, the agent of zoonotic visceral leishmaniasis (ZVL) having infected domestic dogs as the main reservoir hosts. Of them, *Phlebotomus perniciosus* represents one of the most abundant and competent vectors in the western Mediterranean basin, including southern Europe and North Africa. Other routes of transmission have been demonstrated among dogs, including sexual, vertical, and blood transfusion-borne infections, whose importance in ZVL epidemiology, however, is still under investigation. The role of clinically vs. subclinically infected dogs in maintaining the parasite life cycle in ZVL endemic foci is still debated. Dogs' infectiousness to competent vectors has been associated with many factors, the most important being the severity of the disease exhibited by infected animals. In fact, canine leishmaniasis (CanL) is a progressing disease characterized by a broad spectrum of signs, ranging from asymptomatic/subclinical infection—frequently characterized by negative *Leishmania* serology and general healthy status—to severe, fatal disease condition. The latter is associated with elevated humoral T-helper 2 unbalanced response, leading to diffuse immune complex deposition in several organs. The severity of the clinical picture is often characterized by a large variety of skin lesions which has been associated with the ability to transmit *L. infantum* back to the sand fly population. The significance of intact skin in transmission to sand flies is less clear; however, the description of the persistence of the parasite at sand flies deposition site is interesting, described 6 months after experimental infection via the vector. The infectiousness degree to sand flies was also related to the number of parasites measured in the skin by molecular methods and also demonstrated

in dogs with doubtful serology results. Despite inherent limitations due to technical difficulties, xenodiagnosis represents the only method for the definitive assessment of the infectiousness to vectors by *Leishmania*-infected hosts, eventually allowing estimates on the potential transmissibility of the parasite. When compared to the huge amount of information concerning CanL infection and disease, data deriving from xenodiagnosis studies appear limited and, recently, often associated with the New World vector *Lutzomyia longipalpis*. The aim of this study is to increase the knowledge on the significance of the clinical stage and associated parameters in determining the infectiousness of CanL diseased dogs. In particular, the study examines the relationship of different clinical and laboratory parameters, with the infectiousness to colonized *P. perniciosus* sand flies having a blood meal on diseased dogs.

2.2 Materials and methods

2.2.1 Study Design

Data obtained in the present study come from an untreated control group of CanL sick dogs submitted to xenodiagnosis for the anti-feeding and insecticidal efficacy evaluation of a spot-on insecticide solution. The study was carried out in a facility located in the Regional Center for Parasitic Diseases (CREMOPAR) of Campania region, South Italy. This area has long been known to be endemic for CanL. The study protocol was approved by the Ethics Committee of University of Naples Federico II (n. 2016/0106421). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (i.e., Good Clinical Practice, VICHGL9, 2000; Directive 2010/63/UE; National Legislative Decree 26/2014); owners signed an informed consent. Seventeen owned dogs of different sex, breed, and age were enrolled following the diagnosis of canine leishmaniasis. Dogs were included if they were ≥6 months, were not clinically pregnant, and have not been treated with anti-*Leishmania* drugs and/or any adulticide, arthropod growth regulator, or any other pesticide or compound with central nervous system (CNS) activity in the 3 months before admission to the study. Dogs were selected among a larger group, based on their behavior, to allow their quiet exposition to sandflies' bites, for 1.5 h in the exposure chambers, without trouble. Any dog suffering from life-threatening condition or severe discomfort was not included in the study. The animals were kept under constant veterinary care during the study

period, and they were acclimatized to the study site for at least 5 days before the test.

2.2.2 Sampling Materials

From each dog, blood (two tubes with EDTA and a tube with serum separator gel), two conjunctival swabs, and fine-needle aspiration of lymph nodes were collected. Each blood sample with EDTA was analyzed: one for a complete blood count analysis (CBC) and the second for the loop-mediated isothermal amplification (LAMP) assay. The blood sample in the serum separator tube was centrifuged at 360 g for 15 min and divided into two aliquots, one used for biochemical analysis (chemistry panel, protein electrophoresis) and one stored at −20°C until serological analysis.

2.2.3 Diagnosis of CanL

CanL was diagnosed based on clinical signs attributable to *Leishmania* infection, serology result (immunofluorescent antibody test, IFAT), and specific LAMP performed on blood, lymph-node, and conjunctival samples. IFAT was performed following the procedures provided by the National Reference Center for Leishmaniosis (CReNaL, Palermo, Italy) to detect anti-*Leishmania* antibodies. The antigen used by CReNaL consisted of promastigotes of the international reference strain for *L. infantum* MHOM/TN/80/IPT1. Serum samples were considered positive if they showed a titer ≥1:160; positive and negative controls provided by CReNaL were added for each test to verify the validity of the results. DNA extractions from blood and lymph nodes were performed using the *Leishmania* Screen Glow kit (Avantech Group, Angri, SA, Italy) following the manufacturer's instructions. For conjunctival swabs, DNA extraction was performed from one of the two samples using the above kit and procedure with slight modifications. Briefly, the conjunctival swab was put in a 2 ml tube with 500 µl of extraction buffer for 10 min at room temperature. An aliquot of each extracted DNA sample was stored at −20°C until LAMP analysis. LAMP was performed using the above-mentioned kit, following the manufacturer's instructions. In each amplification run, one positive and one negative control (without DNA), both supplied by the kit, was used. Specific primers were used to amplify the 18S small subunit rRNA gene, plus a partial sequence of the internal transcribed spacer 1 (ITS-1).

2.2.4 Disease Severity Score

To be considered eligible to the study, dogs had to be positive by IFAT Leishmania serology and by LAMP-PCR performed on at least one of three different matrices. CanL severity was staged using a numeric value (range: 0–16) derived from eight clinical and parasitological parameters, including clinical signs, clinicopathological alterations, IFAT value, and LAMP result (Table 2.1). The severity of skin pathology was defined by the "skin score" (range: 0–8), obtained by adding scores attributed to each cutaneous sign detected on dogs.

Tab. 2.1 Clinical and parasitological parameters to assess the disease severity score

PARAMETER	SCORE	PARAMETER	SCORE
IFAT value		**Hematological Disorders**	
≤ 1:320	1	Absence	0
> 1:320	2	Presence	1
LAMP-PCR		**Elevated renal parameters**	
1 positive tissue	1	Absence	0
> 1 positive tissue	2	Presence	1
Clinical signs (not cutaneous)		**Elevated liver enzymes**	
Absence	0	Absence	0
Presence	1	Presence	1
Cutaneous Signs		**Abnormal Protidogram**	
Absence	0	Absence	0
Focal/multifocal alopecia	1	Presence	1
Diffuse alopecia	2		
Dermatitis /nodules	2	**Disease severity**	**(0-16)**

Ulcers	3			

2.2.5 Sand Flies and Xenodiagnosis

Adult *P. perniciosus* specimens were taken from a Spanish strain maintained under laboratory conditions since June 2012 at the facilities of Istituto Superiore di Sanità, Italy, and certified for pathogen-free status. Standard rearing conditions were 28 ± 1°C and 75–80% relative humidity (RH), with an inverted photoperiod of 17 h light/7 h dark to facilitate the feeding behavior of females during daytime. Dogs were exposed for 90 min to the bites of 3- to 9-day-old unfed *P. perniciosus* females (a range of 85–141 in different experiments) with the addition of about 10% males to promote biting behavior. The exposure cages have been designed to accommodate individual dogs of different sizes (90 cm high, 100 cm long, 75 cm wide). The bottom and the lower side of the cage's walls were made of polymethyl methacrylate (Perspex®) to avoid breakages by the dogs, whereas the upper side of the walls and the roof were made of fine-gauze cloth. Two fine-gauze tunnels were fixed to the cage to allow dog handling and sand fly release/recovery, respectively. Two hours before the sand fly exposure, Adaptil® Express Calming tablets (CEVA, FRANCE) were administered to help dogs to relax during the test. These tablets solely contain dog-appeasing pheromones (DAPs) which are not considered pharmacological compounds. This product was used instead of sedative drugs to avoid a decrease in the dogs' temperature, which would negatively affect sand-fly biting behavior. The dogs were introduced into the cage 30 min before the release of the sand flies to acclimatize them. During acclimatization and for the entire duration of exposure, the caged animals were maintained in the dark. Standard conditions suitable for sand flies were reproduced in the test room with temperatures in the range of 25–28°C and RH close to 50%. At the end of the test, the light was turned on, the dog brought out of the cage, and the sand flies, both alive and dead, were recovered with the aid of a mouth aspirator. The specimens were pooled in cylindrical plastic pots (400 ml) fitted with a tight lid, which was perforated, with the hole covered with a fine gauze holding a piece of cotton soaked with glucose-saturated solution and maintained thereafter at the usual rearing conditions. Blood feeding rate (no. of engorged specimens/no. recovered) was assessed at 24 h post-exposure, and live engorged sand flies were then individually transferred into 5-ml glass vials. Promastigote detection, in order to calculate sand fly infection rate and hence dog's infectiousness, and assessment of

promastigote burden and stage maturation in positive flies, in order to estimate a potential transmissibility rate, were performed through dissection and microscopic examination of gut samples from live blood-fed sand flies at 96 h post blood meal. This period is necessary for the initial, successful parasite multiplication and foregut migration in the invertebrate host, which are the prerequisites for maturation and subsequent transmission of metacyclic stages. The burden of *Leishmania* infection was evaluated as light (<100 parasites/gut = score 1), moderate (100–500 parasites/gut = score 2), heavy (500–1,000 parasites/gut = score 3) or very heavy (>1,000 parasites/gut = score 4).

2.2.6 Statistical Analysis

The correlation analysis between the dichotomous variables of laboratory score (IFAT, LAMP-PCR, hematological disorders, elevated liver enzymes, elevated creatinine, and abnormal protidogram) and sand fly infection (feeding rate, promastigote detection, and promastigote burden) was evaluated using the Kruskal–Wallis test. The correlation of ordinal variables of clinical/laboratory score (clinical signs except cutaneous ones, cutaneous signs, and disease severity) and quantitative morphometric variables (age, weight, and body surface area) with sand fly infection was assessed by Spearman's rho correlation coefficient. In order to understand how all clinical/laboratory score associate with sand fly infectivity (potential transmissibility rate), the Pearson's chi-squared test for dichotomous and ordinal variables and the Spearman's rho correlation coefficient (rs) for quantitative variables were performed. In addition, the correlation between entomological parameters (feeding rate, promastigote detection, promastigote burden, and potential transmissibility rate) by Spearman's rho correlation coefficient was investigated. All statistical analyses were performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA), and significance level of $p < 0.05$ was applied.

2.3 Results

The disease severity score of the 17 enrolled dogs, summarized in Table 2 2, ranged between 5 and 14 (median: 10). All dogs exhibited clinical signs related to CanL, such as lymph node enlargement, cutaneous lesions, weight loss, muscular hypotrophy, or ocular involvement. Skin lesions were present

in 81% of the cases, classified as focal/multifocal alopecia (35.2%), diffuse alopecia (47.0%), furfuraceous dermatitis (41.2%), and ulcers (29.4%). Hematological disorders, mainly represented by non-regenerative anemia, together with hyperglobulinemia and hypoalbuminemia, were the most frequent clinicopathological findings. The severity of the clinical picture was always associated with elevated IFAT titers that ranged from 1:160–1:5,120. The main entomological findings related to each single dog are reported in Table 2.3. All dogs demonstrated adequate attractiveness to sand flies, as shown by the median value of 55.4% blood feeding rate (range: 14.3–90.7%). Seven dogs were not infectious to sand flies; these dogs were characterized by lower median blood feeding rate, when compared with the remaining 10 dogs (35.6 vs. 69.7%; rs: 0.574, $p < 0.05$). Infectious dogs determined an infection rate ranging from 17.5% (Dog 4) up to 78.9% (Dog 9) of sand flies that had a blood meal on them. Based on the assessment of promastigote development in sand fly gut at 96 h post blood meal, the potential transmissibility rate of *L. infantum* by the infectious dogs ranged from 7.5% (Dog 4) to 63.6% (Dog 6). Statistical analyses of the relationship of canine clinical and laboratory parameters with sand fly blood feeding and sand fly infection parameters are reported in Table 2.4. These findings suggest that severity of clinical score is not only the main determinant for infectiousness of dogs toward the vector, but also for potential infectivity of sand flies that had a blood meal on them. The presence of skin lesions seems to be the main parameter that influences dogs' infectiousness. Interestingly, sand fly infectivity is significantly related to IFAT titer. It is worthy to note that all entomological parameters are strongly interrelated ($p < 0.05$; rs range from 0.574 to 0.971), but apparently none of them is associated with morphometric variables of dogs ($p > 0.05$).

Tab. 2.2. Breed, age, sex and disease severity of the enrolled dogs.

DOG (n.)	***IDENTIFICATION***			***SCORE***					
	Breed	***Age (ys)***	***Sex***	***IFAT***	***PCR***	***Cutaneous signs***	***Clinical signs***	***Laboratory abnormalities***	**DISEASE SEVERITY**
1	Mongrel	3	M	2	2	3	1	2	**10**
2	En. Setter	8	M	2	1	7	1	2	**13**
3	Mongrel	6	M	2	2	4	1	3	**12**
4	Mongrel	6	M	2	1	7	1	2	**13**
5	Mongrel	7	F	2	2	7	1	2	**14**
6	Ep. Breton	4	M	2	2	1	1	2	**8**

7	Mongrel	3	M	2	1	4	1	1	**9**
8	Mongrel	3	F	2	2	6	1	2	**13**
9	Mongrel	1	M	2	1	4	1	2	**10**
10	Mongrel	4	M	1	0	4	1	1	**7**
11	Mongrel	9	F	2	1	1	1	2	**7**
12	Mongrel	2	M	2	1	1	1	1	**6**
13	Mongrel	6	M	2	1	4	1	2	**10**
14	Mongrel	5	M	1	2	0	1	2	**6**
15	En. Setter	5	F	1	1	0	1	2	**5**
16	Mongrel	8	F	2	2	5	1	2	**12**
17	Mongrel	4	F	2	1	0	1	1	**5**

Tab. 2.3. Xenodiagnosis results: sand flies feeding rate and promastigote infection

Dog no.	Feeding rate (%)	Promastigote detection (%)	Promastigote burden (score) *	Potential transmissibility rate (%)
1	90.7	58.3	3	52.8
2	84.9	69.1	3	59.6
3	84.4	25.5	2	19.6
4	82.3	17.5	2	7.5
5	78.0	70.0	3	60.0
6	75.7	72.7	2	63.6
7	72.2	0	0	0
8	63.6	39.3	2	30.3
9	61.9	78.9	2	50.0
10	54.2	0	0	0
11	44.8	0	0	0
12	39.3	50.0	2	36.4
13	36.5	40.7	2	27.8
14	24.4	0	0	0
15	21.1	0	0	0
16	14.4	0	0	0
17	14.3	0	0	0

Tab. 2.4. A and B. Analysis of the relationship of laboratory and clinical dog's parameters with sand flies' parameters

A)

