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# Epidemiological, clinical and molecular characterization of tegumentary leishmaniasis in the Emilia-Romagna region

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# Abstract

Leishmaniasis is a parasitic disease caused by different species of *Leishmania* and transmitted by bites of phlebotomine sandflies. The disease is endemic in over 100 countries, including southern European countries.

The clinical spectrum of the disease depends on parasite species and host characteristic. It ranges from visceral leishmaniasis (VL), the systemic, the most severe and potentially lethal manifestation of the disease, to tegumentary leishmaniasis (TL), where parasites localize in skin and/or mucosal tissues. TL can be sub-divided into three forms, cutaneous (CL), mucosal (ML) and mucocutaneous leishmaniasis (MCL).

As in other part of the Mediterranean region, leishmaniasis is recently emerging in the Emilia Romagna region (RER) (north-eastern Italy) with multiannual upsurge of epidemic foci of VL and TL. Despite the strengthening of surveillance system, the prevalence of TL in the region is only partially known.

The focus of the project was to collect and process epidemiological data and clinical samples in order to: (1) better understand the TL burden in the RER and (2) characterize at species and strain level the *Leishmania* population causing TL in this region from a molecular point of view.

Study I results revealed that between 2017 and 2020 135 cases of TL were diagnosed in the RER, among them the most (84%) were autochthonous and 8% were ML cases. Within the study we organized a multicentric surveillance system that contributed to recover a significant number of unnotified cases and collect data describing the clinical management of TL in the region. In study II we successfully typed the 80% of the tested TL confirmed cases that we collected between 2014 and 2020 by the combined use of two typing tools, ITS1 and hsp70. By this way we identified 8 imported cases of *L. major*, *L. tropica* and *L. donovani* from various countries and 75 autochthonous samples. Among the latter we detected a high variability of the analyzed hsp70 conserved sequence and the

presence of distinct *Leishmania* populations causing TL in the RER. Study III was a phylogenetic study that was performed by using Oxford Nanopore Technologies NGS sequencing in order to obtain the complete conserved sequence of kinetoplast maxicircles in two TL isolates from central and northeastern Italy. We observed a clear phylogenetic divergence between the two isolates and the *L. infantum* strains circulating in southern Europe, confirming the presence of a new dermotropic strain circulating in the area.

Keywords: neglected disease, tegumentary leishmaniasis, molecular epidemiology, *Leishmania infantum* phylogeny, leishmaniasis molecular typing method, hsp70, ITS1, kinetoplast maxicircles coding region.

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# Glossary

**AFLP:** amplified fragment length polymorphism

**AmB:** amphotericin B

**bp:** base pair

**CanL:** canine leishmaniasis

**CL:** cutaneous leishmaniasis

**CMV:** Cytomegalovirus

**CNV:** copy number variation

**CPB:** cysteine proteinase B

**CR:** coding region

**CSB:** conserved sequence block

**CytB:** cytochrome B

**cur-1:** MHOM/IT/2019/cur-1

**DAT:** direct agglutination test

**DR:** divergent region

**DRs**: direct repeats

**EBV:** Epstein-Barr virus

**ELISA:** enzyme-linked immunosorbent assay

**EMTM:** Evans' modified Tobie's medium

**FBS:** fetal bovine serum

**FFPE:** formalin-fixed and paraffin-embedded

**G6PDH (or G6PD):** glucose-6-phosphate dehydrogenase

**GOT:** glutamate-oxaloacetate transaminase

**gp63:** glycoprotein 36

**GPI:** glucose phosphate isomerase

**GFR:** glomerular filtration ratio

**gRNA:** guide RNA

**HIV:** human immunodeficiency virus

**HOMEM:** Hemoflagellate-modified minimum essential medium

**hsp70:** heat shock protein 70

**ICT:** immunochromatographic test

**IFAT:** immunofluorescent antibody test

**IFN-γ:** interferon γ

**IL:** interleukin

**IRs:** inverted repeats

**ITS:** internal transcribed spacer

**kDNA:** kinetoplast DNA

**L-AmB:** liposomal amphotericin B

**LHU:** local health unit

**LPG:** lipophosphoglycan

**MLEE:** multilocus enzyme electrophoresis

**MLST:** multilocus sequence typing

**MLMT:** multilocus microsatellite typing

**MO44:** isolate MHOM/IT/2021/IZSLER-MO44

**MPI:** mannose-phosphate isomerase

**mRNA:** messenger RNA

**MCL:** mucocutaneous leishmaniasis

**ML:** mucosal leishmaniasis

**NCT:** no template control

**NCBI:** national center for biotechnology information

**NGS:** next generation sequencing

**NMDA:** N-methyl-d-aspartate

**NWL:** new world Leishmania

**ONT:** Oxford Nanopore technologies

**OWL:** old world Leishmania

**PBS:** phosphate-buffered saline

**RER:** Emilia-Romagna region

**RFLP:** restriction fragment length polymorphism

**rtPCR:** real-time PCR

**SbV:** pentavalent antimonial

**SDS:** sodium dodecyl sulfate

**SIDER:** short interdispersed degenerate retrotransposon

**SFB: short-fragment buffer**

**SMI: national notification system**

**SNP: single nucleotide polymorphism**

**Sider: short interdispersed degenerate retrotransposon**

**Th1:** T helper-1

**Th2:** T helper-2

**TL:** tegumentary leishmaniasis

**UMS:** universal minicircle sequence

**UMSBP:** universal minicircle sequence binding protein

**WB:** western blot

**WHO:** World Health Organization

**WGS:** whole genome sequencing

# Introduction

Leishmaniases are among the most important neglected diseases. Different species of *Leishmania,* the causative agents of the disease, are endemic in 99 countries, threatening more than 350 million of peoples, with 700,000 to 1 million of estimated cases occurring annually [1-3].

In many areas presence of leishmaniasis is often linked with poor socio-economic conditions, but also environmental and climatic factors influence the disease's epidemiology. Recent studies reported reemergence of the disease in some southern European countries, with focal emergence in previous non-endemic areas [4,5].

Leishmaniasis is caused by protozoa of the genus *Leishmania*, 20 species cause disease in humans [6]. The parasite is transmitted by the bite of infected female sandflies (genera *Phlebotomus* and *Lutzomiya*). The infection, even if often asymptomatic, can manifest with two main clinical form: visceral leishmaniasis (VL) that is a systemic and lethal disease, and tegumentary leishmaniasis (TL), with parasite's replication involving skin and mucosal tissues. TL can be subdivided into cutaneous (CL), mucosal (ML) and mucocutaneous (MCL) leishmaniasis [7,8].

## 1. The parasite

### *1.1 Taxonomy*

*Leishmania* spp. are grouped into the *Leishmania* genus, which is part of the *Trypanosomatidae* family, order *Kinetoplastida*. Protozoa causing American and African trypanosomiasis (respectively *Trypanosoma cruzi, T. brucei* and *T. gambiense*) are part of the same family [8].

*Leishmania* species of clinical importance are divided into two sub-genera based on the parasite development within the vector gut: *Leishmania Leishmania* (developing anterior to the pylorus) and *Leishmania Viannia* (developing in the hindgut and pylorus). Species belonging to *Leishmania Viannia* are exclusively endemic in Latin America while species belonging to *Leishmania Leishmania*

can be found both in Old World (OWL) and New World (NWL) [9]. In addition, *Leishmania* subgenera are divided into complexes of closely related species (Figure 1)[10] .

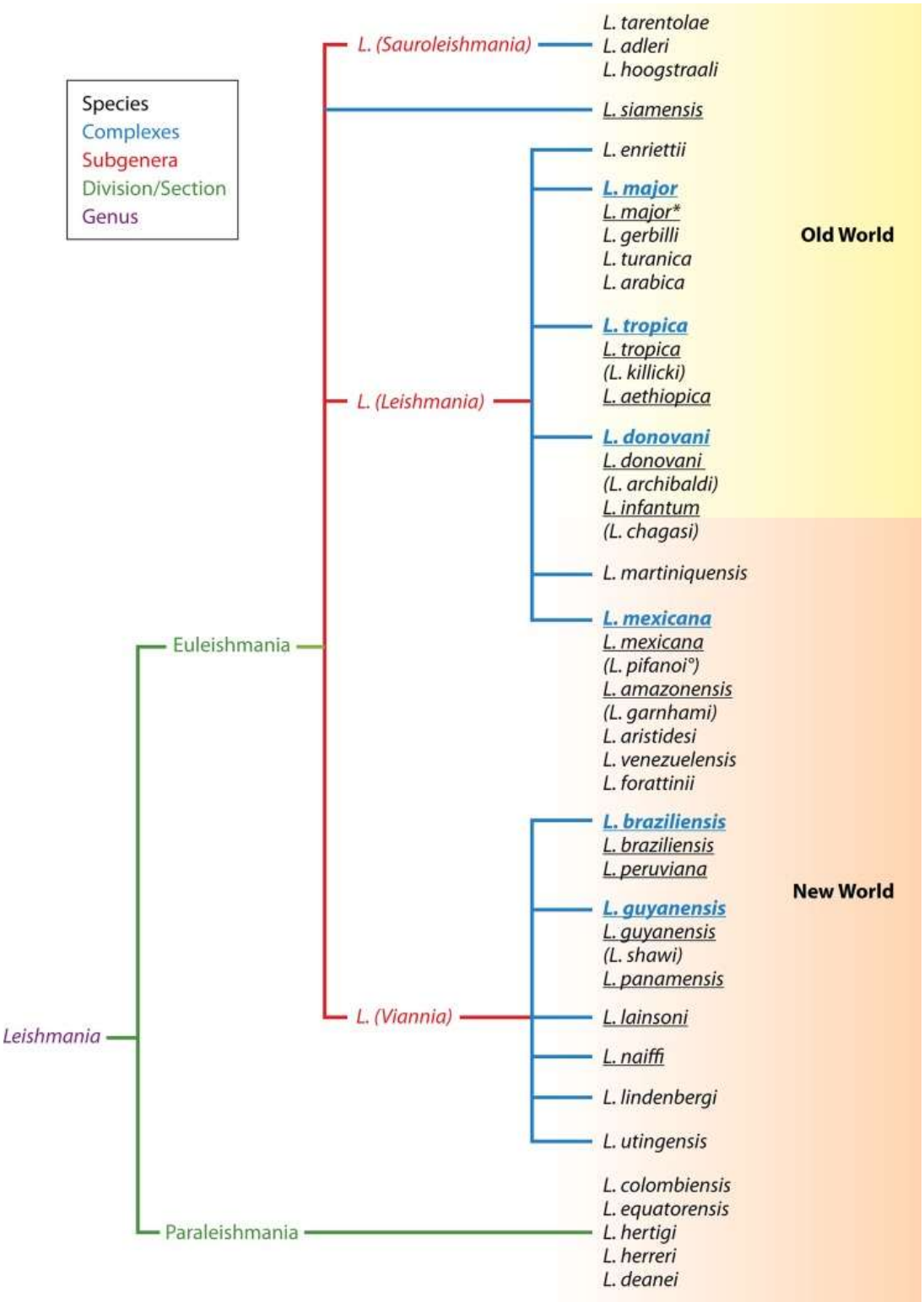


***Figure 1. Taxonomy of the Leishmania genus based on MLEE analysis***. *Modified from Van der Auwera G. et al., 2015 [10].*

*Leishmania Leishmania* subgenus comprehends the following complexes and species [9,10]:

- *L. donovani* complex, (*L. infantum* and *L. donovani*), these species cause visceral and tegumentary forms in both the Old and the New World.
- *L. major* complex, include species causing cutaneous forms in the Old World.
- *L. tropica* complex (*L. aethiopica* and *L. tropica)* these species cause localized and disseminated tegumentary forms in the Old World.
- *L. mexicana* complex (*L. mexicana*, *L. amazoniensis* and *L. ganhami*) causes localized and disseminated CL in the New World.

Leishmania Viannia subgenus includes two complexes both cause of CL and destructive MCL in the New World

- L. braziliensis complex (L. braziliesis and L. peruviana)
- L. guyanensis complex (L. guyanensis and L. panamensis)

Traditionally, the gold standard in species typing is based on multilocus enzyme electrophoresis (MLEE) that was developed in the 1980 and that analyze electrophoretic profile of a series *Leishmania*'s enzymes [11]. Nevertheless, molecular methods are now more widely used for species typing.

### L. donovani complex

Among human pathogenic *Leishmania* spp. those who are almost sole causative agents of VL, excluding sporadic cases due to *L. tropica*, are the ones that clusters into the *L. donovani* complex [6].

Both *L. donovani* and *L. infantum* show viscerotropic tendency, but different capabilities of causing dermal manifestations; *L. donovani* is reported to cause post kala-azar disease leishmaniasis (PKDL) and CL in some foci [6,12,13], while *L. infantum* is described more frequently causing TL, both in the forms of CL and ML [7,14].

Geographically the species part of *L. donovani* complex are widely diffused. *L. infantum* strains cause zoonotic cycles mainly in the Mediterranean basin, parts of the Middle East and Central-South America. *L. donovani* take part mainly in anthroponotic cycles in the Indian sub-continent, east and north-eastern Africa. The Chinese area reports the presence of both the species [15-17].

The first etiological agent discovered as cause of VL, also known as kala-azar disease, was *L. donovani* in 1903, followed by *L. infantum* in 1905, which causes the disease in children in the Mediterranean area. Later on, *L. archibaldi* was named in Sudan in 1919 and *L. chagasi* in South America in 1937 [18].

These four species formed the first grouping of the *L. donovani* complex based on clinical and geographical definitions, this lasted until taxonomical classification based on MLEE analysis was performed in 1980 [11,19].

By MLEE, distinction between *L.* donovani complex species was based on electrophoretic mobility values of a single isoenzyme: the glutamate-oxaloacetate transaminase (GOT). Electrophoretic mobility values of this isoenzymes observed in the four species was 113 for *L. donovani*, 100 for *L. infantum* and *L. chagasi* and 110 for *L. archibaldi*. This classification showed discrepancies with previous taxonomical classification. *L. chagasi* showed no differences with *L. infantum* MLEE profile and then was considered as its synonymous, on other hand while *L. archibaldi* with $GOT_{110}$ intermediate value was temporarily clustered separately [11].

Later studies gave more information about these species: *L. chagasi* classification was enforced by Leblois et al. discovering that this specie was actually *L. infantum* imported in the Americas by European colonizers in the 15$^{th}$ and 16th centuries [20], while *L. archibaldi* intermediate GOT values was discovered to be caused by an heterozygous pattern, successively sub-grouped within *L. donovani* [21].

More recently, genotyping studies based on variation of small number of genetic loci, microsatellite repeats and analysis of genome wide polymorphism provided rich genomic data, improving resolution of *Leishmania* phylogenesis and enabling population genetics analysis [22-26].

A study from 2020 analyzed 151 cultured field isolates of the *L. donovani* complex species from a wide geographic range through Next Generation Sequencing (NGS). This study evidenced that *L. donovani* is divided into 5 distinct lineages coinciding with their geographic positions, while *L. infantum* appears to be a monophyletic group with little differences between strains. In addition, the study confirmed the existence of hybrid strains between the 2 species in Cyprus and Turkey. These new genomic data revealed a grater genetic diversity in the *Leishmania donovani* complex than previously presumed [27].

## *1.2 Morphology and structure*

Many protozoan parasites of *Leishmania* spp. exhibits a large range of cell morphologies and cell types (also called developmental forms) to adapt either to the host or the vector. The parasite shows a digenic life cycle, represented by the promastigote in the sand fly midgut and by the amastigote in the of host macrophages [28,29].

Promastigotes are extracellular, 15-20 µm long motile forms, colonizing the midgut of the sandfly. They show elongated shape with a long motile flagellum. Various types of promastigotes can be observed in the vector. These developmental stages can be distinguished on the base of length/width of the cell body and flagellum and can be found in the vector [30]. The promastigote developmental stage can be summarized as:

(i) procyclic promastigote: with cell body longer than the flagellum, between 6.5 and 11.5 µm;

(ii) nectomonad promastigote: cell body long 12 µm or more;

(iii) leptomonad promastigote: with cell body shorter than the flagellum, between 6.5 and 11.5 µm;

(iv) metacyclic promastigote: cell body shorter than 8 μm long and 1 μm wide; they possess a flagellum longer than the cell body [31];

Amastigotes (also called Leishman-Donovan bodies) are intracellular, 3-5 μm long, replicating inside macrophage phagolysosome. In contrast with the other cell form they shows a round cell shape and a shorter non motile flagellum [31].

Despite the differences between the two main cell forms, *Leishmania* spp. cell structure is conserved and defined by cross-linked sub pellicular corset of microtubules. Housed within cellular structures are conserved: the nucleus, a single mitochondrion and Golgi apparatus. Anteriorly to the nucleolus a kinetoplast contains the concatenated mitochondrial DNA, and is linked to the basal body of the flagellum [32]. At the bottom of the flagellum there is an invagination of the cell membrane, this mouth-like structure is defined as flagellar pocket that is a critical interface between parasite and host environment and the only endo-exocytosis site available in these parasites [33-35]. Essentially there is a cell structure conserved across *Leishmania* spp. formed by a series of modular units: the flagellum, basal body–mitochondrial kinetoplast unit and a Golgi–flagellar pocket neck unit [31,35].

### *1.3 Leishmania genome*

*Leishmania* possesses a diploid genome of 32 Mb containing around 8000 coding genes. Parasite's genome is organized into 2 compartments: the nuclear DNA, which is distributed into 35-36 chromosomes and located in the nucleus, and the kinetoplast DNA (kDNA), which is collected into a specialized part of the mitochondrion and forms a distinguishing feature of the order *Kinetoplastidia* [36-38].

kDNA (Figure 2) represents 30% of *Leishmania* DNA and consists of a network of thousands of interlinked DNA rings divided into two types: maxicircles and minicircles. Maxicircles are 20-40 kb long molecules repeated hundreds of times (depending on the species) that resembles mitochondrial DNA of others eukaryotic organism. Maxicircles contain two different regions: the coding region

(CR) and the divergent region (DR). CR is 16 – 17 kb long and is characterized by highly conserved gene order (synteny among the trypanosomatids) [39-41]. This characteristic and the fact that kDNA is uniparentally inherited and not affected by polymorphisms and recombination events occurring in the *Leishmania* inter-species/-strain hybrids makes maxicircles reliable markers to determine evolutionary relationships among parasitic strains [26,39,42-44].

Minicircles are smaller molecules (0.5-2.5 kb long), numbering thousands of copies per cell and encoding for guide RNA (gRNA) involved in RNA editing mechanism [32,45]. Although minicircles are heterogeneous in size and sequence, they present a conserved region that measured around 150 base pairs (bp) in different *Leishmania* species [44]. Within this region, we found various conserved sequence blocks (CSB): CSB-3, also known as universal minicircle sequence (UMS, 5′GGGGTTGGTGTA-3), CSB-1 (5′-AgGGGCGTTC-3′) and CSB-2 (5′-cCCCGTNC-3′) [46]. CSB-3 is 12 bp conserved sequence signature of this class of molecules, it is the central part of the minicircle replication origin, being the binding site for the UMS binding protein (UMSBP), a protein involved in kDNA replication and segregation [47].

*Leishmania* chromosomes range in size from 0.3 to 2.8 Mb and their genes are organized into larger polycistronic units as observed in other trypanosomatid parasites [36,48,49]. Their gene organization is atypical for eukaryotes; often genes are organized in tandem repeats and many of them are transcribed polycistronically. In addition, long transcription units contain genes with related function, as the operons observed in prokaryotes [50]. Furthermore, regulation of gene expression in this parasite exhibits remarkable differences with other eukaryotes because of the lack of introns and consensus promoter sequences for RNA polymerase II. In contrast, ribosomal DNA (rDNA) genes are the only genes known to possess promoters and that are transcribed by RNA polymerase I [51] . Maturation of messenger RNA (mRNA) requires extensive post-transcriptional processing that includes, trans-splicing of a 39-nucleotide spliced leader (SL, or miniexon-derived) RNA onto the 5′

ends of all mRNA molecules [52,53]. In contrast, no introns have been detected, removing a requirement for cis splicing.

One of the most unique evolutionary features of *Leishmania* is the high degree of plasticity of the genome that often leads to DNA copy number variation (CNV) of various natures (aneuploidy, gene amplification or deletion), thus regulating gene expression [54]. This feature helps the parasite to adapt to stressful situation, and, in particular, some of these genomic rearrangements coincide with drug resistance and environmental adaptations [55-58]. Widespread presence of homologous repeated sequences that covers *Leishmania* DNA contributes to genome plasticity and enhances gene rearrangement [59]. Approximately 2000 low complexity Direct Repeats (DRs) and Inverted Repeats (IRs), defined in relation to their genomic orientation, can be found in the *Leishmania* genome and most (~68%) belong to a family of extinct transposable elements (TE), known as Short Interspersed Degenerate Retroposons (SIDER), that became expanded in *Leishmania*. Two SIDER subfamilies were observed in these parasites, ie SIDER1 and SIDER2 [54]. From these repeated elements, ~3000-4000 unique and selectable extrachromosomal circular or linear amplicons are estimated to be originating from the genome [59]. Amplification of *Leishmania* extrachromosomal DNA was believed to rely on 2 distinct hypothetical pathways: one of linear amplification and one of circular amplification. However, neither pathway has been completely described. It seems that amplification occurs in a stochastic manner with subsequent changes to the abundance of beneficial amplicons. This then leads to alteration in RNA levels that are selected under stressful environments, helping the parasite to adaptation. Furthermore, recent works evidenced that these extrachromosomic elements are involved in horizontal gene exchanges between *Leishmania* cells through extracellular vesicles [60].