Dog variable	Sand fly variable		
Laboratory parameters*	Feeding rate	Promastigote detection	Promastigote burden
IFAT	p = 0.308	p = 0.139	p = 0.139
Hematological disorders	p = 0.308	p = 0.543	p = 0.456
Elevated liver enzymes	p = 0.654	p = 0.765	p = 0.323
Elevated creatinine	p = 0.541	p = 0.478	p = 0.487
Abnormal protidogram	p = 0.534	p = 0.898	p = 0.952
Clinical/lab parameters**			
Absence of cutaneous signs	ND	ND	ND
Cutaneous signs	p = 0.024	p = 0.179	p = 0.152
Disease severity	p = 0.009	p = 0.058	p = 0.044
PCR	p = 0.363	p = 0.323	p = 0.172
Morphometric parameters**			
Age	p = 0.981 rs = - 0.006	p = 0.368 rs = - 0.233	p = 0.539 rs = - 0.160
Weight	p = 0.670 rs = - 0.112	p = 0.485 rs = - 0.182	p = 0.641 rs = - 0.122
Body surface area	p = 0.687 rs = - 0.106	p = 0.466 rs = - 0.190	p = 0.637 rs = - 0.123

B)

Dog variable Laboratory parameters*	Sand fly variable Transmissibility potential
IFAT	p = 0.043 Cramér's V = 0.553
Hematological disorders	p = 0.284 Cramér's V = 0.274
Elevated liver enzymes	p = 0.165 Cramér's V = 0.540
Elevated creatinine	p = 0.585 Cramér's V = 0.299
Abnormal protidogram	p = 0.856 Cramér's V = 0.369
Clinical/lab parameters*	
Absence of cutaneous signs	ND
Cutaneous signs	p = 0.032 Cramér's V = 0.758

Disease severity	p = 0.014 Cramér's V = 0.761
PCR	p = 0.190 Cramér's V = 0.268
Morphometric parameters**	
Age	p = 0.433 rs = 0.245
Weight	p = 0.383 rs = 0.235
Body surface area	p = 0.365 rs = 0.236

2.4 Discussion

Xenodiagnosis is established as the best method to determine the role of domestic and wild animals, and humans, to act as reservoir hosts for *Leishmania* species. Therefore, the accurate evaluation of a dog's infectiousness remains problematic due to such an unpractical approach, which is only possible by specialized institutions. Specific serology and PCR tests that detect *L. infantum* infection in dogs seem to have low usefulness in providing information on the capacity of infected dogs to be infectious, even if some studies positively correlate the dog's skin and blood parasitic load with the capacity to infect sand flies vectors. Several limiting factors, first of all the need for mass breeding of sand fly colonies, make xenodiagnosis not easily applicable to large-scale studies, in particular to evaluate dog's infectiousness during and after the use of preventative and/or therapeutic compounds against *Leishmania* parasites. Because of the complex methodology, different ways to perform xenodiagnosis could make it difficult to compare results from different investigations, which would suggest the need for a consensual standardization. For example, the main differences across published studies are the number of sand flies employed for dog biting, the nature and extension of the body surface exposed to their bite (for example, the whole body or the head or areas limited to the ear, neck, or belly), and the time of exposure. In the present study, we tried to mimic a natural condition of sleeping dogs, for example, by avoiding deep anesthesia or sedation that could influence physiological parameters such as body temperature, and by giving to female sand flies the opportunity to bite on the whole body surface. The use of natural pheromonous to appease our dogs during xenodiagnosis gave very good results. All enrolled dogs were bitten by sand flies with no trouble during their caged period. They appeared

in a sleeping-like condition, similar to what happens during the natural nocturnal activity of phlebotomine sand flies.

Our study included only CanL sick dogs as per trial design, so that we could not investigate on the infectiousness and related entomological parameters of the existing broad spectrum of canine infections. In fact, further limitations in the definition of dog's infectiousness are due to a non-definitive proof of the role of subclinically infected dogs (i.e., asymptomatic) in comparison with clinically ill animals in the contribution of ZVL transmission risk. Some studies pointed out the role of asymptomatic dogs and/or the lack of correlation with clinical signs for determining the dog's infectiousness. However, the majority of studies seem to confirm that sick dogs are more dangerous epidemiologically and that the severity of clinical signs may directly influence the sand fly infection rate. Contradictory interpretation of results from different studies is likely to be due to the lack of standardized definition of the subclinical/asymptomatic condition, as often it is only referred to as the absence of overt clinical signs at external inspection without considering the possible presence of clinicopathological alterations. Analogously, there is no definitive consensus for the definition of clinical stages in diseased dogs, although in the past years the Leishvet and Canine Leishmaniotic Working Group (CLWG) groups classifications 112, have been considered as the most applicable in the clinical practice.

In the present study, we chose a different clinical score for statistical purposes. Our results clearly confirmed that the dog's infectiousness to *P. perniciosus* is significantly determined by the disease's severity, with an elevated antibody level measured through IFAT as the most important marker related to infectiousness toward *P. perniciosus* and associated with high probability of parasite transmissibility. This last finding confirms that the spread of parasite during *Leishmania* infection is strongly associated to the negative role of an unbalanced immunological Th2-humoral response, causing immune-complexes deposition and tissue damage in different body compartments. Dogs' morphometric parameters such as age, body surface, and weight did not influence the reservoir potential of the infected animals, similarly to what resulted for each single clinical parameter. Once again, these results demonstrate how difficult it is to predict the infectiousness of infected dogs based on the host morphological characteristics or one or a few clinical and/or laboratory parameters. Undoubtedly, the skin represents a remarkable parasite's rich tissue during progressive *Leishmania* infection, which explains why parasites can also be isolated from intact skin of

naturally infected dogs. In addition, an experimental model carried out on beagle dogs exposed to infected *L. longipalpis* using custom-made feeders applied to the neck or belly revealed the persistence of the parasite in small-positive lesions or intact skin distal from them for several months. Skin lesions observed in clinically ill dogs have been associated with the ability to transmit *L. infantum* back to the sand fly population. Among these dogs, those with dermatitis proved to be much more infectious in a study evaluating serological test as predictive marker of infectivity. In our study, skin lesions represented the most frequent clinical sign observed in the enrolled dogs. The kind and severity of dermatological alterations are indicative of the chronicity of the disease, as described in the following chapter.

Many animals exhibited the simultaneous presence of more than one skin lesion, usually due to mechanical trauma and/or vascular changes. The most common histologic lesion is referred to as a pyogranulomatous inflammation that affects different structures of the skin and, less often, by immune complex deposition. The presence of parasites in the skin is originated by hematogenous dissemination, whereas nodules and papules are regarded as representing sites of *Leishmania* promastigote deposition. It is not surprising that in our study a marked association between skin lesions and dog's infectiousness was demonstrated. Unfortunately, we did not have owners' consent for skin biopsy that could have added more information on individual parasite loads associated with different lesions. Even though all dogs were bitten by sand flies, 7/17 were not infectious to the vector, independently from their individual clinical status. It is worthy to note that feeding rates as high as 45–72% were recorded for three of these dogs, which means that there were good chances for a parasite to be picked up from any approachable part of the body. The reasons for which it happens in natural disease conditions remain unclear. Our experimental conditions of xenodiagnosis did not permit monitoring of full parasite development in the vector for 7–10 days—i.e., till metacyclogenesis recording—because of elevated insect mortality after 4–5 days post-the-blood meal. Anyhow, *P. perniciosus* infectivity expressed as promastigote multiplication and foregut migration at 96 h from biting (potential transmissibility) was clearly related to the same clinical and parasitological parameters associated with dogs' infectiousness; moreover, all examined entomological parameters were statistically related among them.

2.5 Conclusion

This study confirms that both *P. perniciosus* infection and infectivity can be influenced by a dog's clinical condition, particularly the skin lesions. Veterinarians, public health authorities, and dog owners should be aware of this risk for recommending and applying the necessary measures to prevent *Leishmania* spreading, primarily consisting in the application of sand fly repellent pyrethroid formulations which, unfortunately, are rarely used in sick dogs. The use of anti-*Leishmania* therapeutic protocols is known to reduce the parasite load and hence infectiousness by treated dogs but have only temporary efficacy.

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Chapter 3

Laboratory evidence that dinotefuran, pyriproxyfen and permethrin combination abrogates Leishmania infantum transmissibility by sick dogs

G Bongiorno, A Bosco, R Bianchi, L Rinaldi, V Foglia Manzillo, M Gizzarelli, M P Maurelli, D Giaquinto, **N El Houda Ben Fayala**, M Varloud, A Crippa, G Oliva, L Gradoni, G Cringoli

3.1 Introduction

Dogs are the only confirmed primary reservoir of zoonotic visceral leishmaniasis, a protozoan disease caused by *Leishmania infantum* in the Mediterranean, Middle East and Central Asia in the Old World, and in Latin American countries. Outcomes of canine leishmaniasis (CanL) infection range from a subclinical condition to clinical stages characterized by increasing severity. The earliest signs of the progressive disease are lymph node enlargement and weight loss, followed by cutaneous abnormalities, renal alterations, and ocular signs. Although *L. infantum* infection can be acquired by non-vectorial modes such as venereal and congenital transmission, or via blood transfusion, its main transmission route is by deposition into the host's skin of infective metacyclic promastigotes by the bite of infectious phlebotomine sand flies. All stages of canine infection can be potentially infectious to vectors; however, the duration and severity of CanL are associated with an increased probability of transmission. The potential for *Leishmania* transmissibility by competent phlebotomine vectors is dependent on the capacity of multiplication, migration and maturation of flagellates to infective stages in the foregut about 1 week after an infected blood meal is ingested. Increased transmissibility may occur following reverse metacyclogenesis stimulated by a second ingestion of uninfected blood meal.

Phlebotomus perniciosus is the main competent vector of *L. infantum* throughout the western Mediterranean basin, including southern Europe (Portugal, Spain, southern France, Italy and northwest Croatia) and Maghreb (Morocco, Algeria and Tunisia). Recently, these sand fly species have demonstrated to be experimentally competent to transmit tropical *leishmania*, a secondary channel agent in North Africa and the Middle East. *Phlebotomus perniciosus* is also the main representative of the *Larroussius subgenus*, consisting of morphologically and biologically close-related species proven *L. infantum* vectors in endemic areas of central and eastern Mediterranean, such as *Phlebotomus neglectus*, *Phlebotomus perfiliewi* and *Phlebotomus tobbi*.

Dog protection from sand fly bites is universally considered to be the first-line approach to prevent CanL infections and transmission of zoonotic visceral leishmaniasis by infected dogs. This can be achieved by the use of several topical formulations, such as collars, spot-ons or sprays, containing

synthetic pyrethroids with proven anti-feeding (excito-repellent) and insecticidal effects against phlebotomine sand flies. After being applied to the dog's skin, their active ingredients spread over the entire surface of the body and the hair coat. Although discouraged by manufacturers and regulatory authorities, a combination of devices is commonly used by dogs' owners, being the association of collar plus spot-on the most frequently employed. Preventive measures against sand flies are typically adopted to protect healthy dogs from *L. infantum* infections, less so to avoid parasite spreading by CanL sick dogs, for which anti-leishmanial therapies may lead to decreased infectiousness to vectors but have only temporary efficacy. Analogously, laboratory studies evaluating topical pyrethroid formulations against sand flies make use of naïve purpose-bred Beagles as per consensual international guidelines. Clinical trials of dinotefuran, pyriproxyfen and permethrin spot-on solution (Vectra®3D, Ceva Santé Animale) against *P. perniciosus*, have been found to confer elevated protection from sand-fly bites for 1 month in uninfected dogs under controlled conditions. Consequently, the present study aimed to assess the effect of the spot-on formulation on the potential *L. infantum* transmissibility by infected dogs via the vector *P. perniciosus* Animals naturally affected by clinical CanL were enrolled and xenodiagnosis assays were performed over 1 month using reared sand flies.

3.2 Materials and methods

The study was part of a larger investigation on clinical characteristics of CanL associated with infectiousness to competent
phlebotomine sand flies. Procedures of animals' enrolment and xenodiagnosis test have been reported in detail by Gizzarelli *et al.* (2021). Briefly, naturally infected dogs were identified based on clinical examination and multiple diagnostic assays, including immunofluorescence antibody test (IFAT) on sera (IFAT slides were provided by National Reference Centre for Leishmaniosis-CReNaL; anti-dog IgG used was by Sigma–Aldrich, St. Louis, MO, U.S.A.) and loop-mediated isothermal amplification (LAMP) on blood, fine-needle aspiration of lymph nodes and conjunctival swabs. The study was conducted at the Regional Centre for Monitoring Parasitic Diseases (CREMOPAR), Eboli (SA), Italy, where dogs were housed and handled in accordance with the Animal Welfare and Good Clinical Practice guidelines (VICHGL9, 2000; Directive 2010/63/UE;

National Legislative Decree 26/2014). Dog's owners signed an informed consent.

Dogs were housed individually, observed daily for general health conditions and acclimatized for at least 5 days before pre-treatment test.

Reared sand flies were from a *P. perniciosus* colony maintained at the facilities of Istituto Superiore di Sanità (ISS), Rome, since June 2012 at standard rearing conditions and certified for pathogen-free status. An average of 98 (range 85–141) unfed females ageing 3–9 days, plus about 10% males to promote feeding behaviour, were allowed to feed for 90 min on caged dogs in pre-treatment tests performed 1–4 weeks before enrolment, and on days 1, 7 and 28 after treatment of enrolled dogs. Because of the chronic progression of CanL disease, it was assumed that *Leishmania* infectiousness to vectors did not change much in each dog during 5–8 weeks, so that each animal served as its own control by comparison with pre-treatment xenodiagnosis results. Only dogs infecting >10% of sand flies and with stable renal function (IRIS stage I, without or with mild proteinuria) were included in the study and treated with Vectra®3D on day 0. The product was applied as per label in a line-on. At the end of the study, the dogs were offered a treatment against CanL and were followed-up by the veterinary clinician team.

Xenodiagnosis assays were performed in individual large exposure cages as described by Gizzarelli *et al. (2021)*. Two hours before exposure to the vector, dogs received Adaptil Express Calming tablets (Ceva Santé Animale, 1 tablet/10 kg of body weight) and were allowed to acclimatize inside the exposure cage in the dark for 30 min before the release of sand flies. Cages were located in separate rooms, one for pre-treatment assays, the other for assays on treated animals to avoid contaminations. At the end of the test, live and dead sand flies were recovered with the help of a mouth aspirator and transferred into plastic transport pots equipped with a fine gauze holding a piece of cotton soaked with glucose-saturated solution.

Immediately after collection, the specimens were transported by car (trip duration approximately 2.5 h) inside preheated insulated container at appropriate humidity conditions. The pots were maintained thereafter in the usual rearing conditions at the ISS facilities.

At 24 h from exposure, live or dead blood-fed, and live or dead unfed specimens, were recorded. All live blood-fed sand flies were individually transferred into glass vials and observed daily for blood digestion and mortality. Blood feeding rate (total no. of blood-fed specimens–total no. of recovered specimens), anti-feeding rate (total no. of unfed specimens–total

no. of recovered specimens) and mortality rate (total no. of dead specimens–total no. of recovered specimens) were determined.
At each post-treatment assessment, anti-feeding and insecticidal efficacies were calculated by the comparison with the respective rates recorded for the same dog in the pre-treatment assays, as follows:
[100 × (anti-feeding rate in treated dog–anti-feeding rate in untreated dog)/1−anti-feeding rate in untreated dog] and [100 × (mortality rate in treated dog–mortality rate in untreated dog)/1− mortality rate in untreated dog].