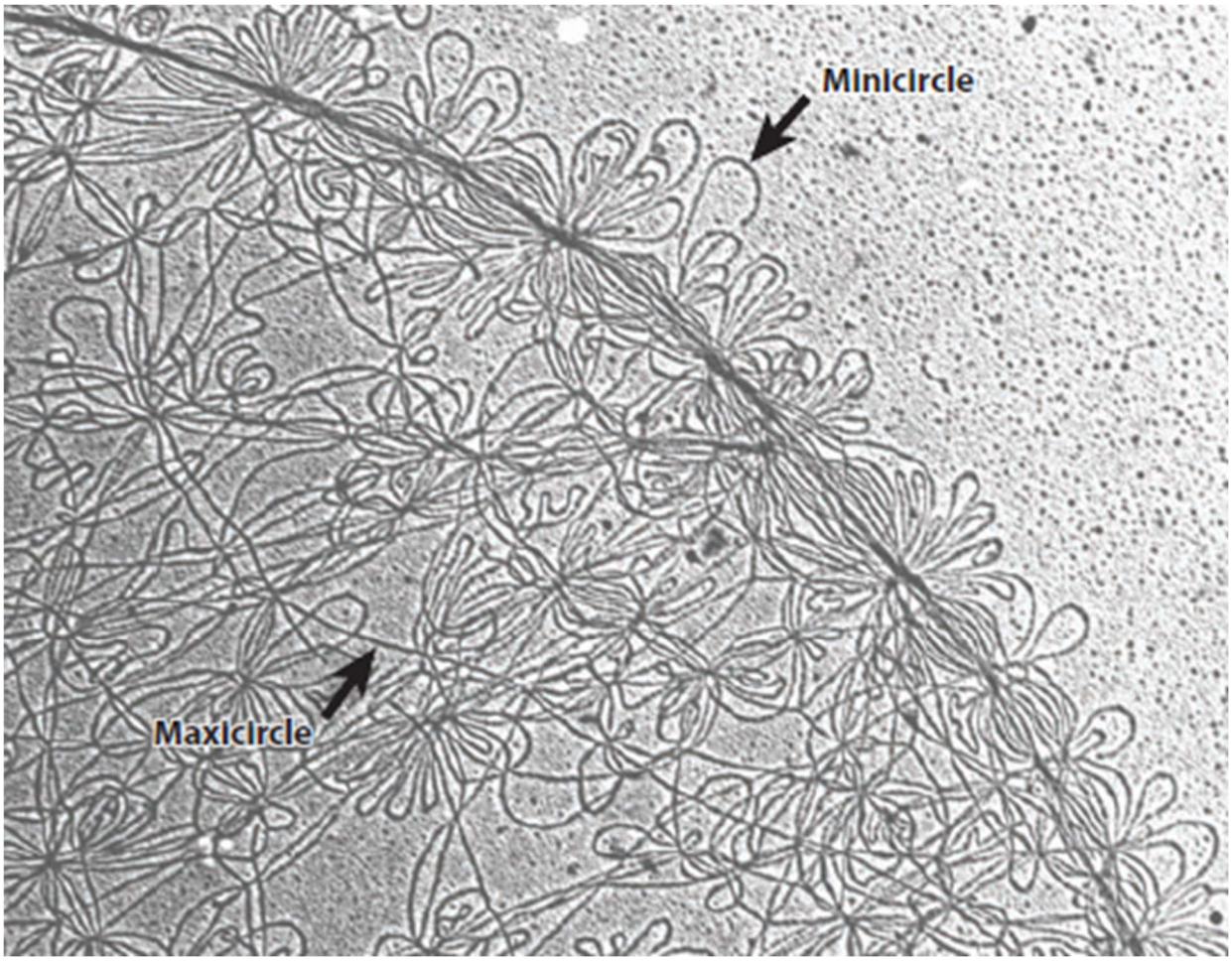


***Figure 2. The kDNA network****. An electronmicroscope image of the edge of an isolated kDNA network. Small DNA loops are minicircles, and long strands are parts of maxicircles. Adapted from Robert E. Jensen and Paul T. Englund 2012 [32].*

## *1.4 Life cycle*

As described above *Leishmania* shows a life cycle shuttling through the sandfly vector and the mammal host [61]. As seen also in other protozoa parasites (as *Plasmodium* spp. or other *Trypanosomes*), *Leishmania* shows different cell morphologies and developmental forms (proliferative or quiescent and pre-adapted for transmission) adapted either to the host or the vector [62-64].

Depending on the species, transmission can be zoonotic or anthroponotic. Anthroponotic cycles have the main reservoir in humans through symptomatic or asymptomatic infections; zoonotic cycles involve wild, domestic, peri-domestic animals as reservoirs and humans as accidental hosts [9].

Starting from the acquisition of the parasite into the vector, *Leishmania* amastigotes within host macrophages are ingested by female sandfly through blood feeding of an infected mammal. Here amastigotes are freed from phagolysosomes into insect's lumen. In this site they start to differentiate into promastigotes driven by the environmental changes, mainly pH and temperature [65]. This progressive differentiation pathway through various promastigote's "developmental stages" is called "metacyclogenesis" and is subdivided into 4 main passages [66] (Figure 3).

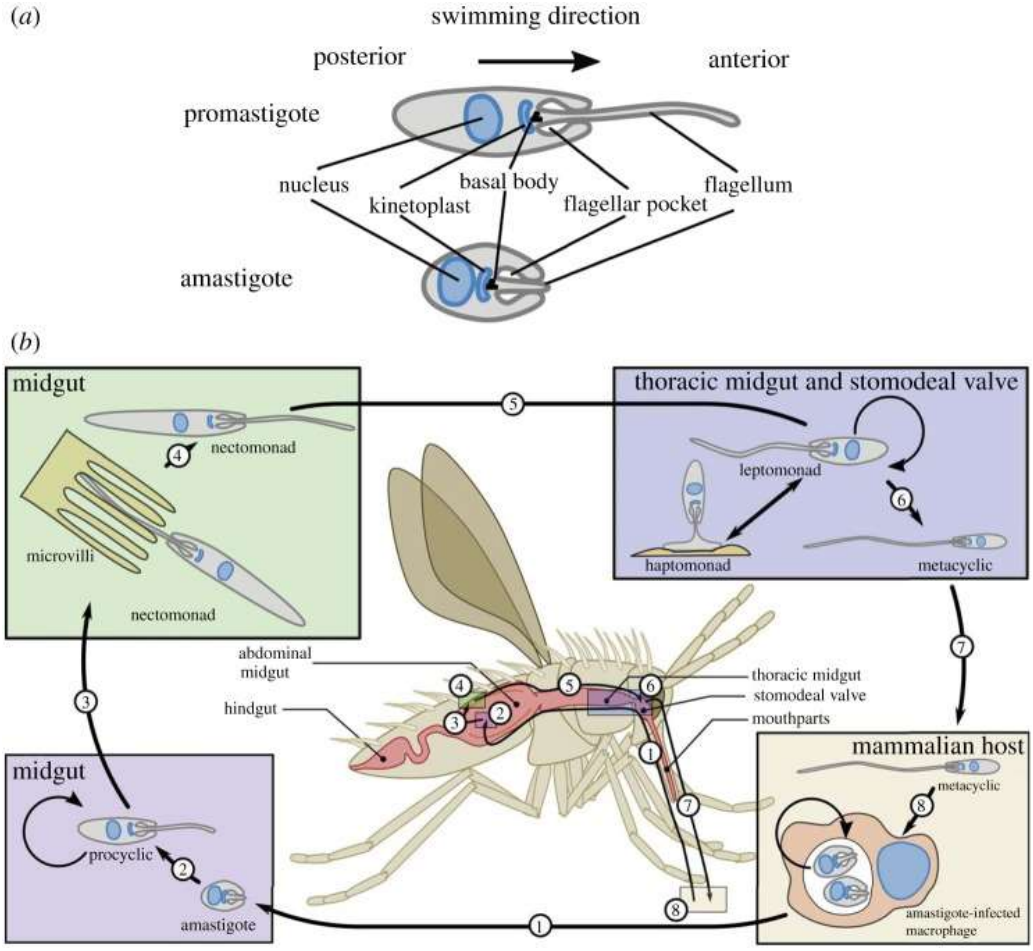


***Figure 3. Leishmania life cycle in the vector.*** *(a) Promastigote and amastigote morphologies aligned along the posterior anterior axis with key structures in the indicated cells; (b)current understanding of the Leishmania life cycle with critical events and different target cells. Modified from Sunter J, Gull K. 2017 [31].*

In the first stage, amastigotes transform into "procyclic" promastigotes, these shows little motility, and replicate cyclically for 48-72 hours. After this step, "procyclic" promastigotes differentiate into "nectomonad" promastigotes migrating to the midgut. Differently, species of the subgenus *Viannia* pass through the hindgut before migrating forward to the midgut. Here, by an interaction between

microvilli and lipophosphoglycan (LPG) on *Leishmania* cell membrane, the "nectomonad" promastigotes attach themselves to the midgut wall. In next step they migrate into stomodeal valve and thoracic midgut, transforming into "leptomonad" promastigotes. In this anatomic section leptomonad promastigotes can start replication or differentiate into attached "haptomonad" promastigotes (a specialized cell type attached to the cuticle via an hemidesmosomal structure in the enlarged tip of the flagellum) or into "metacyclic" promastigotes [31].

Metacyclic promastigotes are highly mobile, non-replicating and infectious, they will be released by the infected vector by regurgitation during blood meal and deposed into the bite's site . Part of metacyclic promastigotes that remain into vector lumen will go through a process of retro-transformation triggered by the contact with blood meal. They will de-differentiate into retroleptomonads, able to rapidly multiply and re-differentiate into metacyclic promastigotes, amplifying the parasitic load by up to 125-fold [67]. In the host, tissue damage caused by the insect, together with inflammatory components deposed with the parasites, will attract macrophages and neutrophils [68]. These will be infected by metacyclic promastigotes via phagocytosis, probably induced by some interaction with the parasite flagellum [69]. Once inside host cell's phagolysosome, promastigotes will escape intracellular killing and transform into intracellular amastigotes and then start to replicate; infected host cell will eventually be lysed releasing the parasites, which are capable of infecting new host cells [70].