At 96 h post blood meal, sand flies were dissected and microscopically examined to detect flagellate parasites in order to determine a promastigote infection rate (total no. of promastigote-positive specimens/total no. of dissected sand flies), being a rough measure of dog's infectiousness.
Promastigote burden was also evaluated, scored as light (<100 parasites/gut), moderate (100–500 parasites/gut), heavy (500–1000 parasites/gut) or very heavy (>1000 parasites/gut).
The 96 h period is necessary for the parasite to develop from the amastigote stage (ingested through the blood meal) to promastigotes in active multiplication, stage maturation and migration towards the foregut after blood digestion, before they transform into the metacyclic infective stage.
The potential transmissibility rate of *L. infantum* by xenodiagnosed dogs (total no. of potentially infectious specimens–total no. of females used in the challenge) was thus determined by a combination of the two entomological parameters, the microscopy evidence for foregut migration of promastigotes, and their 'heavy' or 'very heavy' burden. Hence, an anti-transmissibility effect conferred by treatment could be calculated by the comparison of this parameter from the same dog in pre-treatment tests as follows:
[100 × (potential transmissibility rate in treated dog–potential transmissibility rate in untreated dog)/1−potential transmissibility rate in untreated dog].

The non-parametric test of Kruskall Wallis was used to test entomological parameters from dogs at different days from treatment vs. pre-treatment condition, using SPSS Statistics v.23 (IBM, Armonk, NY, U.S.A.) and significance level of $P<0.05$.

3.3 Results

Among potentially eligible dogs diagnosed as *Leishmania*-positive, six were enrolled because 86-their clinical condition was compatible with inclusion criteria while showing vector infectiousness rates >10% at the xenodiagnosis test. Five dogs were males and only one was pure breed. Ages ranged from 1 to 7 years and body weight 7 to 19 kg; all dogs presented elevated anti-*Leishmania* antibody titres (≥1:2560) at the IFAT assay, a known prognostic marker for the increased probability of infectiousness (Tab. 3.1). In addition, LAMP resulted positive for lymph nodes matrix for all the six dogs, while five of them were positive also for conjunctival swab and one also for blood samples (Tab. 3.1). Clinical signs, each being assigned a score specific for this study, were present in all dogs but of different types and severity. For example, cutaneous alterations ranged from single focal alopecia (score 1, dogs 1 and 3) to generalized dermatitis and ulcers (score 7, dog 5). A number of parasitological and clinical parameters were considered (IFAT value; number of LAMP-positive tissues; cutaneous signs; other non-cutaneous signs; haematological, renal, and hepatic laboratory markers) whose scores were added up to contribute to an 'overall disease severity' score ranging from 6 (dog 3) to 14 (dog 5)

Tab. 3.1. Breed, age, sex and disease severity of the enrolled dogs.

Dog number	Sex	Age (years)	Weight (Kg)	Breed	IFAT titre	LAMP*	Clinical scores		
							Cutaneous signs	Laboratory abnormalities	Overall disease severity
1	M	4	19	Épagneul Breton	1:2560	Positive	1	2	8
2	M	1	10	Mongrel	1:5120	Positive	4	2	10
3	M	2	7	Mongrel	1:10240	Positive	1	1	6
4	M	6	19	Mongrel	1:5120	Positive	4	3	12
5	F	7	15	Mongrel	1:5120	Positive	7	2	14
6	M	3	7	Mongrel	1:2560	Positive	3	2	10

∗Positivity for at least one matrix examined (blood, lymph nodes and conjunctival swabs). Clinical scores were from Gizzarelli et al. (2021). Note that other parameters not included in the table contributed to the 'overall disease severity' score

Tab.3.2 reports on four entomological parameters recorded at the pre-treatment xenodiagnosis test. *Phlebotomus perniciosus* feeding rates ranged

from 39.3% in dog 3 to 90.7% in dog 6. Mortality rates were generally elevated considering the untreated condition of dogs, from 9.1% (dog 4) to 29.1% (dog 5); this much probably reflecting a susceptibility of the reared insects submitted to unusual experimental conditions. Promastigote-infection rates were also elevated, in a range from 25.5% (dog 4) to 78.9% (dog 2); as expected, potentially infectious females were less than the positive ones – indicating a proportion of sand flies in which the parasite failed to multiply and develop further – and so were the rates, which ranged between 19.6% and 63.6%. The ratio 'infectious rate' over 'positive rate' varied from 0.6 (dog 2) to 0.9 (dog 6), but this did not differ significantly between dogs.

Tab. 3.2 Results of the pre-treatment xenodiagnosis test on the enrolled dogs using Phlebotomus perniciosus females.

Dog Number	Weeks before treatment	No. of females recovered	Entomological parameters (%)			
			Feeding rate	Mortality rate	Promastigote infection rate	Potentially infectious rate
1	2	99	75.8	17.9	72.7	63.6
2	2	63	61.9	26.0	78.9	50.0
3	1	89	39.3	30.9	50.0	36.4
4	3	77	84.4	10.0	25.5	19.6
5	3	141	78.0	41.0	70.0	60.0
6	4	108	90.7	30.1	58.3	52.8

Entomological parameters use the number of recovered females as denominator.

Post-treatment changes recorded in entomological parameters were used to calculate the efficacy of Vectra®3D treatment starting from day 1 and to day 28 (Table 3.3). On day 1 the onset of anti-feeding efficacy ranged from 80.6% (dog 2) to 100% (dogs 3 and 4), being >95% in 4 animals. Insecticidal efficacy was in the range from 75.9% (dog 3) to 100% in 4 animals (dogs 1, 4–6). No blood-fed females were found alive at 24 h for any dog, therefore, the entomological parameters associated with *Leishmania* infections at 96 h were set to zero and the anti-transmissibility efficacy resulted 100% for all dogs.

Tab 3.3 Entomological efficacy parameters in post-treatment xenodiagnosis tests performed on Days 1, 7 and 28 using Phlebotomus perniciosus females.

Dog number	No. of females used in cage/recovered	Anti-feeding efficacy (%)	Insecticidal efficacy (%)	Potential transmissibility rate (%)	Anti-transmissibility effect (%)
		Day 1 evaluation at 24h		Day 1 evaluation at 96h	

1	90/82	98.3	100.0	0.0	100.0
2	90/75	80.6	89.2	0.0	100.0
3	90/90	100.0	75.9	0.0	100.0
4	85/83	100.0	100.0	0.0	100.0
5	110/105	84.1	100.0	0.0	100.0
6	90/89	98.8	100.0	0.0	100.0
		Day 7 evaluation at 24h		Day 7 evaluation at 96h	
1	90/88	100.0	69.6	0.0	100.0
2	90/84	78.8	18.0	0.0	100.0
3	97/97	100.0	83.6	0.0	100.0
4	90/77	95.4	100.0	0.0	100.0
5	122/122	79.0	100.0	0.0	100.0
6	91/91	98.8	100.0	0.0	100.0
		Day 28 evaluation at 24h		Day 28 evaluation at 96h	
1	90/78	100.0	62.5	0.0	100.0
2	90/82	64.5	7.7	4.4	78.9
3	90/60	57.6	44.5	0.0	100.0
4	140/137	32.6	65.9	0.7	93.6
5	91/91	71.8	94.4	0.0	100.0
6	100/96	87.4	88.1	0.0	100.0

**Parameter calculations include either the total number of females used in the cage, or the number of recovered females.*

Day 7 assessments resulted in efficacy rates similar to day 1, showing an anti-feeding effect ranging from 78.8% (dog 2) to 100% (dogs 1 and 3), again being >95% in 4 dogs. Insecticidal efficacy was unexpectedly low in dog 2 (18.0%) but it was 100% in 3 animals (dogs 4–6). Only a few blood-fed females survived at 24 h (dog 2) but none at 96 h for any dog, therefore, the anti-transmissibility efficacy of treatment has proved 100% in all animals.

On day 28, the anti-feeding efficacy reached 100% in one animal (dog 1), whereas it was found as low as 32.6% in dog 4. Insecticidal efficacy was highest in dog 5 (94.4%) and confirmed, from day 7 result, to be the lowest in dog 2 (7.7%). Only sand flies, which had a blood meal on 2/6 animals (dogs 2 and 4) were found to be potentially infectious, but the anti-transmissibility efficacy for them was as high as 78.9% and 93.6%, respectively. Altogether, the tested spot-on abrogated by >98% the *Leishmania* transmissibility by the examined pool of infected dogs over 1 month.

3.4 Discussion

To the best of our knowledge, this is the first laboratory study aiming to assess the effect of a topical insecticide treatment against the transmission of *L. infantum* by infectious sick dogs via phlebotomine vectors. The main difference with clinical trials of topical formulations against sand flies carried out according to consensual guidelines lies in the composition of the

canine population. In a standard laboratory study, groups of treated and control purpose-bred dogs are tested in parallel and are homogeneous in breed, sex, weight and naïve status, as well as in the attractiveness to sand fly's bites as determined by preliminary challenges. Such parallel testing allows to reduce confounding effects for feeding and mortality rates calculation that may be associated with different rearing batches, transport and handling of sand flies, and authorizes the calculation of arithmetic means of values from all dogs in each treatment arm and hence provides a statistically robust comparison of effects between arms. Because of the present study design, the sand fly exposure of owned, untreated (healthy or *Leishmania* sick?) dogs in parallel with Vectra®3D-treated dogs at each of the 3 post-treatment individual assessments, was considered unfeasible for both practical and ethical considerations. Therefore, due to the large variations of dog's characteristics, which also included type and severity of CanL signs, any entomological parameter was managed and analysed individually in comparisons between pre-and post-treatment conditions of each dog. The only efficacy parameter, which was assumed a posteriori to be homogeneous among the dogs, and hence treated as a mean, was the anti-transmissibility effect conferred by treatment in all post-treatment days evaluated: this was 100% in 16/18 determinations over 1 month (Tab. 3.3), for an average of 98.5%.

To estimate *Leishmania* transmissibility by our infected dogs, a potentially' infectious condition was determined microscopically in specimens up to 4 days after blood feeding. Morphological metacyclogenesis, the only available marker of 'actual' sand fly infectivity, may take place in the insect foregut starting from about 7 days after parasite ingestion along with the host's blood. Monitoring gut infections over this long period, however, would be hardly achievable because laboratory-reared females of *P. perniciosus* are characterized by high mortality (around 90%) from day 5 post blood meal, immediately after laying eggs. A proxy for the infectivity was therefore a compromise in order to have a sufficient number of live females to dissect after blood digestion. Another technical limitation associated with the fragility of sand flies, was the relatively elevated mortality of specimens released in cages with untreated dogs (Tab. 3.6). This was probably caused by handling and transport of the insects to the study site and back on the same day; on the other hand, sand flies from treated dogs were also exposed to the same experimental conditions, and the

formula used for the insecticidal efficacy rate calculation reduced the background of mortality in controls.

At the last assessment on day 28, dogs 2 and 4 were found to harbour potentially transmissible infections, although at a much lower rate as compared with pre-treatment conditions. Apparently, the cause could not be attributable to the promastigote-infection rates detected in sand flies, which fed on these animals before treatment: actually, dog 2 scored the highest one (78.9%) but dog 4 the lowest one (25.5%) (Tab. 3.1). Rather, a suggested hypothesis could be that in the presence of cutaneous lesions including ulcerative ones – in fact, both animals had the second-highest score among the study dogs – the active ingredients of the product did not spread homogeneously over the body surface and the hair coat. Interestingly, on day 28 the lowest insecticidal efficacy (7.7%) was recorded in dog 2, and the lowest anti-feeding efficacy (32.6%) in dog 4.

It has been assumed that the repellent and insecticidal effects on sand flies (Fig. 3.1 A, B) are due to the activity combination of permethrin, an excito-repellent and moderate insecticidal pyrethroid, and dinotefuran, an insecticidal neonicotinoid. Dinotefuran was indeed shown to be highly toxic to phlebotomine sand flies. Moreover, a synergic activity was demonstrated between the two active ingredients against insects and is expected to provide an improved efficacy. A claimed 3- or 4-week duration of elevated anti-feeding efficacy was reported by a number of commercial spot-on or a spray containing permethrin as an active ingredient (reviewed by Fondati *et al*., 2018). The anti-transmissibility activity recorded by Vectra®3D in our sick dogs was probably due to both effects, which were differently expressed among the dogs on day 28 (Fig. 3.1C). In already infected dogs, the first expected benefit of the product is to avoid the spread of the pathogen from the reservoir host. The product will also prevent the bites from potentially infectious sand flies, limiting new *Leishmania* challenge in those animals, which are already weakened, or from other vector-borne diseases expected to worsen the clinical picture.

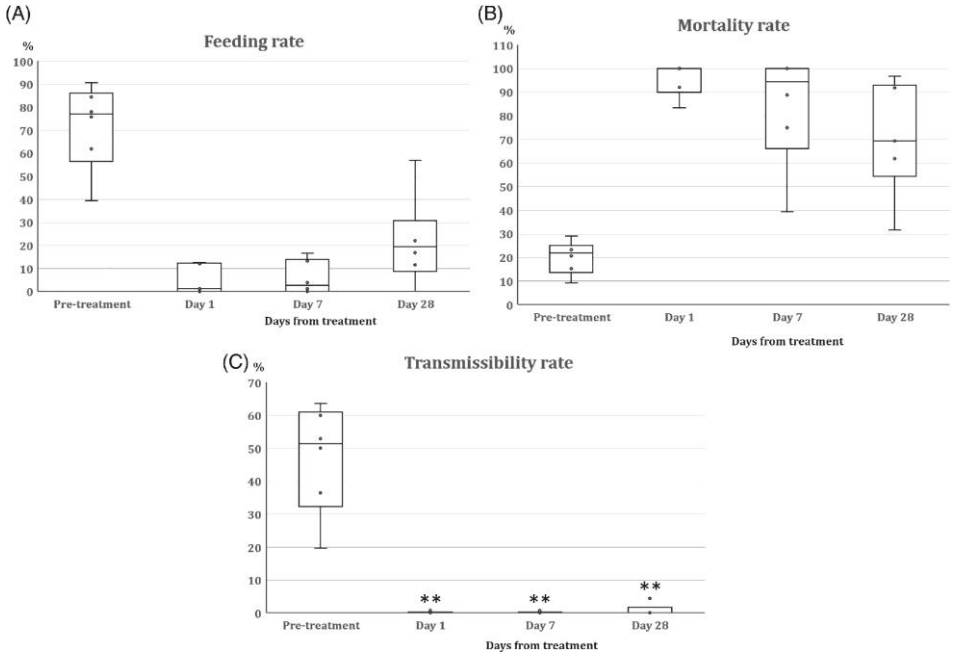

*Fig. 3.1 Box-and-whisker plots showing the feeding (A), mortality (B) and transmissibility (C) rates recorded in 6 dogs before treatment and on days 1, 7 and 28 from treatment. Statistical differences from pre-treatment condition are indicated: *P < 0.05, **P < 0.01.*

Among the available canine topical insecticides with proven efficacy against sand flies, the choice for a spot-on formulation vs. long-lasting deltamethrin-impregnated collars for leishmaniasis control should be based on a number of criteria. They include, among others, a required broader spectrum of activity against arthropod vectors, avoidance of abrupt loss of protection especially in dogs living in groups (e.g. collar loss or disruption in kennelled dogs), and fast onset of action – reached by Vectra®3D within the first 24 h of application, whereas collars require several days before appropriate levels of pyrethroid spread over the dog's body.

3.5 Conclusion

Monthly Vectra®3D topical treatment of the canine population in *L. infantum* endemic areas, including healthy and infected animals, represents a reliable tool for the control of zoonotic disease.

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Chapter 4

Assessment of Circulating Immune Complexes During Natural and Experimental Canine Leishmaniasis

Manuela Gizzarelli, Eleonora Fiorentino, **Nour El Houda Ben Fayala**, Serena Montagnaro, Raquel Torras, Luigi Gradoni, Gaetano Oliva and Valentina Foglia Manzillo.

4.1 Introduction

Canine leishmaniasis is a parasitic infection caused by the *protozoan Leishmania infantum* (*Kinetoplastida: Trypanosomatidae*). Affecting several millions of dogs globally, CanL is most often manifested as a chronic systemic disease characterized by a large variety of clinical signs and clinicopathological alterations, the majority of which due to immune mediated mechanisms. The disease progression depends largely on the immune responses mounted by infected dogs. Animals presenting overt clinical signs exhibit high titers of anti-leishmanial antibodies associated with reduced immune cellular response. In diseased dogs, T lymphocytes undergo depletion in the lymphoid tissues where mainly B-cell, histiocytes and macrophages proliferate, which may contribute to cause generalized lymph node enlargement, splenomegaly and hypergammaglobulinemia. The uncontrolled concentration of antibodies and the large amount of *Leishmania* antigens can give rise to circulating immune complexes (CICs) that determine the reduction of the macrophage ability to kill the parasite and induce vasculitis that activates the complement cascade, which eventually is responsible for tissue necrosis and for some of dermal, visceral, ocular and renal lesions. Deposition of CICs in specific organs, determined by deficient activity by scavenger macrophages, results in glomerulonephritis, vasculitis, uveitis, myositis, and polyarthritis. As regards the pathogenesis of other canine vector-borne diseases (CVBDs) characterized by a progressive course of infection, the role of CICs is also well-described in different stages of infection by *Ehrlichia canis*, whereas this is under discussion in stages of *Anaplasma canis* infection.

Several commercial tests have been developed to detect and measure CICs from serum samples, that exploit different biochemical and biophysical properties such as precipitation, binding to complement fractions or Fc-recognizing molecules, however no standard tests are currently available for dogs, nor for detection of CICs during CVBDs.

The aims of the present study were to measure the serum level of CICs in dogs exposed to CanL infection, both in natural and in experimental conditions, and to assess the usefulness of a commercial ELISA kit for canine CICs detection. The justification for the use of data deriving from experimentally infected dogs is that the course of CanL infection is different when this is caused by deliberate parasite injection or laboratory-controlled

sand fly bites, as compared with natural exposure to field conditions in endemic settings. On the other hand, pathological manifestations deriving from experimental Leishmania infection can only be attributed to the parasite infection alone, due to the use of naïve dogs, bred under vector-borne infection-free conditions in a well-controlled environment.

4.2 Materials and methods

A retrospective study was designed to assess the CICs level in five different groups of naïve beagles (total number: 52). These dogs belonged to untreated control groups previously studied to assess the performance of anti-leishmanial vaccines under natural (no. 22) or experimental (no. 30) transmission conditions. Field studies were performed in Italy during the years 2010–2013 and had been approved by the Veterinary Board of the Italian Ministry of Health. The experimental study was performed in Spain (years 2016–2017) and approved by Health Catalan Authorities (Ethical Committee authorization no. 9099).

4.2.1 Natural Leishmania Infection

As previously reported, dogs were exposed to natural conditions of *Leishmania* transmission in a rural site of southern Italy endemic for CanL, the activity of the local sand fly vector, *Phlebotomus perniciosus*, was typically seasonal (May-October); to ensure exposure to bites, the animals were not treated with repellent products with proven activity against sand flies, whereas tick and flea infestations were avoided by appropriate environmental and mechanical control measures. The dogs received routine vaccinations and were submitted to deworming every 6 months. Starting from the first month of exposure during summer, a follow-up period of 24 months was considered necessary because of the long pre-patent and incubation periods as well as the natural slow course of naturally-acquired *Leishmania* infections. This particular condition allowed to select dogs with chronic infections, most often without evident clinical disease. Dogs were examined and sampled monthly for the detection of clinical signs and clinicopathological alterations, and every 3 months for the laboratory detection and evaluation of infection.

4.2.2 Experimental Leishmania Infection

Beagle dogs submitted to *Leishmania* experimental infection were housed at ISOQUIMEN S.L. (St. Feliu de Codres, Spain). The animals were bred under controlled conditions aimed at preventing vector-borne infections, including leishmaniasis, by mechanical measures. All dogs were under constant veterinary care, received their routine vaccinations and periodical anthelmintic treatment. The inoculum for the experimental *L. infantum* infection was prepared at the Section of Parasitology, Faculty of Farmacy of Barcelona University (Dr. Montserrat Gállego Culleré). The laboratory strain MCRI/ES/2016/BCN-890 was obtained through passage to hamster of the canine strain MCAN/ES/1992/BCN-83 (zymodeme *MON-1*). Parasites cultured from heavily infected hamster's spleen were used. The infection was performed by intravenous injection of recently-transformed promastigotes at the dose of 5×10^7 in 1 ml physiologic saline solution. In contrast to what happens in natural Leishmania infections, experimentally-infected dogs usually develop infections already detectable a few weeks after receiving an intravenous injection of parasites, followed by rapid development of disease signs in most of the infected dogs. Dogs were examined and sampled monthly for detection of early clinical signs and clinicopathological alterations, and every 3 months for the evaluation of the infection burden.

4.2.3 Serological and Parasitological Diagnosis

An in-house quantitative IFAT assay was used to detect and provide titration of anti-leishmanial immunoglobulins G, according to the technical recommendations of the Office International des Epizooties. Threshold for positivity (cut-off) had been previously determined at the serum dilution of 1:160. BM aspirates were subject to nested-PCR (n-PCR) analysis for *Leishmania spp*. DNA using the sets of R221/R332 and R223/R333 primers in two consecutive runs.

4.2.4 Group Composition for CICs Level Assessment

Fifty-two sera stored at −80°C were examined for the detection of CICs level. Sera were classified in 5 groups according to the dog's health condition, IFAT result and titer, and the BM nested (n)-PCR result. A: no.10 healthy dogs before the experimental infection; B: no.10 clinically healthy dogs infected experimentally, IFAT negative (= reciprocal titer <160) and

n-PCR positive; C: no.10 clinically healthy dogs naturally infected, IFAT positive at titers 160–320 and n-PCR negative; D: no.10 sick dogs experimentally infected, IFAT positive at titer >320 and n-PCR positive; E: no.12 sick dogs naturally infected, IFAT positive at titer >320 and n-PCR positive. Because the longitudinal feature of these studies allowed to follow the dogs from their negative status through the detection of the infection till the appearance of clinical signs, the selected sera belonged to dogs with different monthly (M) periods of follow-up, respectively: Group A: M0; Group B: M3 (infection); Group C: M19 (infection); Group D: M6 (disease); Group E: M16 (disease).

CICs levels (μg/ml) were assessed in duplicate using the ELISA method (canine CIC assay—Cloude-Clone Corporation, USA), according to the manufacturer's instructions. The kit consists of a competitive inhibition enzyme immunoassay technique for the in- vitro quantitative measurement of CICs in dog serum, plasma, and other biological fluids, made possible by pre-coating ELISA test plates with a canine-specific mAb. The resulting competition between biotin-labeled (standard) and unlabeled CICs (sample) is revealed and measured by horseradish peroxidase-conjugated avidin, taking into account that the color intensity developed after substrate addition is reverse proportional to the CIC concentration in the sample.

Statistical analysis was performed with MedCalc software (Frank Shoonjans, V.7.2.1.0) by the Tukey's multiple comparison test.

4.3 Results

Individual CICs and IFAT values determined in sera from all dogs were plotted and analyzed for a relationship (Figure 4.1A). A statistical correlation was assessed by R-squared method, resulting in a significant positive correlation explaining about half of the increased CICs values associated with elevation of IFAT titers (r^2 0.46: moderate correlation; $p < 0.0001$).
The median levels of CICs associated to parasitological and antibody responses of each of the A-E groups, are shown in Table 4.1. In the same table, the type and frequency of clinical and clinicopathological findings recorded in the two symptomatic groups, D and E, are also shown. Graphical Abstract of CICs levels by dog group is shown in the graph of Figure 4.1 B.

The values of single parameters referred to each dog are supplied in a separate file.
The two groups characterized by negative IFAT (A and B) had the lowest median level of CICs (16.09 and 12.78 μg/ml, respectively). Median CICs values increased progressively in the group C and reached the highest levels in the groups D and E, both characterized by high anti-leishmanial antibodies titer and severe disease, regardless of the mode of *Leishmania* infection.

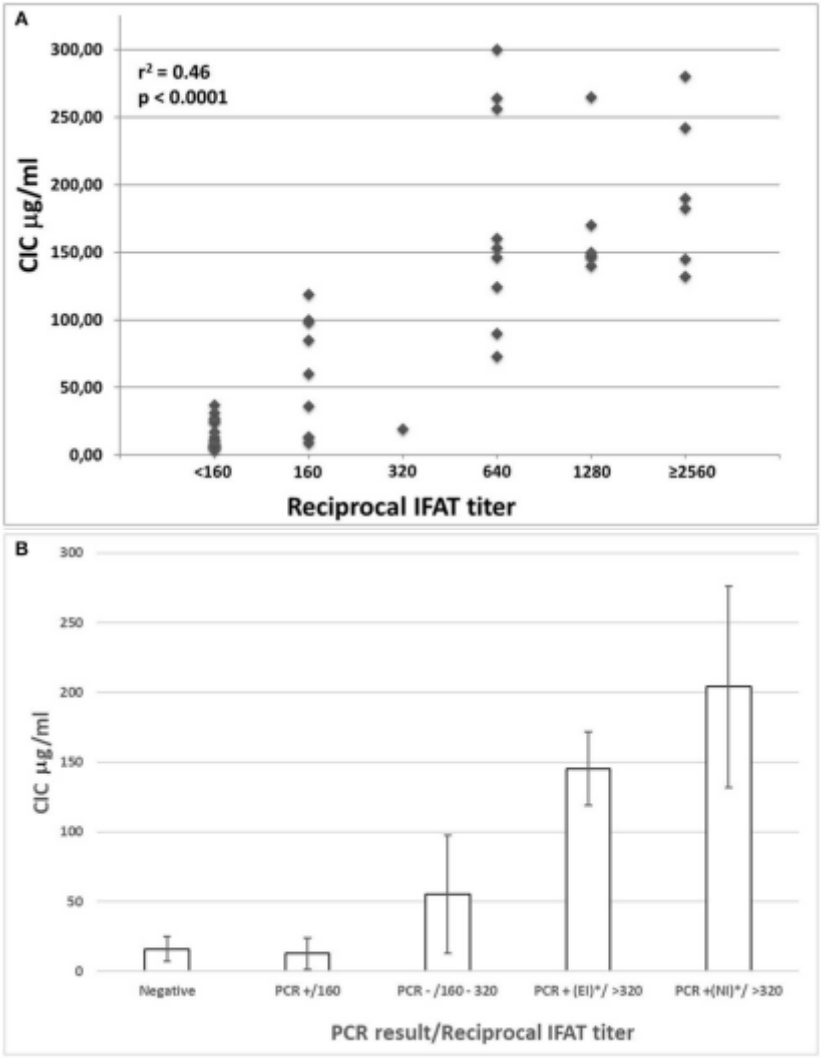

Fig. 4.1 (A) Correlation between IFAT titers and CICs levels in sera from 52 non-infected or Leishmania infantum-infected beagles; (B) CICs serum levels (median ± SD) in five groups of beagles classified by negative condition or by Leishmania infantum infection characterized by different serological and parasitological parameters. EI, experimental infection; NI, natural infection.

Significant differences in CICs concentration were demonstrated between A, B and C groups when compared with D and E groups ($p < 0.0001$). Whereas, no differences were recorded within the first three groups, CICs concentration differed significantly between D and E dogs ($p = 0.0002$).
In these groups of symptomatic dogs, clinical examination revealed typical signs related to CanL such as lymph node enlargement, apathy and mild weight loss, along with frequent clinicopathological alterations in most of the dogs, such as normocytic normochromic anemia, thrombocytopenia, hyperproteinemia, reduction of serum albumin/globulin ratio. Proteinuria, as assessed by urinary protein/creatinine ratio, was found in 3/10 and 3/12 dogs of groups D and E, respectively. However, as shown in Table 3.8, not all dogs of groups D and E exhibited the same clinical signs and clinicopathological alterations, despite an elevated background level of CICs.

4.4 Discussion

In several infectious diseases of humans and animals, especially those characterized by a prolonged chronic phase, the formation of CICs is promoted as a deleterious side effect of the humoral immune responses, and can cause glomerulonephritis, polyarthritis or uveitis.

Tab. 4.1 CICs serum levels (median ± SD) in Leishmania infantum negative (Group A), infected (Groups B and C), and diseased (Groups D and E) dogs, as classified by serological (IFAT) and parasitological (bone-marrow nested-PCR) parameters, and clinical findings (proportion of dogs that expressed the alterations).

Group (no dogs)	Infection	CICs µg/ml	IFAT	BM n-PCR	Lymph node enlargement	Weight loss	Anemia	Decreases in platelets	Increased BUN	Increased serum protein	A/G inversion	Proteinuria
A (10)	Naïve NoI	16,09 ± 8,94	Neg	Neg	-	-	-	-	-	-	-	-
B (10)	EI	12,78 ± 11,48	Neg	Pos	-	-	-	-	-	-	-	-
C (10)	NI	55,21 ± 42,41	≤1/320	Neg	-	-	-	-	-	-	-	-
D (10)	EI	145,4 ± 26,31	>1/320	Pos	1/10	3/10	2/10	2/10	-	-	6/10	3/10
E (12)	NI	204,16 ± 72,27	>1/320	Pos	3/12	3/12	6/12	4/12	1/12	3/12	5/12	3/12

NoI, no infection; EI, experimental infection; NI, natural infection.

Clinical manifestations of CanL also result from both, a direct intracellular parasite activity and the dog immune response associated with deposition of CICs in different body compartments. Immune-mediated mechanisms play a pivotal role in the pathogenesis of many clinical manifestations of CanL, particularly in the development of four principal types of glomerulonephritis, characterized by different grade of proteinuria and responsible for the development of renal failure, the most severe complication of CanL. Previous studies found a significant correlation between the level of antibody titers when assessed by IFAT and the severity of the disease, while a recent paper demonstrated that CICs concentration is clearly related to the progression of CanL in naturally infected dogs. In this study, remarkable was the demonstration of larger-size CICs (ranging from 100 to 400 nm) formed during the worsening of the disease, which was also characterized by an increase of IFAT titers. In our study too there was a clear correlation between the CICs and IFAT values, with CICs concentration being low in clinically healthy infected dogs with undetectable or borderline anti-leishmanial antibodies. The comparison of CICs levels between the two groups of sick animals revealed that sera from naturally infected dogs were characterized by significantly higher CICs than those from experimental infections, despite no significant differences were found in both IFAT value and clinical outcome. This could be explained by the different time course necessary to express overt clinical signs and clinicopathological alterations by the two modes of infection. As described in a previous study, any non-resistant *L. infantum*–infected dogs show a similar slow progress of disease

during 2 years post infection, with the first detection of early clinical and clinicopathological signs in the range of 6–12 months from the exposure to natural sand fly bites. In the present study, naturally sick animals showed severe clinical manifestations of CanL in a median time of 16 months, compared with the experimentally sick dogs that manifested the disease in median period of 6 months. This difference may be attributed to the chronic stimulation of immune system caused by repeated inoculations with metacyclic parasites (in the order of hundreds) from infective bites during two seasons of transmission, with the consequent T cell exhaustion. This is quite different if compared with the probable acute stimulation due to the single intravenous injection of millions of parasites typical of an experimental infection. For this reason we believe important not only to assess the level of CICs during different stages of disease, but also to correlate the observed CICs levels with the time spent from the first demonstration of infection. The present study demonstrated also the usefulness of the ELISA kit for CICs detection. This test is easy to perform and does not require sophisticated instruments. A limit could be related to the generic (aspecific) assessment of CICs, independently from the pathogen that caused them or their molecular size. This limitation could be important, mainly in case of concomitant infectious disease(s). In our study, however, we had good evidence that the ELISA test we used for the *Leishmania* CICs detection, worked in an appropriate manner in experimental infected dogs that had no exposure to other infectious pathogens, which instead could have happened in the group of naturally infected dogs. Ultimately, the inclusion of a laboratory infected canine model has represented a control for the ELISA assay specificity of CICs measurement in CanL. Further limitation of the present study is represented by the retrospective design that did not allow to follow further the dogs after the end of each study protocol. The detection of a low level of CICs in naïve and anti-*Leishmania* antibody negative dogs confirms that apparently aspecific immune complexes could be present in healthy individuals. In our case, because of the young age of the naïve beagles, we hypothesized that the low CICs amount could have been related to the core vaccinations.