## 2. The vector

*Leishmania* vector is represented by insects part of the *Diptera* order, subfamily *Phlebotominae*, commonly called sandflies. Sandflies are 2-3 mm long insects, with colors ranging from white to grey and wings characteristically positioned at an angle to the abdomen in contrast with the other *Diptera* [9,71]. These insects are mainly active at early morning, evening and night. They exhibit a noiseless disgraced flight resolved generally in short hops, close to the ground with flight range of about 300 meters, starting from the larval developmental site. Larval habitat requires a warm and moist environment, in contrast with standing water needed by mosquitoes [72]. Among 800 species of sandflies, only 93 were proven to be competent vectors of *Leishmania* species of clinical interest, of them the ones part of *Lutzomiya* subfamily can be found in the New World and *Phlebotomous* subfamily in the Old World [8]. Sandflies cover territories of Central and South America, Africa and Europe. The suitable habitat of these arthropods is strongly linked with various elements such as altitude, land cover, vegetation, climatic factors and to the availability of suitable hosts [72]. In the Mediterranean habitat, the sandfly species that can be found are *P. perniciosus*, *P. tobbi*, *P. ariasi*, *P. papatasi*, *P. perfiliewi, P. sergenti* and *P. neglectus* [73]. In Italy, autochthonous species are *P. perniciosus* and *P. neglectus* as most prevalent species in the central- southern part of the peninsula and pre-Alpine territories, *P. ariasi*, which is found near to the French border and *P. perfiliewi*, which is the most prevalent species in northeastern Italy [74].

## 3. The reservoir hosts

Leishmaniasis transmission cycle type can be grouped into two categories: anthroponotic, where the primary host and reservoir are humans, and zoonotic, where there is a primary animal reservoir and humans are infected as accidental host [75]. In addition, zoonotic leishmaniasis can be distinguished between domestic and sylvatic cycles; the first involves domestic or peri-domestic animals and the second involves wild animals [9]. Canids (both wild and domesticated) act as main reservoir for *L. infantum*, but also other animals such as cats, donkey, horses and rodents were found to be infected [76-78]. Additional known animal reservoir are rocky hyrax in Ethiopia as reservoir of *L. aethiopica*, rodents in Middle East as reservoir for *L. major*, rodents and sloth in the New World as reservoir for various parasitic species [79-81].

*L. donovani* and *L. tropica* are mainly anthroponotic species but some studies evidenced circulation of these species in animals [82]. At the same level, other *Leishmania* spp. known for zoonotic cycles, such as *L. infantum* and *L. major*, can have humans as main infections source f [75]. Other possible transmission routes are parenteral transmission *via* blood transfusion, transplant or promiscuous use of needle in drug addicted individuals. In addition, vertical transmission was reported [75,83].

# 4. Pathogenesis of leishmaniasis

Clinical manifestations of leishmaniasis are various, ranging from localized skin lesions to systemic VL, as consequence of the interplay between the host's immune response and the infecting parasitic species. Parasites like *L. major* primarily affect the skin and remain localized in the insect's bite site, whereas *L. infantum* and *L. donovani* can spread to internal organs [84,85].

When sandflies feed, they disrupt blood vessels and introduce parasites intradermally. Neutrophils are the first cells to arrive at the sandfly bite site and are the initial cell type to be infected. Dendritic cells and macrophages phagocyte the parasites and then, leaving the lesion site, allow them to disseminate. The specific type of macrophage population infected by dermotropic and viscerotropic *Leishmania* spp. differs, this is possibly due to the parasite's affinity for specific macrophage cell surface receptors, although this mechanism remains poorly understood [86].

Antileishmanial immunity involves components of both innate (neutrophils, macrophages and dendritic cells) and adaptive (T cells) immunity. Among the latter, the CD4+ T-cells shows a crucial role for host defense response. Additionally, cytokines response induces and regulates immune responses. Interestingly, despite a robust humoral response was observed during infection, antibodies seem to have no protective role [87].

Experimental studies in murine models, demonstrated that efficient resistance to the disease is linked to the expansion of T-helper-1 (Th1) cells that release IL-2 and IFN-γ enhancing macrophages activation to suppress parasite growth. On the contrary, the development of T-helper-2 (Th2) response, resulting in the production of IL-4, IL-5 and IL-10, or a mixed Th1/Th2 response, is associated with disseminated infection [87].

Polymorphisms of genes involved in these immune interactions may contribute to the clinical diversity observed in leishmaniasis [9]. Furthermore, acquired immunosuppressive conditions can affect parasite replication. For example, HIV-1 infected patients have an increased the risk of 100–

2230 times of developing VL, disseminated CL or ML. HIV/*Leishmania* coinfections can lead to chronic evolution of the disease, with a high relapse rate and poor response to treatment [88].

# 5. Clinical presentation of leishmaniasis

The range of leishmaniasis clinical manifestations is determined by the type of parasite species involved and the strength of the host's immune response. It spans from asymptomatic infections, which occurs in approximately 80-95% of cases for *L. infantum* infection, to three primary clinical presentations: VL (also known as kala-azar) which is the most severe form of the disease, and TL; which includes CL, ML and MCL [7,14]. The incubation, time between the initial sandfly bite and the onset of symptoms, can vary; 2-6 months for VL and 2 weeks-2 months for CL; however in some cases, it can extend to a year or even longer [75].

## *5.1 Visceral leishmaniasis (VL)*

VL is caused by parasites belonging to the *L. donovani* complex and sporadically by *L. tropica* [6,89]. This disease form is endemic in 80 countries in Europe, Asia, Africa and Latin America but the most of the cases (90%) are notified in just 10 countries: as previously mentioned [2] .

VL stands as the most severe and life-threatening form of this disease, potentially fatal if left untreated. It is characterized by a combination of symptoms, including irregular fever, weight loss, enlargement of the spleen (splenomegaly), enlargement of the liver (hepatomegaly), and anemia [75]. Parasite's proliferation occurs within cells of the mononuclear phagocyte system, most commonly within macrophages. Consequently, the infection extends to bone marrow cells, leading to a condition known as pancytopenia, a reduction in all types of blood cells, and immunosuppression, leaving the patient susceptible to superinfection [17].

## *5.2 Tegumentary leishmaniasis (TL)*

Tegumentary leishmaniasis encompasses both cutaneous leishmaniasis and the mucosal forms of the disease (ML and MCL), different *Leishmania* species can cause diverse clinical features of TL (Figure 4). In the initial stage of the infection, there is a significant localized presence of parasitized macrophages at the site of inoculation followed by an increasing infiltration of lymphocytes and

plasma cells. As the infection progresses, host macrophages are eventually destroyed, releasing amastigotes and causing the breakdown of the basal layer, which can lead to ulcer formation [75].

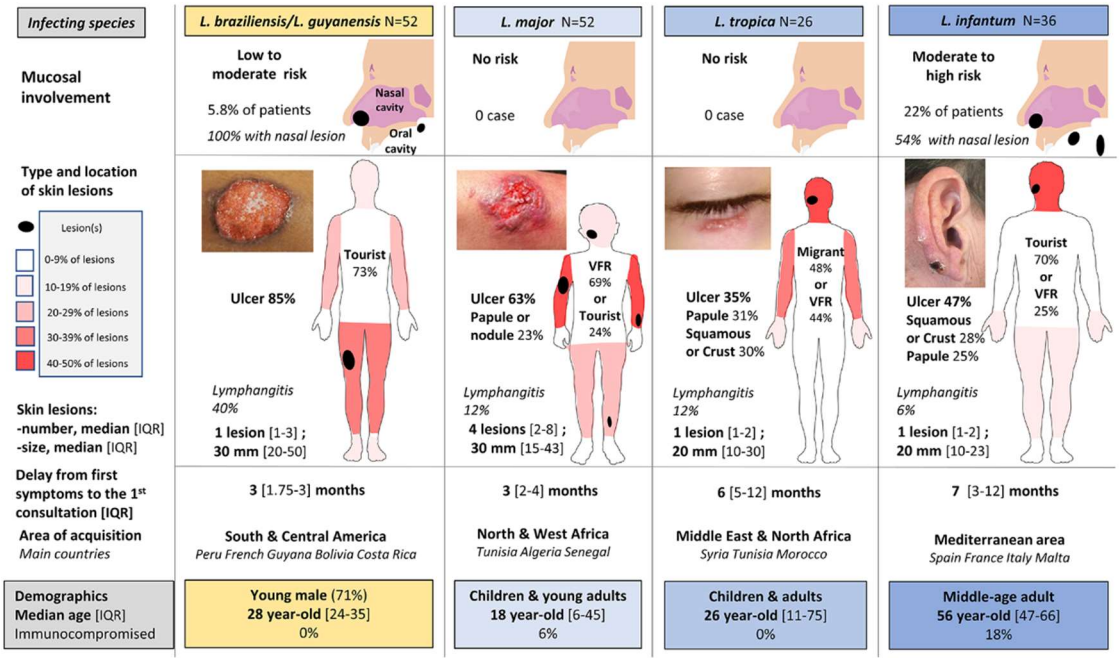


*Figure 4.* ***Comparative features of cases of tegumentary leishmaniasis by main infecting species****. Adapted from Guery et al 2021 [7].*

## Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis typically affects body exposed areas like the face, neck and limbs, where sandflies have readily access. In the Old World, CL is s caused by parasites from *the Leishmania major* complex, L. *tropica* complex, and *L. donovani* complex. In the Americas, *L. mexicana, L. braziliensis*, and *L. panamensis* are the most common [7]. Notably, CL of the New World tends more often to require treatment for resolution[14].

CL is not life-threatening; however, the healing process typically spans several months and can result in scarring and permanent changes in skin pigmentation. Initially, lesions may appear as small red papules and can evolve into various forms, including erythematous nodules, indurated plaques, scaly plaques, or ulcers with raised, rolled, dusky borders. These lesions might be dry and crusted or accompanied by exudates. Different *Leishmania* spp. can lead to different skin lesion evolutions s [9,14] as summarized in Table 1.