Both symptomatic groups expressed clinical signs and clinicopathological alterations, typical of the disease. The most frequent sign related to CICs was proteinuria, that however was detected in a small proportion of sick animals, regardless the mode of infection. This indicates that proteinuria in CanL could not exclusively be related to CICs level and size, but also to

other pathogenic mechanisms. The study indirectly supports the evidence that other well-known clinical immune-mediated signs such as vasculitis, uveitis and arthritis, usually observed in aged dogs affected by CanL, require longer time from the initial infection to manifest than our follow up duration.

4.5 Conclusion

In summary, this study corroborates the direct correlation between IFAT value and CICs level in the progression of the *Leishmania* infection. Nevertheless, further researches are required to determine the importance of CICs as a biomarker for diagnostic and prognostic purposes. What is clear is that CICs monitoring should be used and interpreted in different ways, depending on the status of dogs suffering from CanL. If confirmed by further studies involving more animals, CICs measurements could represent an important correlate for sick dogs at different stages of the disease.
Furthermore, they could be included in the preliminary biochemical panel of analyses for clinically healthy dogs detected infected by *L. infantum*, in order to monitor any variations in their value across time and/or after specific treatment.

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Chapter 5

Simultaneous detection of parasitic vector borne diseases: a robust cross-sectional survey in hunting, stray and sheep dogs in a mediterranean area

Manuela Gizzarelli, Valentina Foglia Manzillo, Lavinia Ciuca, Maria Elena Morgoglione, **Nour El Houda Ben Fayala**, Giuseppe Cringoli, Gaetano Oliva, Laura Rinaldi and Maria Paola Maurelli

5.1 Introduction

Canine vector-borne diseases (CVBDs) are a spectrum of diseases caused by different infectious/parasitic pathogens transmitted by blood-feeding arthropoda such as fleas, lice, mosquitoes, phlebotomine sand flies and ticks. The most common CBVDs are anaplasmosis, babesiosis, bartonellosis, borreliosis, dirofilariosis, ehrlichiosis, leishmaniosis, rickettsiosis, dipylidosis, and thelaziosis. Most of these CVBDs are important not only for animal health and welfare, but also because they are of major zoonotic concern (e.g., *Babesia venatorum*, *Babesia microti*, *Dirofilaria immitis*, *Dirofilaria repens*, *Leishmania spp*.). The interest on CVBDs has grown in the last two decades and therefore an increased number of studies have been published in the recent few years. The epidemiology of CVBDs (i.e., geographical distribution, prevalence, and pathogenicity) is changing due to several factors, especially climatic changes, ecosystem changes, increased mobility of dogs and humans and developing phenomena of chemoresistance to insecticides and acaricides. Consequently, CVBDs are spreading into areas considered non-endemic until recently.

Often, CVBDs cause chronic and asymptomatic infections and their diagnosis requires specific tests. Furthermore, co-infections are common, especially in areas suitable for many vector species, thus changing clinical manifestations and complicating diagnosis, therapy and prognosis. If dogs are not properly treated for CVBDs, they may act as reservoir of them, representing a zoonotic risk especially as the consequence of the increasing phenomenon of cohabitation with humans in urban and rural environments. Therefore, diagnosis and control of CVBDs are highly complex and challenging.

The epidemiological scenario of canine and feline vector-borne diseases in Italy has been recently reviewed. While most of regions have been investigated for CVBDs, a dearth of data is present for some regions of central-southern Italy. The aim of the present study was to investigate three parasitic CVBDs (leishmaniosis, babesiosis and filarial infections) in dogs with three different life styles (hunting, stray and sheep dogs) in Molise, the smallest region of southern Italy where data available about these parasitic infections are very scant.

5.2 Materials and methods

5.2.1 Study Area and Collection of Samples

A cross-sectional survey was conducted between June 2017 and June 2018 in the Molise region of southern Italy (Latitude = 41°40′00″N; Longitude = 14°30′00″E) which extends over an area of 4,438 km2. The region is mainly mountainous and extends from 0 to 2,185 m above sea level. The climate is cold-temperate in the western part and Mediterranean in the eastern part of the region.

A grid-based approach within a Geographical Information System (GIS) was used in order to uniformly sample the dogs throughout the entire region (15). For this purpose, a grid representing quadrants of 10 × 10 km was overlaid on the regional map within the GIS. Thus, the Molise region was divided into 55 quadrants and the study was designed to sample 6 hunting, 6 stray and 6 sheep dogs in each quadrant (Fig. 5.1) for a total of 990 dogs. From each dog, blood samples were collected as follows: 2 ml in tubes with EDTA and 2 ml in tubes with serum separator gel. Serum was separated by centrifugation at 360 g for 15 min and stored at −20°C until analysis.
All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Data on age, sex and living conditions (hunting, stray, sheep dogs) were registered. Moreover, dogs were submitted for physical examination and a clinical form was completed.

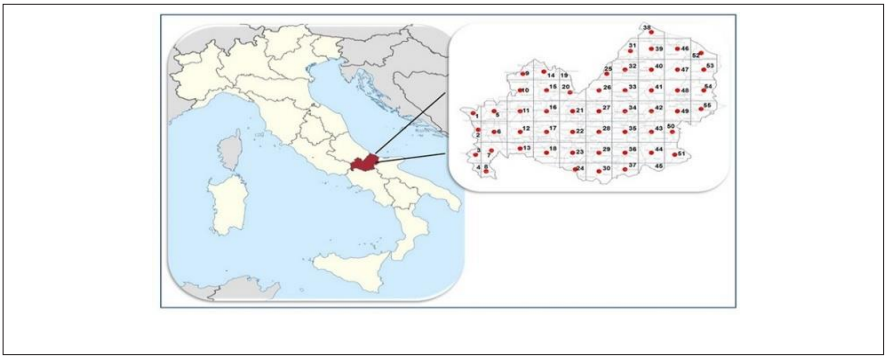

Fig. 5.1 Study area. Molise region, Italy.

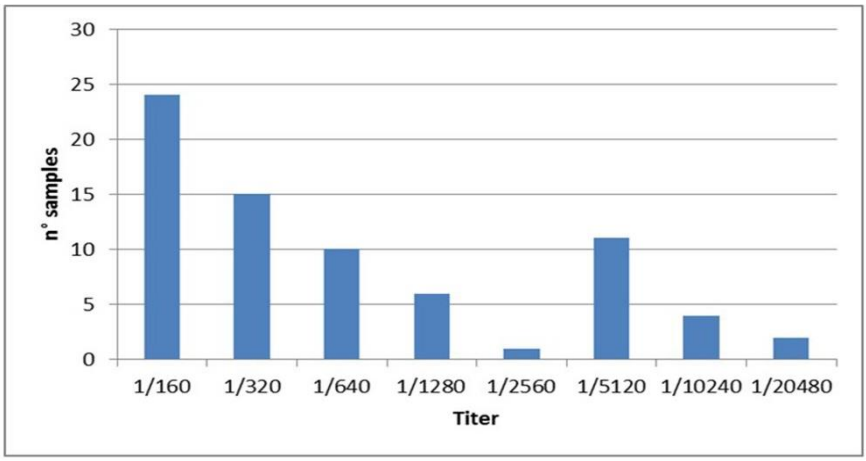

Fig. 5.2 Nº. of dogs positive to Leishmania distributed by antibody titres.

5.2.2 Detection of Antibodies to Leishmania infantum

Serum samples were analyzed by an immunofluorescence antibody test (IFAT) provided by the National Reference Center for Leishmaniosis (CReNaL, Palermo, Italy) to detect anti-*Leishmania* antibodies (sensitivity = 96% and a specificity = 98%). Antigens used by CReNaL were promastigotes of strain MHOM/TN/80/IPT1. Samples were considered positive, if they showed a titer ≥1:160. Reading was performed using a fluorescence microscope (Leica DM 2500, Germany) by three independent technicians.

5.2.3 Detection of Babesia

Blood smears were prepared using blood samples in EDTA and stained using Differential Quick Stain kit (Modified Giemsa) (Polysciences, Inc. Warrington, PA, USA), according to the manufacturer's instructions for detection of *Babesia* protozoa. Samples were analyzed by an expert examiner. Positive samples were then analyzed by molecular techniques. DNA was extracted by blood samples using the DNeasy Blood & Tissue Kit (QIAGEN, Germany). A semi-nested Polymerase chain reaction (PCR) was performed to amplify the 18S ribosomal RNA gene. The PCR products were detected on 2% ethidium bromide-stained low melting agarose gel (BIO-RAD, Spain). Positive amplicons were purified by QIAquick PCR purification kit (QIAGEN, Germany). The purified PCR products were sequenced in both forward and reverse directions and were analyzed by the

Chromas version 2.1 software and finally compared with the NCBI/GenBank database using the Basic Local Alignment Search Tool (BLAST) and ClustalW software.

5.2.4 .Detection of Microfilariae

Blood samples in EDTA were analyzed by the modified Knott's test to detect microfilariae of *Dirofilaria immitis, D. repens* and *Acanthocheilonema reconditum.* The circulating microfilariae (mf) were identified based on their morphology and morphometry and counted (mf/ml of blood) in 20 ul of blood. Morphometric analyses of the mf were then performed with a standard microscope equipped with calibrated measuring eyepieces at final magnification of 200–400×. Body length and diameter of 10 randomly selected mf were determined.

5.2.5 Statistical Analysis

Chi-squared test was performed using SPSS 23.0 software (IBM, Armonk, NY, USA) to study the association between positivity to CVBDs and dog's characteristics (age, sex, living conditions). Differences were considered significant at $P < 0.05$.

In addition, a spatial analysis was conducted to detect possible clusters of positive samples, using the average nearest neighbor (ANNI) index (20). If the index is < 1, the pattern exhibits clustering, while index of > 1 indicates a trend toward dispersion. A significance level of 99% was chosen in the analysis. Moreover, to confirm results obtained, the Moran's I test was used. A low negative z-score indicates a statistically significant spatial data outlier. A significance level of 95% was chosen for this test.

5.3 Results

Tab. 5.1 Prevalence and 95% CI of positive samples to Leishmania infantum, Babesia canis canis and filarial infection for each class of age tested.

Class	Age of dogs (months)	No. samples analyzed	*Leishmania infantum*		*Babesia canis canis*		Filarial infection				
			No. samples with *Leishmania*-IFAT titer ≥ 1:160	Prevalence % (95% CI)	No. positive samples	Prevalence % (95% CI)	*D. immitis*	*A. reconditum*	*D. repens*	Co-infection	Prevalence% (95% CI)
A	3–12	146	7	4.8 (2.1–10.0)	0	0	0	2	1	0	2.1 (0.5–6.4)
B	13–36	238	29	12.2 (8.4–17.2)	1	0.4 (0.02–2.7)	1	3	2	1	1.7 (0.5–4.5)
C	37–72	194	20	10.3 (6.6–15.7)	1	0.5 (0.03–3.3)	1	3	1	1	2.1 (0.7–5.5)
D	73–120	110	12	10.9 (6.0–18.6)	0	0	0	3	2	1	3.7 (1.2–9.6)
E	121–204	28	5	17.9 (6.8–37.6)	0	0	0	0	0	0	0
	Total positive samples	716	73	10.2 (8.1–12.7)	2	0.3 (0.1–1.1)	2	10	6	3	2.1 (1.2–3.5)

Tab. 5.2 Prevalence and 95% CI of positive samples to Leishmania infantum, Babesia canis canis and filarial infection for sex.

Sex	No. samples analyzed	*Leishmania infantum*		*Babesia canis canis*		Filarial infection				
		No. samples with *Leishmania*-IFAT titer ≥ 1:160	Prevalence % (95% CI)	No. positive samples	Prevalence % (95% CI)	*D. immitis*	*A. reconditum*	*D. repens*	Co-infection	Prevalence% (95% CI)
Male	388	42	10.8 (8.0–14.5)	1	0.3 (0.01–1.7)	0	6	3	0	2.3 (1.1–4.5)
Female	328	31	9.5 (6.6–13.3)	1	0.3 (0.02–2.0)	2	4	3	3	1.8 (0.8–4.1)
Total positive samples	716	73	10.2 (8.1–12.7)	2	0.3 (0.05–1.1)	2	10	6	3	2.1 (1.2–3.5)

Tab. 5.3 Prevalence and 95% CI of positive samples to Leishmania infantum, Babesia canis canis and filarial infection for each living condition tested (hunting, stray and sheep dogs).

Living condition of dogs	No. samples analyzed	*Leishmania infantum*		*Babesia canis canis*		Filarial infection				
		No. samples with *Leishmania*-IFAT titer ≥ 1:160	Prevalence % (95% CI)	No. positive samples	Prevalence % (95% CI)	*D. immitis*	*A. reconditum*	*D. repens*	Co-infection	Prevalence % (95% CI)
Hunting	318	29	9.1 (6.3–13.0)	2	0.3% (0.1–1.1%)	2	8	5	3	3.8 (2.1–6.7)*
Stray	180	23	12.8 (8.4–18.8)	0	0	0	2	1	0	1.7 (0.4–5.2)
Sheep	218	21	9.6 (6.2–14.5)	0	0	0	0	0	0	0
Total positive samples	716	73	10.2 (8.1–12.7)	2	0.3% (0.1–1.1%)	2	10	6	3	2.1 (1.2–3.5)

* *Significantly different* $\chi 2 = 8.2$; $P = 0.017$.

5.3.1 Detection of CVBDs

A total of 716 blood and serum samples (72.3% of the expected sample) were collected: 318 from hunting, 180 from stray, and 218 from sheep dogs.

The age of animals ranged from 3 months to 17 years (median age = 3 years). Based on their age, dogs were divided into 5 classes: (a) 3–12 months; (b) 13–36 months; (c) 37–72 months; (d) 73–120 months; (e) 121–204 months. Regarding sex, 328/716 dogs were females (45.8, 95%, CI = 42.1–49.5%) and 388 males (54.2%, 95% CI = 50.5–57.9%).

An overall prevalence of 12.3% (95% CI = 10.0–15.0%) was detected for CVBDs caused by *Leishmania infantum, Babesia spp.* or filarial nematodes.

Specifically, a total of 73 dogs resulted positive for leishmaniosis (10.2%, 95% CI = 8.1–12.7%) with titers ≥ 1:160 up to 1:20480 (Fig. 3.4; Tab. 3.9–11). The positivity was not significantly associated with age, sex and living conditions (P > 0.05).
Results of microscopic analysis of blood smear for *Babesia spp.* showed that 2 dogs had large intraerythrocytic piroplasms compatible with a large *Babesia*. These samples were analyzed also by PCR and sequencing and an identity of 100% with *B. canis canis* sequence (GenBank accession number KX236456.1) was found. The two positive dogs for *B. canis canis* (0.3%, 95% CI = 0.1–1.1%) were both hunting dogs: 1 male of 40 months old and 1 female of 36 months old (Fig. 5.1; Tab 5.1–3).
Moreover, a total of 15 dogs (2.1%, 95% CI = 1.2–3.5%) resulted positive for filarial infections (Fig. 5.2). Specifically, 10 samples (1.3%; 95% CI = 0.7–2.5) were positive for *A. reconditum*, 6 (0.8%; 95% CI = 0.3–1.9) for *D. repens* and 2 (0.3%; 95% CI = 0.1–1.1) for *D. immitis*, with a higher significantly associated prevalence (P = 0.017) in hunting dogs (3.8%; 95% CI = 2.1–6.7), but positivity was not associated with age and sex (P > 0.05) (Tab 5.1–3). Two samples were co-infected with *A. reconditum* and *D. immitis*, while 1 sample with *A. reconditum* and *D. repens.* Finally, 2 samples showed co-infections of *L. infantum* and *A. reconditum*, whilst 1 sample showed a co-infection of *L. infantum* and *B. canis canis.*

5.3.2 Clinical Signs

Only 37% (95% CI = 26.2–49.1%) of positive dogs showed clinical signs for leishmaniosis and babesiosis. The most frequent clinical signs detected in positive dogs for these CVBDs were: loss of weight, depression, lymph-nodes and spleen enlargement, conjuntivitis, keratitis, blepharitis, alopecia, ulcers/nodules, and exfoliative dermatitis. No other clinical signs were recorded.

Only two positive dogs for filarial infections (13.3%; 95% CI = 2.3–41.6%) showed clinical signs, but unspecific for clinical diagnosis of this CVBD. Both dogs showed loss of weight, whilst only one of these, positive also for leishmaniosis, had blepharitis, and conjunctivitis.
At physical examination, thelaziosis was not found in any of the tested dogs. It should be noted that the 716 dogs were analyzed also for intestinal nematodes (*Trichuris*, *Toxocara*, *Toxascaris* and *Ancylostomidae*), cardiopulmonary nematodes (*Angiostrongylus* and *Capillaria*) and cestoda (*Dipylidium* and other *Taeniidae*) (unpublished data), but no significantly association was found between them and vector borne diseases investigated in this study ($P > 0.05$).

5.4 Discussion

The limited extension of the Molise region territory, the small size of its dog population (56,729; www.lav.it) and the availability of an efficient veterinary system of active surveillance made it possible for us to have an accurate scenario of leishmaniosis and other CVBDs in dogs in this poorly investigated region.

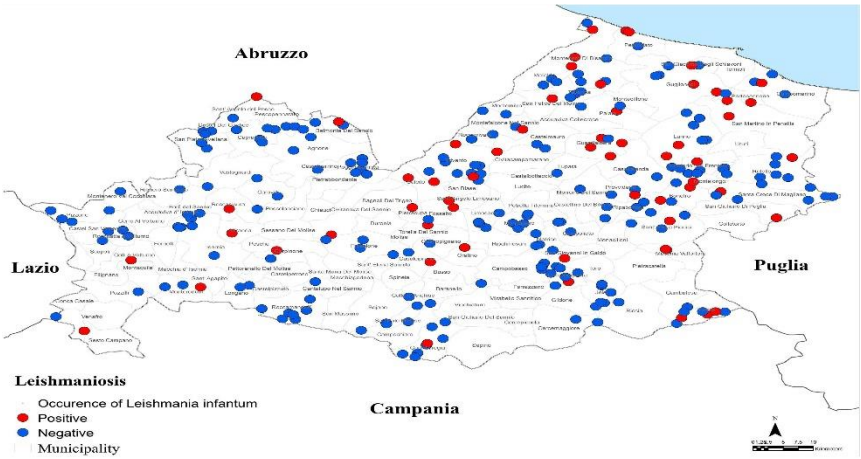

Fig. 5.3 Distribution of dogs positive to Leishmania infantum.

Specifically, a prevalence of 10.2% of leishmaniosis, 2.1% for filarial infections and 0.3% for *B. canis canis* was found.

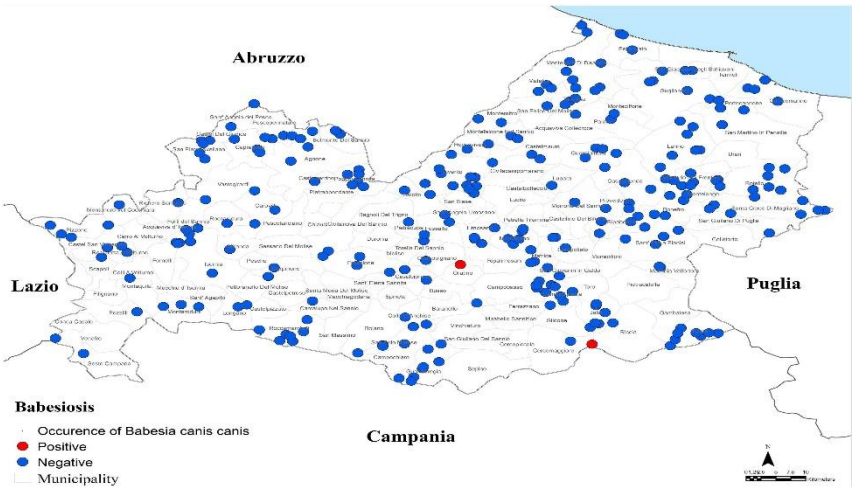

Fig. 5.4 Distribution of dogs positive to Babesia canis canis.

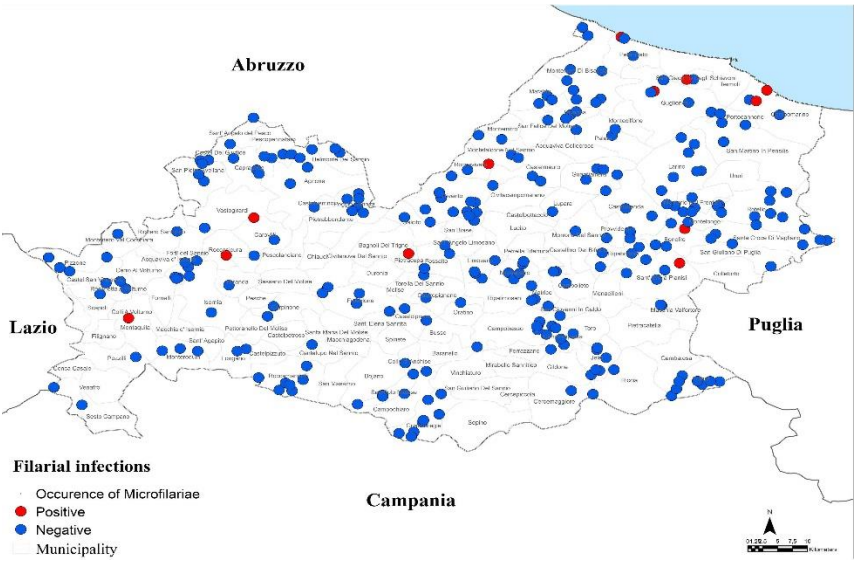

Fig. 5.6 Distribution of dogs positive to filarial infections

Canine leishmaniosis caused by *L. infantum* is endemic in all countries of the Mediterranean basin, but it is spreading northwards. In Italy, the median seroprevalence of leishmaniosis in 377 studies on dogs since 1971 to 2006 was 17.7% (range 10.7–21.1%), but prevalence of leishmaniosis is increasing over 40% in different zones not only of southern Italy, but also of northern Italy.

In the present study, the prevalence recorded in Molise was 10.2%. Not many data are available about canine leishmaniosis in this region, but the more recent indicate an average prevalence of 18% in 2016 (unpublished data provided by CReNaL). The lower prevalence reported in our study can be correlated with enrolled dogs that were chosen at random, whilst the sera of dogs detected by CReNaL were all symptomatic for leishmaniosis.

This is the first time that *B. canis canis* is reported in the Molise region. PCR-positive prevalence (0.3%) for this parasite was higher than prevalence reported in hunting dogs from southern Italy (0.15%) and from northern Italy (0%) but lower than a study conducted in central Italy with a prevalence of 2.3%. Higher prevalence values were reported in a study performed on 103 dogs from northern, 43 form central and 18 form southern Italy with PCR-positive prevalence of 29.1, 4.7, and 11.1% respectively, but all enrolled dogs in this study had clinicopathological findings compatible with tick-borne diseases.

Prevalence of filarial infections reported in this study (2.1%) was not very high. *A. reconditum* was the most prevalent (1.3%) filarial species and noteworthy was reported for the first time in the Molise region. The zoonotic *D. repens* was found in 0.8% of the tested dogs. Interestingly, a case of subcutaneous human filariosis caused by *D. repens* was described by Pampiglione *et al*. in the arm of a children in Campobasso, one of the two provinces of the Molise region. In the same study the Knott's test was performed also on blood from 135 dogs, over 2 years old, confirming the presence of *D. repens* (3.0%) in dogs living few km from the clinical study. *D. immitis* was found in this study in 2 dogs (0.3%). For a long time *D. immitis* was recorded only in the Po River Valley, but more recently this parasite has spread to previously non-endemic areas of central and southern Italy. The two positive dogs were autochthonous hunting dogs that had never been abroad. A significantly higher association between hunting dogs and filarial infections was shown, according to literature.

In this study, 3 dogs showed co-infections of *L. infantum* and *B. canis canis* (0.1%) or *A. reconditum* (0.3%). No associations were found between intestinal and cardio-pulmonary parasites and vector borne diseases investigated in this study. Recently, Baxiaras *et al.* showed the severity of clinical signs of leishmaniosis is increased where a co-infection with other vector-borne pathogens is present. In some studies, a predisposition to leishmaniosis in dogs with other vector-borne pathogens has been described.

5.5 Conclusion

The detection of CVBDs in dogs with or without clinical signs urges the need to the high importance of increasing awareness of the veterinary community, owners and public health authorities about the risk of infection. It is even more necessary to highlight the need to plan effective control programs in order to guarantee the health and welfare of pets, and to enhance the safety of people.

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Chapter 6

Investigation on the possible role of dogs in the epidemiology of *L. major* infection

6.1 Introduction

Leishmaniasis represents a zoonotic vector-borne disease of great important public health concerns. In Tunisia, human and canine leishmaniasis are endemic. Three *Leishmania* species are responsible for human leishmaniasis*: L. infantum*, *L. major*, and *L. killicki* (*syn. tropica*); widely distributed in three nosogeographic forms: cutaneous, visceral and mucocutaneous according to the ecological and socio-economic conditions which favour the survival of the vector and the reservoir.
Canine Leishmaniasis due to *L. infantum* plays a key role in the incidence of human leishmaniasis in the country since dogs represent the main domestic reservoir of the parasite. It has been reported by Bouattour *et al* that prevalence in canine population across 8 different Tunisian bioclimatic zones was 58.3% (ranging from 6.8% to 84.6%). In the North, *L. infantum* is the causative agent of Visceral (VL) and Sporadic cutaneous leishmaniasis (SCL). However, in the Center and the South, *L. major* represents the most widespread dermotropic species, causing the zoonotic cutaneous form (ZCL) in both rural and suburban areas. In rural area, human prevalence was estimated at 71%.

From the last few years, an increased incidence of HL cases due to *Leishmania major* in the North has been observed and continues to spread. Despite, *Phlebotomus papatasi* and wild small mammals, especially rodents of the *Psammomys spp.* and *Meriones spp.* genera, (both described as the vectors and reservoir hosts of the parasite life cycle) were mostly observed in the center and the southern regions. This suggests that there may be another factor in the epidemiological cycle of the parasite.

Previous reports highlighted the presence of *Leishmania major* infection in dogs, based on molecular and enzymatic biochemical characterization. This raises several questions about the role of dogs in the life cycle of the parasite.

Our research aims to evaluate the prevalence of canine leishmaniasis due to *Leishmania infantum* already described in Tunisia and investigate the possible role of dogs in the life cycle of *L. major* as well as to compare the prevalence of Canine vector-borne diseases between two groups of dogs living in two different endemic areas of *L. infantum* and, *L. major*.

6.2 Material and methods

6.2.1 Study site

Regions were selected based on data provided by the ministry of health according to human cases of visceral and especially zoonotic cutaneous leishmaniasis. Sample collections were performed in foci with the highest prevalence of human leishmaniasis and particularly when dogs belong to families with ZCL

Zaghouan Governorate: Located in North-Eastern Tunisia, it has a mild, continental climate constituting a fundamental asset in the development of the region. The "semi-arid" bioclimatic stage is dominant with a few islands of sub-humidity. The region became one of the main Centers of agricultural production in the country, especially for vegetables and arboriculture (olive and almond trees and milk production). Zaghouan governorate disposes of rich natural potentialities: high mountains, large forest areas, wildlife, miscellaneous fauna and flora, an abundance of water, and thermal springs. This region is characterized by the presence of *L. infantum* Mon-1, Mon-80, and Mon-24 responsible for VL and SCL, with a prevalence rate in canine population ranging from 9.4 to 42.4%.

Kairouan Governorate: Located in the Central-West region of the country, is a territory of plains and hills. It belongs to the natural steppe region and is characterized by a semi-arid bioclimate. The aridity gradient increases from North to South, characterized by significant rainfall irregularities with large thermal amplitudes.
Despite these handicaps, agriculture is the main economic activity in the region. It is dominated by animal husbandry and cereal growing with a marked increase in polyculture and arboriculture which rely on increasingly efficient irrigation systems. The prevalence rate in canine population ranges from 12.16 % to 35.8 %.

Six of its districts have been studied:

- ✓ Sbikha: It is part of the upper arid bioclimatic stage; an area of low plains with an average altitude of about 100m which is in direct contact with the sebkhas (temporary lacustrine systems). The production system of this area is based on livestock farming in steppe rangelands, irrigated crops, and arboriculture. The region is distinguished by the presence of VL.