*Table 1.* ***Cutaneous leishmaniasis caused by different Old World species****. Adapted from Gradoni et al 2017 [14]*

| Species | CL clinical characteristics |
|---|---|
| *L. tropica* | Often present as a painless, dry skin ulcer with a thick crust. It typically heals by his own spontaneously within about one year, sometimes longer, often leading to a disfiguring scar. The incubation period is usually two to eight months long. |
| *L. major* | Frequently cause severely inflamed and ulcerated skin. Usually heals spontaneously within two to eight months. There is possibility of presence of multiple lesions associated with multiple sandfly bites in natural biotopes, or in non-immune patients, which can lead to disfiguring scars. The incubation time take less than four months. |
| *L. infantum* | This frequently presents as a single nodular lesion on the face, although other parts of the body can be affected, and multiple lesions may occur. There is little crust in the lesion and it usually does not ulcerate. Except for the induration and colour, the superficial layer of the lesion's skin looks almost normal. Clinical variation has been described, including ulcerative lesions and plaques. |

## Mucosal leishmaniasis (ML)

ML is a condition in which localized *Leishmania* lesions occurs in buccal, pharyngeal or laryngeal mucosa without primary skin involvement. ML can be seen in immunocompetent individuals as well as in immunocompromised patients. This rare form of disease is present in the Mediterranean basin and it represents about 2% of autochthonous TL cases of leishmaniasis, as observed in France [90], even though a higher frequency has been recently observed [7]. *L. infantum* is the typical causative agent of ML, although in elderly and immunocompromised patients, *L. major* and *L. tropica* can also lead to this form of the disease. Differently from MCL, these lesions are primary and not associated with previous cutaneous lesions. ML and MCL pathogenesis are still poorly understood [14,90].

## Muco-cutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (MCL) is a form of leishmaniasis primarily found in the New World and is predominantly caused by *L. panamensis* and *L. braziliensis*. Most cases are reported in regions such as Bolivia, Brazil, and Peru [3]. MCL results from the metastatic spread of the infection from a previous cutaneous lesion and can manifest months to several years after the initial cutaneous episode. A common presentation of MCL involves nasal lesions, which can cause nostril blockage and disfiguring nasal collapse. Additionally, the upper lip, palate, and throat can also be affected [9,75].

## *5.3 Leishmaniasis in immunocompromised patients*

Immunosuppression is a major risk factor in developing leishmaniasis both for susceptibility against initial infections and the reactivation of latent disease. Immunosuppressive conditions can also influence the severity of the disease and the responsiveness to treatment. While immunosuppression has traditionally been found in HIV-infected individuals, also other non-HIV-related immunosuppressive conditions are becoming increasingly common worldwide. This is primarily due to improved medical care for patients with chronic illnesses, the therapeutic use of immunosuppressive drugs for autoimmune diseases [88] as well as after organ transplantation [91].

It has been demonstrated that HIV and *Leishmania* infections exacerbates each other virulence. HIV-infected patients are more likely to develop VL, either due to the reactivation of a dormant infection or as a clinical manifestation following the primary Leishmania infection [75]. Simultaneously, VL accelerates the clinical progression of HIV disease, and negatively impacts the immune recovery upon antiretroviral treatment [92]. Patients with these dual infections often exhibit disseminated parasites, and the infection proves challenging to cure [93].

## *5.4 Asymptomatic infection*

Clinical manifestation of the disease caused by *Leishmania* represent only a minor fraction of the total infections, while the majority of infected individuals do not manifest evident symptoms. Numerous prospective studies have highlighted the prevalence of asymptomatic infections compared to incident clinical cases. These studies suggests that many individuals infected with *Leishmania* develop an effective Th1-mediated immune response [94].

# 6. Epidemiology of leishmaniasis

Leishmaniasis still represents one of the world‘s most neglected diseases, with a total prevalence of 12 million of cases and rate of new cases per year estimated to be between 50,000 and 90,000 for VL and between 600,000 and 1 million for CL [3,95]. As reported by the World Health Organization (WHO), in 2021 leishmaniasis affected 4 eco-epidemiological regions of the world: the Americas, East Africa, North Africa and West and South-East Asia, where 99 of 200 countries, most of them of low- o middle- income countries, reported endemic cases. In these areas a population of 350 million people at risk of contracting the disease was estimated [2,96]. VL occurred for the majority of cases (90%) in 10 countries: Brazil, Ethiopia, Eritrea, India, Iraq, Kenya, Nepal, Somalia, Sudan and South Sudan; 80% of CL cases were identified in the WHO Eastern Mediterranean Region and 90% of MCL cases occurred in Bolivia, Brazil, Ethiopia and Peru [1,3].

Various factors impact leishmaniasis epidemiology, from environmental conditions to host characteristics. Environmental and climate conditions strongly influence vector distribution. Humidity and temperature have effects on the activity, reproduction rate and parasite development in sandflies, even small changes can significantly influence vector’s activity [5,97]. A particular phenomenon is the domestication of the zoonotic transmission cycle caused by urbanization and deforestation, which disrupt borders between sylvatic and anthropic areas [75]. Climate change is having a significant impact in the evolution of leishmaniasis’s epidemiological patterns in recent years, permitting colonization of new territories for the vector [75,98]. Some host characteristics correlate positively with higher risk of contracting leishmaniasis, both human behavior (such as poverty, living near sylvatic areas, habit to sleep outdoor and migration of peoples from non-endemic areas to endemic ones) and intrinsic characteristics such as malnutrition and immunosuppression conditions, with a particular emphasis on HIV coinfection [3,96]. Despite these high numbers,

incidence data are probably underestimated because of unrecognition and under notification of cases [99,100].

Both VL and TL are endemic in various countries of southern Europe: Spain, Portugal, Greece, France, Italy and the Balkans. As in other part of the world, incidence data are underestimated, particularly for CL; in fact, CL underreporting rate has been estimated to be 2.8–4.6 fold in the Mediterranean basin, and ML are often underrecognized by clinicians [99,101,102]. The parasitic species circulating in this area are *L. infantum* (cause of VL and TL) and *L. tropica* (cause of sporadic cases of CL in Greece) [5]. *L. donovani* cases were reported in Cyprus, but a precise taxonomical definition of this strain is still under debate [27,103]. Leishmaniasis in European region appears to be no more limited to the Mediterranean basin, various studies evidenced a northward spread of cases in northern Italian regions, further a colonization of sandflies and the first autochthonous cases in central European countries were also reported [5,104,105].

## *6.1 Epidemiology of leishmaniasis in Italy*

In Italy both human and canine leishmaniasis (CanL) are caused by *L. infantum;* this species is responsible for VL and TL in humans. *P. perniciosus* is the main vector in the Italian peninsula, but other sandfly species are also observed, such as *P. neglectus* (center- south of Italy and pre-Alps), *P. ariasi* (border with France) and *P. perfiliewi*, (north-eastern Italy) [106]. The main reservoir hosts in *L. infantum* zoonotic cycle are dogs [98], but recent studies evidenced the presence the parasites in rodents, lagomorphs and other species [107]. Until the 80's, VL incidence in Italy was low, with less than 40 cases/year, occurring in the classical endemic areas as the islands of Sicily and Sardinia, the southern part of the peninsula and the Tyrrhenian littoral [106]. Since 1990, a steadily increase of human VL incidence was observed, reaching 200 cases/year in 2000 [108]. In the same period, leishmaniasis spread towards northern provinces previously non endemic for the disease [98]. After 2000 leishmaniasis incidence raised among most of the historically endemic areas of the peninsula

with active foci in Tuscany around the province of Siena, in the area near Naples-Caserta in Campania region and in Sicily (province of Agrigento) [4,109-111].

Considering northeastern Italy, new stable focus of CanL was identified in the coastal Adriatic area of the Emilia-Romagna Region in 1998 [112]. After this event, in the last decade, an increased incidence of human leishmaniasis was observed, with a multiannual outbreak of VL in the province of Modena and Bologna, and a raised incidence of TL cases, which were characterized by presence of autochthonous ML cases [113-118].

# 7. Diagnosis and surveillance of human leishmaniasis

Microbiological diagnosis of leishmaniasis is essential because the symptoms are not distinctive, and other diseases can exhibit similar clinical presentation. To confirm a case of leishmaniasis, clinical suspicions must be supported by a positive result from parasitological and/or serological tests.

## *07.1 Diagnosis of visceral leishmaniasis*

VL must be suspected in subject affected by persistent fever, splenomegaly accompanied by cytopenia polyclonal hypergammaglobulinemia and residing in, originating from or travelling to endemic areas[96]. The differential diagnosis for these symptoms includes: malaria, systemic infections (such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV), brucellosis, and typhus), onco-hematological conditions and autoimmune diseases [75].

There is not a defined gold standard test for VL diagnosis [14]. In the past, microscopy of bone marrow or spleen aspirates has been favored showing variable sensitivity; higher for spleen biopsy (above 90%), than in bone marrow aspirates (50-80%) [6]. However, splenic sampling carries a 0.1% risk of fatal bleeding, especially in the presence of thrombocytopenia, often present in VL patients [9]. A bone marrow aspirate can also be used to exclude hematological conditions in the differential diagnosis. Parasite culture isolation can be performed but is rarely done due to long growth times and moderate sensitivity (60-85%) [14].

PCR assays have been progressively adopted, especially in Europe and the Americas. Molecular techniques show very high sensitivity (above 90% in peripheral blood and bone marrow aspirates) [119]. Yet, PCR might give positive results in some asymptomatic carriers, reducing its specificity. It requires a compatible clinical context and possibly other positive tests for confirmation. PCR is valuable for monitoring VL during and after treatment [120].

Katex, a test based on latex agglutination detecting *Leishmania* antigens, is highly specific but has low sensitivity (below 70%) and is mostly used in resource-limited contexts. It can be employed to monitor treated VL cases, as it becomes negative after successful treatment [6,9,96].

Serology is commonly used, showing moderate sensitivity and specificity. However, it cannot distinguish between active infection and past/asymptomatic infection and varies in performance depending on the geographical area. Moreover, since antibodies persist after treatment, serology is less useful in diagnosing VL relapses [6,14]. Western blot (WB), immunofluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA) are the preferred methods, although they are costly and complex. Direct agglutination test (DAT) exhibits good sensitivity and specificity but has some limitations such as overnight incubation and cross-reactivity issues. Rapid immunochromatographic test (ICT) based on the rk39 antigen show good performance, especially in the Indian subcontinent, but their sensitivity depends on the region, dropping in other areas like Eastern Africa and southern Europe. Combining ICT and ELISA can raise the performance of the serological approach in detection of VL [121].

## *7.2 Diagnosis of tegumentary leishmaniasis*

The suspicion of TL is typically based on clinical evaluation, but it is essential to confirm the diagnosis through parasitological methods since various other conditions share similar symptoms. These conditions may include bacterial infections, superinfections from arthropod bites, mycobacterial ulcers, cutaneous tuberculosis, leprosy, impetigo, fungal infections, and skin malignancies, among others [9,14]. On the other hand, particular forms of tegumentary leishmaniasis, such as MCL and PKDL often raise a higher level of clinical suspicion due to distinctive lesion characteristics and additional information from the patient's history (as previous episode of VL or travel in endemic areas for *L. Viannia* species [75].