- ✓ Nasrallah, Bouhajla, El Makhsouma, Haffuz, and Echrarda districts: they represent the delegations from the South of Kairouan. It is an area of high plains with an average altitude between 200 and 300 m. They have rich water reserves but average to poor quality (high salinity). Moreover, they're little or not affected by erosion because of their flat relief. The production system is versatile (based on arboriculture and large dry and irrigated crops). Located on the low steppes area and characterized by dams, (*Chenopodiaceae* flourish there) especially saltbush Atriplex which are a source of food for the *Psammomys spp.* and thus for *Leishmania major*, and the jujube tree, which is the traditional habitat of the *Meriones spp.*

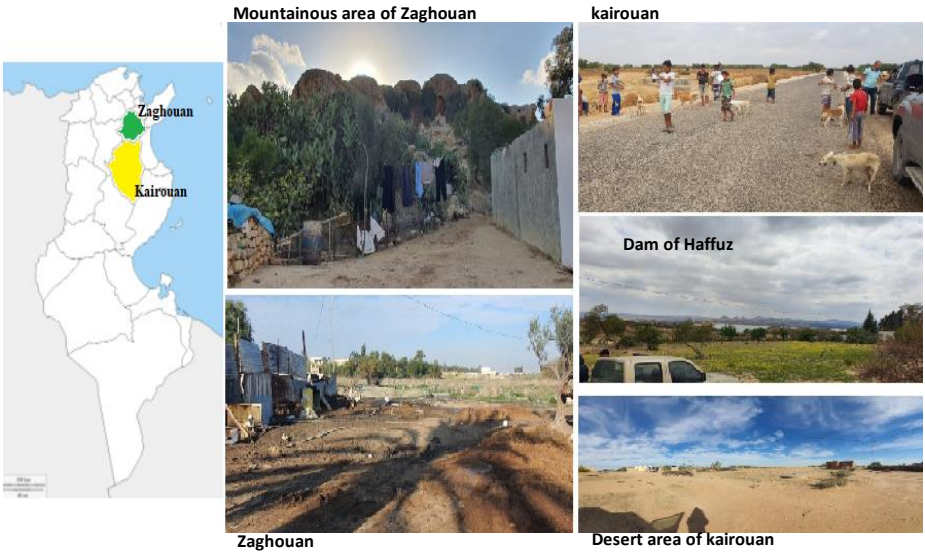

Fig. 6.1 the different geographical localisations

The investigated regions were divided into 2 geographical areas, North and South, in relation to central Kairouan and based on the nosogeographic distribution of human leishmaniasis. The governorate of Zaghouan and Sbikha district belong to the Northern region while Kairouan southern districts belong to the Southern region.

6.2.2 Animal identification and sample collection

The research study was conducted from September 2019 to March 2022. Tether and free-roaming owned dogs were sampled jointly to the rabies vaccination campaigns conducted by the regional veterinary services. Dogs selected were over 6 months old, and generally were in bad clinical condition mainly caused by poor owner management.

Each dog was subjected to a general physical examination, and a clinical form was completed in which a unique code was assigned, reported both on the card and the samples. The form reported, in order, the owner's /holder's data, the dog's signalment, clinical signs derived from the main and most frequent manifestations of leishmaniasis described in the literature, including BCS, lymph nodes enlargement, lethargy, onychogryphosis, and cutaneous lesions (alopecia, ulcers and nodules).

Dogs were divided into 2 groups: Northern dogs living in Zaghouan governorate and Sbikha district and Southern dogs living in Bouhajla, Echrarda, El Makhsouma, Nasrallah and Haffuz.

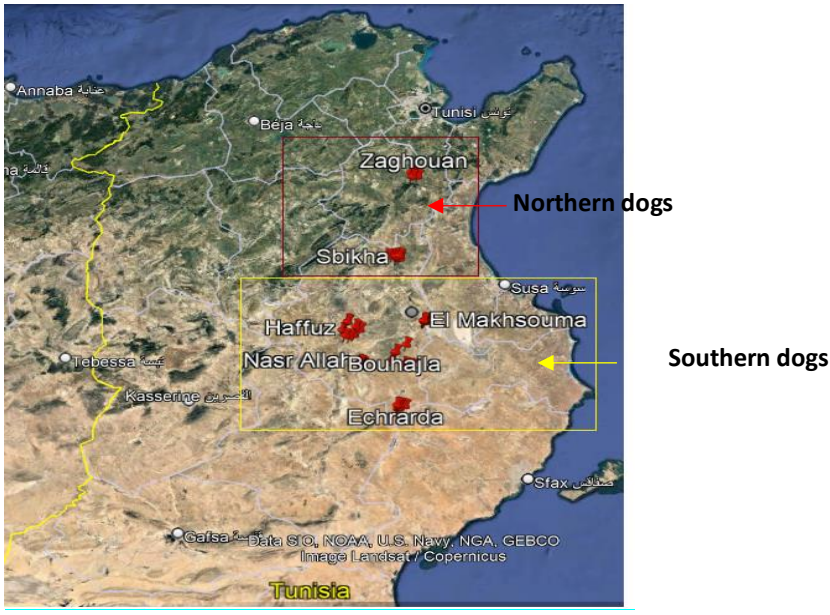

Fig. 6.2 location of the studied foci with the two dog groups geographically distinct. The red dots indicate dogs' distribution in the different regions.

Samples collected from each dog were distributed as follows:

- Blood collection: With aseptic precautions, we have collected peripheral blood from each dog in tubes containing Ethylenediaminetetraacetic acid (EDTA), and used them for serological (ELISA, IFAT, 4DX) and molecular tests (PCR).
- Lymph node fine-needle aspiration: Whenever dogs allow it, and Lymph Nodes were enlarged, we performed FNA of peripheral lymph nodes (LNs). We used half the quantity for the culture of the parasite onto Novi, McNeal, and Nicolle medium, and we added the remaining half to 200µL of sterile PBS for DNA purification to perform a subsequent *Leishmania* species' identification.
- Skin scrapping: In the presence of ulcerated, crusty, or nodular lesions, the used technique was Skin scraping. We transferred the first fraction to sterile tubes with 200µL of lysis buffer for DNA purification and PCR identification whereas we spread the second fraction on a slide and viewed it after coloration in order to detect the presence of amastigotes of *Leishmania spp.*

➢ All samples were stored at -20°C until use.

6.2.3 Identification of Leishmania species

The laboratory work was conducted at two different laboratories: the laboratory of the Department of Parasitology and Mycology of Pasteur Institute of Tunis and the laboratory of the Department of Veterinary Medicine and Animal Production of the University Federico II of Naples.

- **Detection of the parasite**

The presence of parasites was investigated using a fraction of the lymph nodes added to the Novi, McNeal, and Nicolle medium. The cultures were examined by optical microscopy and were sub-cultured, added urine to promote the growth and antifungals if they were contaminated, Over the period of 7 to 10 days the cultures were examined three times.

In addition, slide smears were prepared from skin samples. The slides were stained using the May-Grunwald Giemsa stain and subsequently observed under an optical microscope at x100.

- **Detection of Antibodies to *Leishmania infantum***

The commercial leishmaniasis indirect screening test (Indirect ELISA ID Screen®) was performed according to the manufacturer's manual (LEISHS ver 0814FR).

Absorbance was measured at 450 nm wavelength in the Thermo Scientific, Multiskan FC Microplate Photometer ELISA reader. The results were considered valid only if optical density of a positive control serum (OD PC) was higher than 0.350 and (OD PC) was more than three times higher than optical density of a negative control serum (OD NC).
Then the optical density of a serum sample (OD sample) was calculated into percentage with the formula:

$$S/P\% = \frac{OD\ (sample) - OD\ (NC)}{OD(PC) - OD(NC)} \times 100$$

Plasma having a S/P% value ≤ 40 % were considered negative. While plasma S/P% value ≥ 50% were considered positive.
In the case of plasma S/P% value between 40 % and 50% were considered as doubtful. The latter were serially diluted and tested by immunofluorescence antibody test (IFAT).
IFAT was considered positive at a serum dilution of 1:100 or higher.

- **Detection of DNA**

DNA was extracted from the Buffy coat, lymph node, and cutaneous scraps samples using the QIAamp DNA mini kit (Qiagen, Hilden Germany). The protocol was performed following the manufacturer's recommendations. DNA was eluted on 50 µL and extracts were stored at −20°C until use. PBS buffer was included as a negative control to test possible contamination.

- **Real-time PCR**

At the laboratory of the Pasteur institute, only the buffy coat DNA of the seropositive dogs and the LN FNA were tested by qPCR described previously by Mary *et al.*, a quantitative technique targeting *Leishmania* kinetoplast DNA. The DNA amplification was performed in a Taq Man Applied Biosystems 1 apparatus (Applied Biosystems, Foster City, California). A standard curve, plotted from a dilution series of *Leishmania* DNA extracted from $5x10^6$ *L. infantum* promastigotes allowed parasite

quantification. Then, all DNA of seropositive and seronegative dogs have been elaborated again at the laboratory of parasitology of the Department of Veterinary Medicine and Animal Production of the University Federico II; samples were subjected to qPCR targeting kDNA according to the method of *Vitale et al*. and were conducted by TaqMan universal master mixture technology. The result was expressed by the value of the threshold cycle (Ct). The parasite load of each sample was calculated using a standard range prepared from a serial dilution of the DNA extract of 1×10^6 promastigotes of *Leishmania infantum* and *Leishmania major*. Amplification was conducted in an C1000 Touch Thermal Cycler (CFX96 Real-Time System) for 50 and 45 cycles at 60°C and 95°C respectively.

- **Sequencing**

For the sequencing, we performed a second method at the laboratory of the Pasteur institute; all positive kDNA qPCR specimens from seropositive dogs were amplified by ITS1-PCR (internal transcribed spacer 1 genes) according to Shönian *et al*. method.

For the detection and the sizing of DNA, we used 2% Agarose Gel by electrophoresis and used ultraviolet light for DNA visualisation. The sample was considered positive when there was an adequate resolution of bands in the range of 300-350 base pairs. ITS 1 PCR amplicons were purified using ExoSAP (ThermoScientific, USA).

The DNA sequencing was performed with same primers as ITS1 PCR using an ABI Prism1 Big Dye ™ Terminator Cycle Sequencing Ready Reaction Kit (USA) and an AB1 3130 sequencing system (ABI, PE Applied Biosystems, USA). DNA sequences of 2 strands were aligned and edited using Staden software package (http://staden.sourceforge.net/). Sequences were compared with those of GenBank using Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov) as well as those of 2 isolates of *L. infantum*, one human (LV) from Kairouan region and other canine from Tunis region.

6.2.4 Identification of co-infections/co-exposures

The identification of co-infections and co-exposures were carried out by direct and indirect techniques:

- **Detection of the antigen of *Dirofilaria immitis* and the antibodies of *Anaplasma spp.* (*A. phagocytophilum/A. platys*), *B. burgdorferi sensu lato,* and *Ehrlichia spp.* (*E. canis*/*E. ewingii*)**

Plasma samples from *Leishmania infantum* infected dogs were tested using an in-house test (SNAP® 4Dx®Plus; IDEXX Laboratories, Inc., Westbrook, ME, USA) for the detection of co-infection/co-exposure of dogs. The test was performed according to the manufacturer's instructions.

- **Detection of Microfilariae**

In order to detect microfilariae of *Dirofilaria immitis* and *Dirofilaria repens*, we analysed 79 Blood samples which were collected from southern Kairouan, using the modified Knott's test, as follows:

In the conical centrifuge tube, we added 9 ml of 2% formalin to 1 ml of blood after centrifugation (at 15000 rpm for 10 min), the supernatant was discarded, and the precipitate was stained with methylene blue 1%. Then, we smeared a drop of the mixture onto a slide. We performed a microscopic examination of the entire smear at a low magnification (x10), and identified the circulating microfilariae based on their morphology and morphometry.

- **Detection of *Babesia spp.* DNA**

The DNA extracted from the buffy coat was submitted for an end-point PCR protocol, that was described by Bajer *et al.* as *Babesia spp*. preparing a total of 25 µL PCR volume (22 µL of PCR mix + 3 µL of the extracted DNA sample) for each sample with 1x buffer (EcoTaq PLUS, Lucigen, WI, USA) and 0.5 µM of each primer.

6.2.5 Statistical analysis

We used the Chi-square test in order to perform statistical analysis with a commercially available statistical program. Observed risk factors were considered significant when the resulting *p-value* ≤ 0.05.

6.3 Results

The total number of samples were 229 dogs, whose average age was 2.7 years (ranging from 6 months to 15 years). As per the sex ratio, it was 1:1.8 (83 females (36%) and 146 males (63%)). The majority of dog breeds were

Mongrel breed while only 7% were hunting dogs. A total of seventy-eight percent manifested at least one clinical sign compatible with canine leishmaniasis.
Each group of dogs belonged to a distinct nosogeographic area; the Northern group consisted of 115 dogs from Zaghouan and Sbikha living in an epidemiologic area of Visceral and SCL. The Southern group consisted of 114 dogs from South Kairouan living in endemic area of ZCL.
The two groups were not statistically different in terms of number, sex, and age.

The main detected clinical signs were lymph nodes' enlargement (45%), weight loss (40%), and cutaneous lesions (31%) especially diffuse alopecia (21%) and ulcers/nodules (13, 5%). No other clinical signs were recorded (Tab. 6.1).

Tab. 6.1 Percentage of clinical signs

Clinical signs		**Prevalence%**
Lymph nodes' enlargement		45
Cutaneous lesions	Alopecia	21
	Ulcers/Nodules	13,5
Weight loss		40

We observed an overall detection of *Leishmania infantum* by ELISA and/or qPCR in fifty dogs (21,8%), among which 39 dogs were serologically positive and 11 were serologically negative with a positive Rt-PCR.

Seropositive dogs had an ELISA antibody titer above 50% .Only nine results were suspected with a titer of 40% to 50% and have been confirmed by IFAT at 2 dilutions 1:50 and 1:100 (the last one was the cut-off). Among these latter, two were confirmed positive. This was probably due to Elisa's sensitivity and specificity compared to the IFAT, that is considered the gold standard for the diagnosis of canine leishmaniasis. Experience demonstrated that forty-three of infected dogs (86%) were symptomatic and had at least one symptom suggestive of CanL.
A total of 31 (62%) infected dogs were positive by Rt-PCR, Kinetoplast DNA detected by real-time quantitative PCR were also tested by ITS1-PCR. Band profile was obtained around 320 bp in 5 extracts (4 LN and 1 BC).
Sequencing identified four DNA bands as *L. infantum*, which further confirmed previous reports of the diffusion of canine leishmaniasis due to

L. infantum in the north and the south of Tunisia. A sequence corresponding to a weak band was inconclusive. The sampling of different types of tissue for PCR-based diagnosis allowed to detect infection in seronegative subject.

Tab. 6.2 Infected dogs' results

Infected dogs	Sero positive	PCR positive	Clinical signs
50	39	31	43

Male sex was a risk factor for canine leishmaniasis (p-value =0.04), as seventy-six percent of infected dogs were males (Tab 6.3). The age of dogs was not a significant risk factor for the infection since the p value > 0,05 but we can note a difference in the distribution of infected cases.
In fact, 88% of the infected dogs were adults between 1 and 5 years old. We can note that 77% of *L. infantum* noninfected dogs were symptomatic and this was probably due to the poor owners' management and other parasitic bacterial and worm infections.

Tab. 6.3 Risk Factors for Canine leishmaniasis

Variables		Infection		*p* value
		Positive	Negative	
Age	Young (< 1 years)	3	13	0.8576
	Adult (1-5years)	44	152	
	Senior (>5 years)	3	14	
Sex	Male	38	108	0.04163
	Female	12	71	
Region	North	31	84	0.059
	South	19	95	
Skin lesions	Yes	16	55	0.8633
	No	34	124	
Health status	Asymt	7	42	0.102
	Sympt	43	137	

The detection of co-infection/co-exposure by qualitative serology showed that the prevalence of *Ehrlichia spp*. in infected dogs was about 36% and

16% for *Anaplasma spp.* Six infected dogs (12%) showed exposure to both bacteria. The co-infection could not be confirmed because the definitive diagnosis was obtained only after direct evidence of the bacteria or its DNA.