Clinical tissue samples can be collected by scraping (for CL only) or by biopsy of the lesion. These samples can be processed in different ways; microscopic visualization of amastigotes in the sample is confirmatory [9] but the sensitivity of microscopy can be modest in conditions whit scarce parasites presence, like ML and PKDL [75].

Parasite detection based on PCR performed on tegumentary leishmaniasis samples shows excellent sensitivity and specificity. The employment of molecular methods in TL diagnosis is particularly important to obtain species identification, which is useful as different Leishmania species are associated with different

prognoses and outcomes [10]. The role of serology in TL diagnosis is generally limited, due to its low sensitivity [96].

## *7.3 Identification of species and strains of Leishmania*

Species identification cannot rely on microscopy nor on clinical features of the disease [10] Furthermore, surveillance of the circulating *Leishmania* populations is recommended as different *Leishmania* species could need different clinical management [122]. In addition, it is important to monitor the potential introduction of allochthonous *Leishmania* species/strains in new territories to allow a rapid public health response [123].

In this context, various assays have been designed for *Leishmania* species discrimination, and are in continuous development. Different methods can be grouped by the technology and/or biological target used [10].

### Multilocus enzyme electrophoresis (MLEE)

MLEE is still considered by the WHO and many others [75] to be the gold standard in *Leishmania* spp. typing. Being developed in the late 80's this method shows several remarkably disadvantages because it is laborious, time consuming, expensive and requires cultured parasites. In addition, MLEE suffers from a critical lack of resolution when compared to molecular based methods [124].

This technique is a biochemical characterization analyzing the pH-dependent electrophoretic mobility of a predetermined set of *Leishmania* proteins (generally around 10 or 15). Combined electrophoretic profiles of these targets represents a so-called zymodeme, which is used for species assignment [125-128]. Different laboratories designed different set of enzymes, and consequently identification systems, such as MON (Montpellier, France), widely used for classification of the *L. donovani* complex subspecies [11], LON (London, United Kingdom) [129], and IOC (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) [130]. As only a few laboratories nowadays persist in performing these analyses, and being MLEE a method that relies on parasite isolation and culture, MLEE is not suitable

as a typing method for an everyday use. In addition, more recent typing methods analyzing *Leishmania* DNA surpassed MLEE typing because they possess better *Leishmania spp* phylogenetic resolution and enable population genetic studies [10].

### PCR based methods

Most of the methods for species typing involve the use of PCR. Specific amplification reaction of *Leishmania* DNA sequence made these methods applicable directly on clinical samples with no need of parasite isolation. The technique can rely on either primers capable of amplify DNA targets of any *Leishmania* spp. or other more specifically designed for targeting a single or multiple species, species complexes, or subgenera. PCR products can be visualized and verified either by conventional agarose gel or by a real-time PCR (rt-PCR). Information on the amplified sequence obtained by PCR can be obtained by RFLP (restriction fragment length polymorphism) or by sequencing , on the other hand rt-PCR can rely on the use of specific probes or the analysis of the melting curves to obtain information about the sequence (Figure 5) [10].

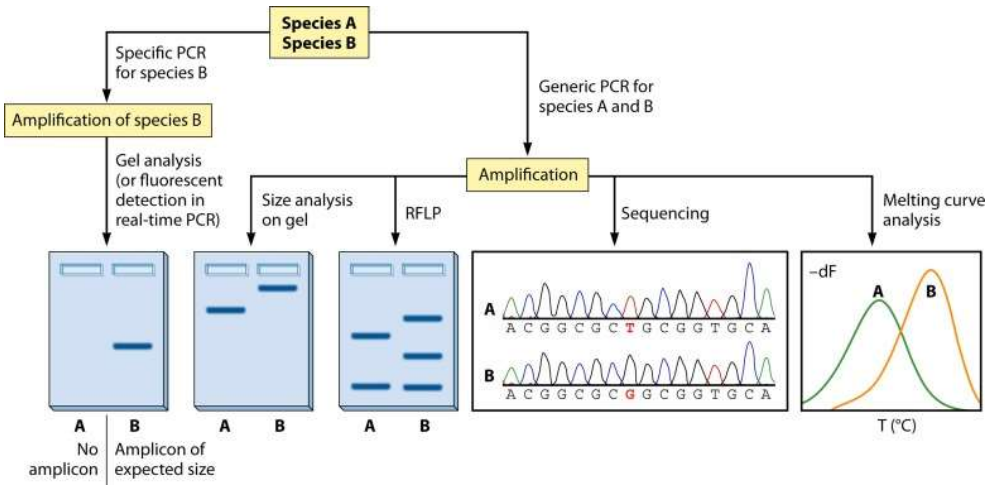


*Figure 5.* ***Schematic overview of common techniques to discriminate between two species****. Adapted from Van der Auwera G, Dujardin JC. 2015 [10].*

### rDNA array

rDNA sequence is one of the most popular target for species typing in leishmaniasis. It is estimated that the *Leishmania* genome display 10 to 20 copies of this sequence, revealing it suitable for direct amplification in clinical samples. Every repeated unit is composed of coding sequence and spacers [131,132], the second ones contain enough variability for species and subspecies typing [133,134].

Of the various designed PCR the one called LITSR-L5,8S developed by el Tai et al [134] and then applied for species identification via RFLP analysis of the ITS (internal transcribed spacer)-1 sequence by Shönian was proved able to discriminate between species of the *L. Leishmania* subgenus, but not between the ones of *L. Viannia* [135,136]. Other studies used this sequence to developed various typing PCR; one with a set of 4 fragments [137], one based on highly resolution melt analysis [138], another based exclusively on ITS2 fragment analysis [139].

### Kinetoplast DNA (kDNA) array

#### *Minicircles*

These are small molecules of circular DNA with average length of 800 base pairs and conserved region of around 150 bp. They are located into the kinetoplast, and thousand times repeated in each parasite making them a very sensitive target both for typing and diagnosis. However, minicircles within one strain are not all identical, and they can be divided into minicircle classes [40].

This variability cause difficulty in sequencing and assays identify *Leishmania* subgenera and species using size discrimination [140], hybridization with specific probes, or species-specific PCRs [38].

#### *Maxicircles*

Maxicircles are circular molecules larger than minicircles, located inside the kinetoplast and resembling mitochondrial genome [32]. Target typing sequence used is the cytochrome B gene (CytB). This target is repeated 25-30 time per cell, helping its use in analysis of clinical samples.

Sequencing of the coding region of CytB, which show no difference in size between species, allow to discriminate almost all species except *L. infantum*, *L. donovani*, *L. braziliensis* and *L. peruviana* [38,141,142].

### Antigen coding sequences

#### *Gp63*

Glycoprotein 63 (Gp63) is a metalloprotease whose gene is organized in tandem repeated units, comprehending the intra- and intergenic regions, used for RFLP analysis directly on clinical samples [143]. RFLP analysis was proven to be able to discriminate between all tested species, both from the New and Old World. On the other hand, in Old World species, intra and intergenic regions of this gene were primarily used for looking at intraspecies variability. One main seatback of this target is that it is impractical to be sequenced because of presence of gene variation within the same parasite [144,145].

#### *CPB*

Cysteine Proteinase B (CPB) gene is a multicopy target arranged in several copies in tandem. Not all these copies show identical sequences and are classified into various subgroups [146,147], and include coding and intergenic gene sequence. CPB was sequence analysis can be performed by RFLP analysis or by specific PCR for identification of one or few species [146,148].

#### *hsp70*

Heat shock protein 70 (hsp70) is a gene coding for a parasitic protein, with a role as molecular chaperone in protein folding and transport [149]. The gene is organized in tandem repeated units of 5 – 10 elements and show limited variation among strains [150]. Fraga et al analyzed the hsp70 coding sequence to study *Leishmania* spp. evolutionary pattern [151]. Garcia et al was the first developing a PCR-RLFP method based on a fragment of the hsp70 gene that was able to discriminate between all *Leishmania* species circulating in Brazil. Subsequently, Van der Auwera et al. sequenced and reported

over 200 hsp70 sequences from across the globe and from all medically relevant species [152,153]. From the hsp70 fragment used by Garcia et al, other studies designed more sensitive PCR using smaller fragments that were applied in Brazil, Peru, and several Old World countries (Figure 6) [154-156]. hsp70 typing results were observed to be nearly perfectly congruent with MLEE typing results, which makes hsp70 one of the most widely validated target for species typing [10].

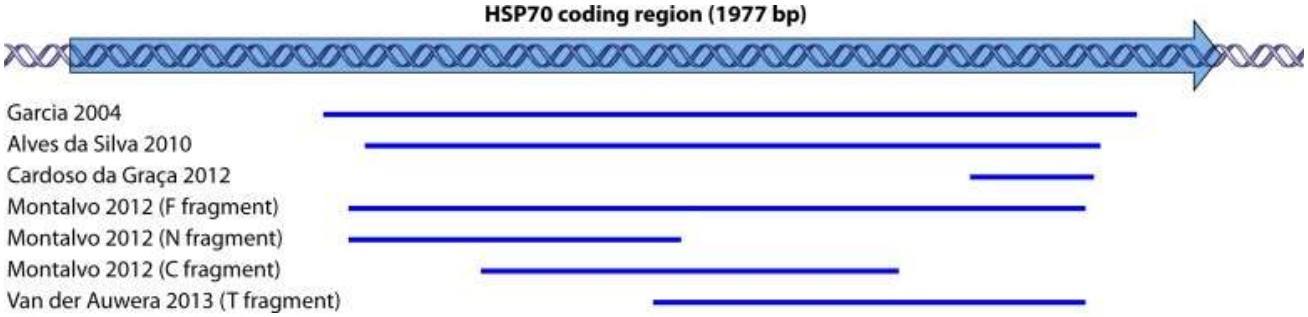


*Figure 6. Mapping of hsp70 fragments used in different studies to the complete coding region of the hsp70 gene (GenBank accession number XM001684512.1) (the arrow indicates the sense direction). These regions were used in either RFLP or sequence analysis. Adapted from Van der Auwera G. et al., 2015 [10].*

*Miniexon*

Also called "splicing leader" this sequence encodes for RNA motif added to the 5' end of each protein-encoding transcript during maturation. This sequence numbers 100-200 copies per genome making this target easily amplified even in clinical samples [157].

While it's true that certain groups of species can be differentiated solely by size, the use of RFLP analysis is still necessary for distinguishing between individual species [158]. Lately, Van der Auwera et al performed a sequence analysis of large number of strains from around the world, using the same PCR-amplified region. Their findings revealed that the miniexon was capable of distinguishing all species, identifying species (four of these species only at the complex level) from both the Old and New World [158].