Moreover, a case of *Dirofilaria* infection has been diagnosed through Knott's test with a prevalence of 1,4%. The identified Filaria was *Dirofilaria repens*. The infected dog had a cutaneous lesion on his muzzle and LNE, which has suggested Canine Leishmania infection (Fig. 6.3)

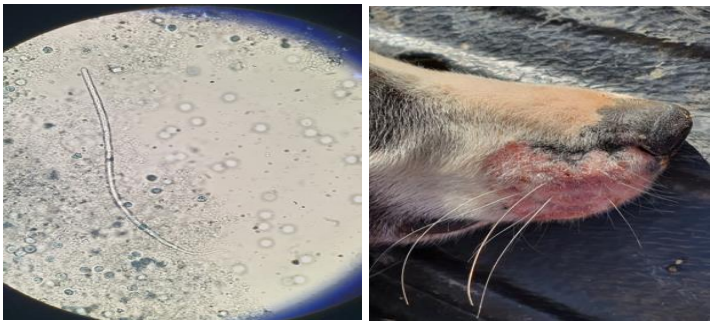

Fig. 6.3. Dirofilaria repens infection in a dog

The PCR-based detection of *Babesia spp*. was inconclusive for the observation of smeared DNA bands. This was probably due to a cross-contamination in the blood samples. A few other PCRs are going to be carried out for the samples taken during the last campaign (Fig. 6.4).

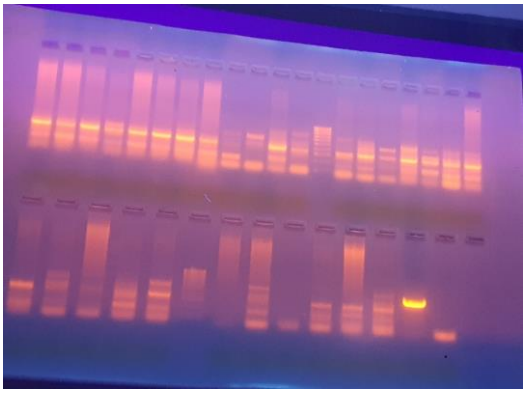

Fig. 6.4 Results of PCR cross-contamination

All DNA extracts had negative results for *Leishmania major* Rt-PCR and ITS1-PCR, but the results of Isoenzyme analysis of 3 cultured parasites (Fig

6.5) sent to the National Reference Center for *Leishmania* in Montpellier (France) are still pending.

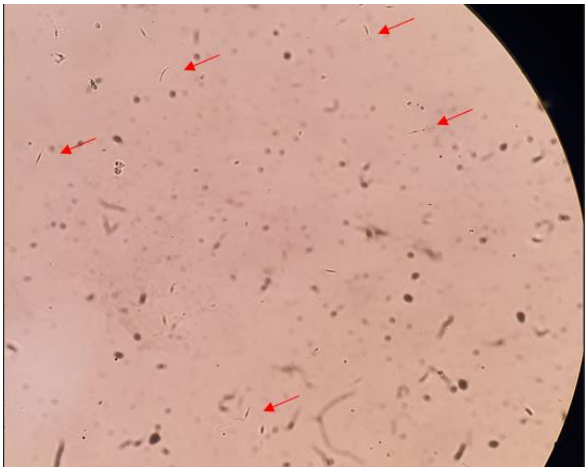

Fig. 6.5 *Leishmania* promastigotes grown in NNN medium. Density after the 7th day observed under light microscope (40X magnification).

Comparative analysis between the two groups of dogs:

The Northern group contained 115 dogs with an average age of 2.7 years (ranging from 6 months to 15 years). The sex ratio was 1:1.8 (41 females (35,6%) and 74 males (64,4%)).
The prevalence of seropositive in the screening test for anti-*Leishmania* antibodies was 20,8%. *L. infantum* DNA was detected in 19% of samples submitted to qPCR. Total prevalence of *L. infantum* in the group was 27%.
Regarding qPCR sensitivity, we detected *Leishmania* kDNA in 16 out of 48 LN of Northern dogs (33,3%), the rate was higher than that obtained with skin (22%) and buffy coat (6%).

Tab. 6.4 Symptomatology association with ELISA, IFAT and Rt-PCR of BC, LN, and cutaneous scraps. for the diagnosis of canine L. infantum infection in endemic area of L. infantum

Northern dogs	**ELISA**	**IFAT**	**Rt-PCR**		
	plasma	plasma	BC	LN	Scraps
symptomatic	19	1	6	16	2
asymptomatic	4	0	1	0	0

Among the infected dogs 87% presented clinical signs, 24 were seropositive and 22 had a positive qPCR, 4 of them had 2 positive matrices BC/lymph node and lymph node /skin scraps.
Clinical signs of infected dogs were LNE 58%, Cutaneous lesions 29% and Weight loss 25,8%

The co-exposure of *L. infantum* infected dogs to *Ehrlichia spp*. was 22.5% and 13% for *Anaplasma spp.* while it was 9.6% for both bacterial infections

Tab. 6.5 Co exposure in northern dogs

Co-exposure	Infected dogs
Ehrlichia spp.	7
Anaplasma spp.	4
E.spp./A.spp.	3

The Southern group contained 114 dogs with an average age of 2.7 years (ranging from 6 months to 10.5years). The sex ratio was 1:1.8 (41 females (35,6%) and 74 males (64,4%)). The seroprevalence was 13,1%. Among seropositive dogs nine had negative qPCRs.
Cutaneous scraps Rt-PCR were negative for detecting *Leishmania* infection in symptomatic and asymptomatic dogs while lymph node Rt-PCR and buffy coat Rt-PCR were positive in only symptomatic dogs (Tab. 6.6).

Tab. 6.6 Symptomatology association with ELISA, IFAT and Rt-PCR of BC, LN, and cutaneous scraps. for the diagnosis of canine L. infantum infection in endemic area of L. major

Southern dogs	**ELISA**	**IFAT**	**Rt-PCR**		
	plasma	plasma	BC	LN	Scraps
symptomatic	11	1	3	7	0
asymptomatic	3	0	0	0	0

Clinical manifestations were classified as follows LNE 73%, Cutaneous lesions 36,8% and severe weight loss 10,5%

The prevalence of co-exposure to CVBP was 57,8% for *Ehrlichia spp*. 21% for *Anaplasma spp.* and 15,7% for both bacteriosis. (Tab 6.7)

Tab. 6.7 co-exposure in southern dogs

Co-exposure	Infected dogs
Ehrlichia spp.	11
Anaplasma spp.	4
E.spp./A.spp.	3

A statistical analysis was performed to compare the prevalence for CanL considering the group of dogs evaluated in the study, the chi-square statistical test was applied, and no significant difference was observed between them (northern dogs 27% vs southern dogs 16%: p-value = 0.059). While the co-exposure rate was higher in Southern dogs (24%) than Northern dogs (14%).

6.4 Discussion

CanL due to *L. infantum* occurs endemically in Tunisia where its distribution was confined to the Northern parts of the country. But it is gaining ground throughout the territory. The parasite first found to be endemic in the sub-humid and upper semi-arid zones of Northern Tunisia, has slowly expanded to the South. and this is well noted in recent studies reported by Bouattour *et al* which showed that seroprevalence had increased 30 times in 50 years of difference (49% vs 1.6%). Our study showed a prevalence of 26.9% in the Northern group and 16.5% in the Southern group this was particularly associated of the parasite distribution in the country

The same nosogeographic distribution change was observed in the ZCL due to *L. major* which is more widespread in the south but continues to spread northward. Despite the fact that this species of *Leishmania* is widespread in the country, its isolation in dogs has never been studied. In our study, the only parasite responsible of canine leishmaniasis in an area of high endemicity to *L. major* was *L. infantum*. This indicates that probably the dog does not play a role in the epidemiology of the parasite because it cannot be a reservoir of this parasitosis but can become infected as an accidental host. In other countries, Canine leishmaniasis due to *L. major* was reported in different cases but still little studied.

The majority of infected dogs were not receiving preventive treatments against ectoparasites, indicating a low level of veterinary care. This explains the high frequency of polysymptomatic CanL clinical cases (86%). This has

also been noted in noninfected cases; symptoms may be due to other CVBDs or intestinal worm infections. The most frequent symptom observed was lymph node enlargement, cutaneous lesions, and weight loss, but animals presented also bad conditions (like cachexia, parasitic hyper-infestation, onychogryphosis) which have not been reported.

According to previous studies in Tunisia, the CVBPs seroprevalence rates were estimated at 54.2% and 25.2% for *Ehrlichia spp.* and *Anaplasma spp.*, respectively, and 22.4% for both pathogens.
To our knowledge this is the first report of canine leishmaniasis co-infections with other VBPs in Tunisia; indeed, 36% of dogs had co-infection with ehrlichiosis, 16% with anaplasmosis, and 12% with both bacteriosis. All these results seem difficult to compare since no previous references related to canine leishmaniasis co-infections in Tunisia were available. However, previous studies have reported seroprevalence for *Ehrlichia canis* ranging from 42% to 85%, which is higher in dogs from humid and semi-arid areas compared to dogs from arid areas 24.6% even though 76.2% of dogs were infected with *Rhipicephalus sanguineus* and this confirms our data that the higher rate of co-infection with ehrlichiosis is described in Northern dogs.

The molecular prevalence of *D. repens* was reported at 3 %, the single case identified in our study belonged to the southern group living in Echrarda district from Southern Kairouan, where human cases have been reported. This Subcutaneous dirofilariasis is rare in Tunisia and not well described in the literature. The prevalence is probably underestimated in human and animal infection since clinical signs are non-specific and spontaneous cure is common.

Different direct and indirect methods have been used for the diagnosis of canine leishmaniasis, as well as different matrices. CanL prevalence of 60% and 14% of symptomatic and asymptomatic dogs, respectively was diagnosed by ELISA technique, while 4% of clinically sick infected dogs were diagnosed by IFAT after a doubtful ELISA. The IFAT technique has traditionally been considered a gold standard for the serological diagnosis of *L. infantum* infection, with optimal performance measures regarding sensitivity and specificity.

Three molecular techniques have been used for the diagnosis of leishmaniasis; the ITS1 PCR showed lower sensitivity than the qPCR: Four out of seven *Leishmania* DNA extracts from infected dogs allowed species identification by using ITS1-sequencing method. Three extracts gave no amplification.
Northern dogs have a higher prevalence than southern, this is due to the geographic and bioclimatic distribution of VL in Tunisia which since the 80s has been spreading from north to south, indeed VL in Tunisia was limited to the humid, sub-humid, and semi-arid bioclimatic stages and more recently has extended to the arid areas in central and southern Tunisia. These data are in perfect agreement with the reported cases of HL.

We gathered information provided by molecular analysis, along with the data obtained from clinical signs and serological evaluations, since, as reported by other authors, positive PCR indicates a *Leishmania infantum* infection, but not necessarily the development of the disease itself.

The qPCR used by Vitale *et al* allowed us to detect a parasitic load equal to 1 x106 compared to 5x106 of *L. infantum* promastigotes quantification with the method by Mary *et al* with these latter parasitic loads equal to 2,3 and 4 *Leishmania* / ml have been identified.

Dogs that were qPCR positive but seronegative might have been recently infected and have not yet developed humoral immunity detectable by the ELISA test, since seroconversion can take several months to appear, although some dogs may remain seronegative for their entire lives.

In both groups, lymph nodes were the best matrices for PCR diagnosis. In fact, the most lymph node examined samples were taken from dogs displaying some signs of infection, because in these animals, the examinable lymph nodes were enlarged, and the skin and blood gave positive results with a lower parasite identification. Blood samples had lower sensitivity, although the collection was easier, which confirmed what has been described in previous studies.

No statistically significant difference was found between the Northern group living in an endemic foci of *L. infantum* and the Southern group living in an endemic focus of *Leihmania major*, therefore, we can conclude that CanL

due to *Leishmania infantum* is equally diffused in the North as well as at the Center

Regarding the dog's age as being a risk factor, this research demonstrated that dogs younger than two years and seniors were less frequently infected, while the most infected age group was from 1 to 5 years old.

This result was similar to what has been previously described in that seroprevalence followed a bimodal age distribution, with two peaks appearing in adult dogs.

We observed a statistically significant difference in prevalence between male and female dogs, which was in agreement with other authors since we have found a higher prevalence in males, due to the greater roaming behavior of male dogs.

Yet, some of the limitations encountered in this research was mainly due to the lack of cooperation of the dogs' owners and even the dogs, which explains the limited number of performed samples. Thereby, this work is still being under progress and still carried out to increase the number of cases and to investigate more regions.

6.5 Conclusion

This study revealed that even in the areas of highest endemicity for *L. major* the dog appears to be an important reservoir for only *L. infantum*, while our study has some limitations in sampling for *L. major* research and that co-infections in those areas need to be more studied and clarified.

The present study demonstrates the widespread distribution of canine leishmaniasis due to *L. infantum* in the country and the need for public health authorities to focus on these zoonoses and to consider the necessity of the one health concept since preserving the health of all living beings and especially the health of an animal as close to humans as the dog means preserving human health.

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Chapter 7

General conclusion

The research aimed at studying several aspects of canine leishmaniasis, through comparing previous works in order to better understand the distribution, examine the pathogenesis and analyse the clinic of this parasitosis. In fact, our study consisted mainly in investigating the role of the dog in the life cycle of *Leishmania major* as well as examining the various aspects of canine leishmaniasis due to *Leishmania infantum*.

In the first stage, the study allowed through an untreated group of *Leishmania* sick dogs submitted to xenodiagnosis for the evaluation of a spot-on insecticide solution to evaluate the statistical relationship between the clinical score and entomological parameters. This experiment confirmed that both *P. perniciosus* infection and infectivity were influenced by a dog's clinical condition especially the presence of skin lesions, and in a later phase, it is recommended to treat sick dogs as well as healthy dogs with effective repellent molecules that can protect the animal as well as those around him.

Then, this theory was more confirmed through assessment of the serum level of CICs in dogs exposed to natural and experimental infection, the direct relationship between the IFAT value and the level of CICs in the progression of *Leishmania* infection and that this data can be used as a clinical diagnostic tool for the monitoring of sick dogs.

Later, a robust cross-sectional survey in hunting, stray and sheep dogs in a Mediterranean area, allowed the investigation of CVBDs with three different dog lifestyles in Molise region and demonstrate a significant association for filarial infection in hunting dogs and the necessity to plan effective control programs to guarantee the health and welfare of pets and inhabitants of Molise region. The next phase consisted in expanding the study in Tunisia that demonstrated that canine leishmaniasis widespread in the country was only due to *Leishmania infantum* also in dogs living in the endemic area of *Leishmania major* and that dogs did not play a role in the epidemiological cycle of ZCL. Moreover, we had demonstrated the presence of co-infections of canine leishmaniasis with other CVBPs.

Yet, we encountered some challenges in our work, mainly to find the laboratory methodology that matches our objectives in the field and to ensure that the clinical and serological aspects can help us better understand the pathogenesis of the disease in order to be able to control and prevent it.

The other challenges consisted in obtaining the samples and biological materials that facilitate and match our research's objectives which was not always obvious with the field's conditions and the COVID-19-related travel and movement restrictions.
Above all to pursue our primary research objective of better understanding of the transmission of *Leishmania major* and the role of dogs in its life cycle, we had to do other skin sampling.

Finally, this study has shown the importance of pursuing research on the different aspects of canine leishmaniasis, since this parasitosis is very diffuse and can reach humans, by being preserved and spread through the dog. Thereby, it was mandatory to study its pathogenesis, as well as the methods to diagnose and prevent it to control it.

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