7SL-RNA

The 7SL-RNA is sequence with a length ranging from 250 to 300 nucleotides, and it plays a crucial role in the translocation of proteins across the endoplasmic reticulum. Due to its significant variability and high abundance, Zelazny and colleagues suggested to perform sequencing of a 140-base pair PCR-amplified fragment for typing purposes. They conducted tests using this target on 30 strains, and their analysis was augmented by Van der Auwera and collaborators, who employed a larger sample of 71 isolates. The results showed that 7SL-RNA sequences could identify species complexes rather than individual species, except in the case of *L. major* and *L. lainsoni*, which could be typed individually [158,159].

Carbohydrate Metabolism Enzymes

Glucose-6-phosphate dehydrogenase (G6PDH or G6PD) was firstly used to distinguish between the subgenera *L. Viannia* and *L. Leishmania* and could distinguish different species of the *L. braziliensis* complex. Despite being less sensitive compared to other methods based on multicopy targets it was employed for clinical specimens [125].

Mannose-phosphate isomerase (MPI) has been employed in PCR assays to differentiate *L. peruviana* from *L. braziliensis*, either through a specific PCR or via RFLP analysis [10]. This assay can be useful as second step option when G6PDH analysis indicates the presence of *L. braziliensis* complex. Target amplification has been also applied directly to clinical samples [160].

Glucose phosphate isomerase (GPI) was employed to differentiate the *L. Viannia* subgenus and some species complexes within the *L. Leishmania* subgenus. Analysis of clinical samples is possible by using specific rt-PCR. However, GPI was not evaluated on all species, and the proper capability of analyzing intraspecies variability remains unclear [161]. Additionally, Weirather et al. employed this target in an evaluation of rt-PCR and melting assays [38].

Multilocus Typing

Combining different genomic targets offers a distinct advantage: it gives information from multiple genetic loci providing more precise evidence for the identification of a particular species or strain. This approach can take the form of a targeted strategy, involving the characterization of several predetermined loci, or a non-targeted one, where random genome variations are analyzed. However, these methods often have limited utility in species typing because they could generate excessive information, which may be redundant, while they are more suitable for population-based studies that aim to understand intraspecies variation. In the context of species typing, multilocus methods can be of great value for distinguishing species within the same species complex, such as *L. infantum-L. donovani , L. guyanensis-L. panamensis* and *L. braziliensis-L. peruviana* [162,163].

Techniques that use set predefined target approaches include: multilocus sequence typing (MLST), and multilocus microsatellite typing (MLMT).

MLEE can also be considered a multilocus typing technique without genomic data analysis. In this context, MLST, analyzing sets of housekeeping gene coding sequences, could be considered an upgraded version of MLEE, able to visualize sequence variations that do not affect enzyme electrophoretic mobility [164-166]. MLST is primarily employed for establishing the genus's phylogeny, but it is not suitable for application on clinical samples [167]. In fact, MLST relies mostly on single-copy genes, which limits its sensitivity. In addition, the cost and time required for amplifying and sequencing several genes in parallel are prohibitive.

MLMT analyzes various microsatellite regions across the genome. Microsatellites are regions with repetitive di- or trinucleotides motif. The variable number of repeats generates a fingerprint pattern used for typing and in population studies [24,168]. MLMT has been applied to clinical samples, but it necessitates multiple PCR amplifications and precise size analysis [169]. It is primarily suited for studying variability within one complex, as the microsatellite sequence diverge too rapidly for typing at the species complex level, and the markers are complex-dependent [25].

Non predefined target strategy used in *Leishmania* typing are represented by randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). RAPD analysis uses permissive primers hybridizing with various unspecified genomic loci under specific PCR conditions, generating a fingerprint pattern for species typing [170]. AFLP analysis rely on a selection of restriction endonuclease-generated genome fragments that once amplified, create a fingerprint pattern for species discrimination [171]. These untargeted methods are not suitable for typing clinical samples, as they are not *Leishmania*-specific and can amplify host DNA. Therefore, they can only be applied to axenically cultured parasites.

Some combinations of single locus assays described above have been proposed by various authors [172,173]. These methods can not be precisely defined as multilocus assays asthey are applied in a sequential manner, amplifying one distinct target in each step [174].

### WGS studies

Recently, next generation sequencing (NGS) technologies allowed to study *Leishmania* genomes in greater detail, consequently NGS has become an indispensable tool to evaluate the molecular and evolutionary biology of the parasite [175-177]. Among the genetic variations of Leishmania such as single nucleotide polymorphisms (SNP), CNV, aneuploidy, and structural variations, aneuploidy is one of the most striking features and occurs frequently in cultured promastigotes [177,178], while disomy is more common in amastigotes [179].

In this context, the advancement in NGS technologies and the presence of databases containing reference genome of most of the clinically relevant Leishmania species [180-182] render whole genome sequencing (WGS) data on this parasite accessible to a plethora of laboratories across the world [27]. This is allowing researchers to better understand Leishmania genome and to proper apply new sequencing techniques and phylogenetic targets to better describe the population genetic of *Leishmania* [39,183,184].

# 8. Treatment of leishmaniasis

Various compounds were proven to be effective against leishmaniasis however, most of them are associated with toxicity and difficult to use [185]. The presence of various *Leishmania* species with diverse clinical manifestations makes developing a universally effective therapeutic approach a significant challenge. While there are several anti-leishmanial agents available (Table 2), finding the optimal drug remains elusive. In fact, nearly all the existing compounds have several limitations, including high costs, severe side effects, toxicity, or a noticeable decrease in therapeutic effectiveness caused by drug resistant strains in some geographical areas [186,187].

*Table 2.* ***Chemotherapeutic agents for leishmaniasis****. Adapted from S. Pradhan et al 2022 [188].*

| Drug | Administration route | Dose | AEs | Advantages | Disadvantages |
|---|---|---|---|---|---|
| Pentavalent antimonial | IM, IV or IL | 20 mg/kg/day for 28–30 days | Cardiotoxicity, pancreatitis; nephrotoxicity; hepatotoxicity | Easy availability (in endemic areas); low cost | Prolonged treatment duration; pain during injection, toxic AE; drug resistance |
| Amphotericin B | IV | 0.75–1 mg/kg/day for 15–20 days, daily or alternate daily | Renal toxicity; injection related reactions; hypokalaemia | Primary resistance is not common | Requires hospitalization for administration; nephrotoxicity; heat; instability |
| Liposomal - amphotericin B | IV | 10–30 mg/kg total dose (single dose; 3–5 mg/ kg/dose) | Chills and rigors during injection; mild nephrotoxicity | High efficacy; low toxicity | High cost; need for slow IV infusion |
| Miltefosine | Oral | 100–150 mg/day for 28 day | GI AE; renal and liver toxicity; teratogenicity | Effective | High cost; possibly teratogenic; drug resistance; poor compliance |
| Paromomycin | IM (VL) or topical (CL) | 15 mg/day for 21 days or 20 mg/kg for 17 days | Renal, ear and liver toxicity | Effective; relatively cheap | Varied efficacy according to geographical area; potential for resistance |
| Pentamidine | IM | 3 mg/kg/day IM alternate daily for 4 injections | Hyperglycemia; hypotension; tachycardia; electrocardiographic changes | Short course needed | Efficacy depends on Leishmania species |

*AE, adverse effect; CL, cutaneous leishmaniasis; GI, gastrointestinal; IL, intralesional; IM, intramuscular; IV, intravenous; VL, visceral leishmaniasis.*

## *8.1 Pentavalent antimonial (SbV)*

Pentavalent antimonial (SbV) has a long history as the first choice for leishmaniasis treatment, dating back to its introduction by Gaspar Viannia in 1912. Sodium stibogluconate, which has been used since 1937, and meglumine antimoniate (commercially known as Glucantime®) are common antimonial drugs used in leishmaniasis treatment [189]. These molecules can be administered through intravenous, intramuscular, or intralesional injection [188]. Their mechanism of action is quite broad, it relies on the conversion to trivalent antimonial within the phagolysosome and amastigote cell, generating reactive oxygen species and affecting the redox balance of amastigotes, thus targeting molecules with sulfhydryl groups. They are also known to cause direct damage to the parasite's genome [190]. Clinically, the effectiveness of antimonial appears to rely on the presence of an effective cell-mediated immune response in the patient [191].

Despite their efficacy, antimonials exhibit various side effects, including anorexia, gastrointestinal symptoms such as nausea and vomiting, malaise, myalgia, arthralgia, and a metallic taste and toxic effect on heart, liver, pancreas, and kidneys, cytopenia has also been reported [192]. In HIV-coinfected individuals, the toxic effects of antimonials are more pronounced, making their use less advisable when alternative treatment options are available [88]. These drugs are primarily excreted by the kidneys, and their dosage should be adjusted when the glomerular filtration rate (GFR) is below 15 ml/min. Additionally, they are metabolized by hepatic cytochrome P450 and can potentially increase serum levels of cyclosporin, an immunosuppressive drug [193].

It's important to note that resistance to antimonials is well-documented, in example emergence of large-scale antimony resistance in North Bihar, India, has been the most outstanding development in recent years, where now SbV therapy treats successfully only 35% of patients in this region [190,194].

## *8.2 Amphotericin B (AmB) and Liposomal -amphotericin B (L-AmB)*

Amphotericin B (AmB) is a polyene antibiotic primarily developed as an antifungal drug, but that also possesses excellent antileishmanial activity. Its mechanism of action is based on its high affinity for 24-substituted sterols found in the membranes of *Leishmania* parasites. This affinity results in the formation of aqueous pores in the parasite membrane, which leads to increased membrane permeability and ultimately causes the destruction of the *Leishmania* parasites. This property makes AmB an effective treatment option for leishmaniasis [195]. AmB, however, also binds human cholesterol, albeit with a lower affinity, and is therefore highly toxic in its conventional formulation [189].

Since the 1980s, lipid formulations of AmB have been introduced, and the most successful among them is liposomal AmB (L-AmB) [196]. Liposomes are phospholipid nanocapsules that enclose an aqueous space, and they can be used to deliver both hydrophilic and hydrophobic substances. L-AmB is more effective and significantly less toxic than conventional AmB [189].

L-AmB is now considered the primary choice among anti-leishmanial drugs, especially for visceral leishmaniasis (VL), and the only WHO-approved preparation is AmBisome® (Gilead Sciences; San Dimas, California, United States of America). However, it is important to note that L-AmB availability can be limited in low-resource settings due to the high costs [196]. L-AmB is administered *via* slow intravenous infusion over a 2-hour period [96]. Side effects can include chills, fever, sometimes as an infusion-related reaction, as well as potential impairment of renal function., Despite a case of L-AmB resistant strain of *L. donovani* was reported in India [197]. L-AmB resistance is considered at very low risk, even in fungal infection [198], this makes L-AmB a crucial treatment option, especially for cases of leishmaniasis where other medications may be less effective or more toxic [199].

## *8.3 Miltefosine*

Miltefosine is an alkyl phospholipid and was developed as an antitumor agent [96]. Oral formulation used for this drug shows relative safety and good efficacy. These features made miltefosine one of the favorites first- and second-line treatment in leishmaniasis [189]. Studies on topical formulation of this drug have also shown promising results as TL treatment [200].

Miltefosine possible mechanism of action could be related to impairment of the lipid metabolism of *Leishmania,* but stimulation of the nitric oxide synthetase 2 in the host's macrophages was also observed, thus facilitating the killing of amastigotes inside the macrophage [189].

The most frequently reported side effects of miltefosine treatment are mild gastrointestinal symptoms such as nausea and vomiting, which usually diminish as the treatment progresses. However, miltefosine has been confirmed to be teratogenic. Consequently, it is contraindicated during pregnancy and lactation, and caution should be exercised when prescribing it to women of childbearing age [188].

## *8.4 Paromomycin*

Paromomycin is an aminoglycoside molecule with a notably wide-ranging antiparasitic activity. It can be administered through various routes, including topical application, intravenous or intramuscular injection [188]. Mechanism of action exerts by binding to ribosomal proteins and inhibition of endocytic pathway trafficking, leading to the disruption of protein synthesis [201].

In the VL treatment, paromomycin is often used in combination with antimonial drugs [202]. This drug shows good effectiveness, although it may vary depending on the geographical area, with higher efficacy typically observed in the Indian Subcontinent compared to Eastern Africa. Consequently, paromomycin is rarely utilized, if at all available, outside of these specific regions [196]. In addition, paromomycin is the most cost-effective among anti-leishmanial drugs [92]. Adverse reactions to paromomycin are infrequent but can be potentially serious. These reactions primarily involve

ototoxicity (damage to the ear) and nephrotoxicity (kidney damage) [188]. Resistance to paromomycin can be experimentally induced in vitro, but no cases of resistance have been reported in vivo so far [14].

### *8.5 Pentamidine*

Pentamidine is an aromatic diamidine with a wide-ranging spectrum of activities, including antiprotozoal, antifungal, antibacterial, antiviral, and antitumoral properties. Its mechanism of action against protozoa involves various sites: RNA polymerase activity inhibition and prevents the synthesis of protein, nucleic acids, phospholipids, and folate. Additionally, it is known to be an anti-inflammatory agent, xenobiotic, and an antagonist of the N-methyl-D-aspartate (NMDA) receptor, histone acetyltransferase, and calmodulin [203].

Introduced as a second-line treatment in 1952, pentamidine use is now discouraged when other alternative treatments are available due to its significant toxicity and declining efficacy as a result of increasing resistance. Adverse effects associated with pentamidine include hypoglycemia, hypotension, myocarditis, and renal toxicity. An important toxic effect linked to pentamidine is the development of irreversible diabetes mellitus, observed in 4-12% of cases [188,203]. However, its use has recently been proposed in Eastern Africa, in combination with other anti-leishmanial agents [204], as well as for the treatment of tegumentary leishmaniasis in Europe [90].

### *8.6 Fluconazole*

*Fluco*nazole is part of the family of the azoles, like ketoconazole and triazole. This molecule exerts it antileishmanial effect by inhibiting the ergosterol synthesis [195].

A double-blind, placebo-controlled trial conducted in Saudi Arabia shed light on the potential role of fluconazole as a treatment for CL caused by *L. major* [205]. Other studies asserted its efficacy against *L. braziliensis* and *L. infantum* [206,207] .

## *8.7 Treatment of VL*

For over six decades, intravenous administration of antimonial drugs has been the standard treatment for VL [193]. However, at present time, L-AmB is regarded as the most effective and safe treatment for VL, making it the first-choice treatment. Nevertheless, the actual effectiveness of individual drugs can vary depending on the geographic region, leading to differences in treatment guidelines from one region to another [75].

L-AmB has been highly effective in the Indian subcontinent, with a cure rate of 99% reported using a total dose of 20 mg/kg, administered in 4 doses over 4-10 days

In contrast, L-AmB is less effective in Eastern Africa, where higher doses (30-40 mg/kg total dose) may be required for successful treatment. In Eastern Africa, where L-AmB may not always be available, other therapeutic regimens, including drug combinations, have been studied. For instance, a combination therapy of antimonials and paromomycin (20+15 mg/kg/day for 17 days) has shown promising results [196].

The treatment guidelines for the WHO European Region recommend a total L-AmB dose of 18-21 mg/kg over 3-6 days. Second-line options include intramuscular antimonials (20 mg/kg/day for 28 days) or intravenous amphotericin B deoxycholate (1 mg/kg/day, daily or on alternate days for 20-30 doses). Drug resistance in Europe is rare [14].

## *8.8 Treatment of TL*

The treatment of TL depends on the severity of the condition and the specific type of TL and/ or causing species [208].

Mild cases of CL caused by Old World species could heal spontaneously, however self-healing of the lesion can lead to scar [14]. In case of localized accessible lesion, treatment can be based on cryotherapy and intralesional antimonial or topical paromomycin [14].

CL caused by New World Leishmania spp. show low tendency to heal spontaneously, differently to Old World CL [14]. Systemic treatment is always indicated in case of MCL, ML, severe CL (defined when the lesion is larger than 4 cm, is a plaque or we found more than 4 lesion at the same time) and if the patient is immunocompromised [9,14].

# Aims of the study

Human leishmaniasis is recently reemerging in focal areas of southern Europe. However, this parasitic disease is still neglected with limited resources invested in diagnosis, treatment and control measures [5]. TL, particularly, the most common form of the disease, is often misdiagnosed and /or unnotified; the underreporting rate has been estimated to be 2.8–4.6 fold in the Mediterranean basin [101,102].

In recent years, multiple epidemiological foci of leishmaniasis were observed in various provinces of the Emilia- Romagna region (RER) [209]. Particularly, a twelve-fold increase of TL incidence was registered in the province of Bologna in 2013-2015 in comparison to 2008-2012 years [113] and autochthonous ML cases were identified in the last decade [90]. This upsurge of human leishmaniasis cases in RER led to the establishment of a regional reference laboratory (RRL) to confirm human cases by molecular methods within the Unit of Microbiology of the University Hospital of Bologna [210,211].

In this scenario, the aims of this project were:

to analyze the incidence, the clinical features, and the geographic distribution of TL cases in the RER. This was achieved by employing data collected from RRL and from a newly developed regional network of specialists.

to perform a molecular surveillance of TL cases that were diagnosed in the RER, allowing to distinguish *Leishmania* populations by employing two genetic markers.

to phylogenetically characterize dermotropic strains of *L. infantum* circulating in northeastern and central Italy by NGS.

# Materials and methods

## 1. Study I

### *1.1 Study design*

In study I, we collected data regarding all TL cases diagnosed in RER from 2017 to 2020. The aim of the study was to evaluate the real prevalence of the disease in the region and it's clinical and epidemiological features. Data collection was performed through the Skin_Leish_RER_network, a newly formed multicentric network connecting 10 diagnostic units covering the regional area and coordinated by the RRL in the U.O. Microbiology of the Sant' Orsola-Malpighi Hospital.

### *1.2 Study area*

Area of the study coincide with the RER. The region location is in northeastern Italy and covers 22,445 $km^2$ [212]. Inside RER resides 4,438,937 inhabitants with a population density of 197.63 inhabitants/$km^2$ [213]. Regional area is formed by the Apennine Mountains (5677 km2; 25%), hills (6202 km2; 27%) and an alluvial plain (10,573 km2; 48%).

### *1.3 Case definition*

TL case definition was based on the WHO directives [7,14]: presence of suggestive cutaneous and/or mucosal lesion/s, detection of Leishmania by histology and/or by molecular methods. Definition of different TL types was based on following criteria established by the European LeishMan consortium [7]; CL refers to the presence of skin lesions, ML refers to the presence of mucosal lesion(s) without skin involvement, MCL refers to the simultaneous presence of both mucosal and skin lesions. Minor/mild disease was defined when lesions (papules or nodules) were less than 4, and small (<4 cm diameter), on contrary severe disease was characterized by presence of plaque/s and/or 4 or more lesions were present on the same patient and/or lesion large 4 cm or more [14]. Presence of ML

always identify the case as severe. Successful lesion healing was defined as complete disappearance of induration for a popular lesion or re-epithelialization in case of ulcer [7]. On contrary treatment failure events were defined as relapse or persistence of lesions after 6-month treatment. Patients were usually followed up for 1 year after treatment. Possible origin of TL cases clustered into three groups: (i) autochthonous, when the place of infection was within RER; (ii) Italian extra-RER, when the place of infection was in Italy but outside RER; and (iii) imported, when the place of infection was outside Italy.

### *1.4 Surveillance of TL in Italy and RER*

Notification of human leishmaniasis mandatory in Italy through with infectious disease reporting system, which report notified cases to the national Ministry of Health [99,102]. Physicians are required to report all confirmed cases of leishmaniasis to the public health service of the Local Health Unit (LHU). Epidemiological investigations and data collection related to the infectious event are carried out by the LHU; these are subsequently communicated to the regional health authorities and to the Ministry of Health [214]. Following the RER surveillance program for leishmaniasis [210,211], TL molecular confirmation is performed by the RRL, which is located within the Microbiology Unit of the University Hospital of Bologna. RRL receives biopsy samples from dermatologists, infectious disease specialists, and/or pathologists all over RER.

### *1.5 Skin_Leish_RER network*

The Skin_Leish_RER network was established to enhance the surveillance of TL cases within the RER region. Through the network centralized collection of TL epidemiological data, clinical information, as well as data regarding anti-leishmanial therapy and patient outcomes. It consists of ten diagnostic units Piacenza, Parma, Reggio-Emilia, Modena, Bologna, Ferrara, Ravenna, Forlì, Cesena, and Rimini, each of which included specialists in Parasitology, Dermatology, Infectious

Diseases, and Pathology (Table 3). The Skin_Leish_RER network is coordinated by the diagnostic unit at the University Hospital of Bologna, which comprises the RRL and the Dermatology Unit.

*Table 3.Network of diagnostic centers for the surveillance of tegumentary leishmaniasis, Emilia-Romagna region (northeastern Italy), 2017-2020. Adapted from Gaspari et al 2022 [117].*

| Diagnostic Unit | Hospital Name | Number of cases |
|---|---|---|
| Piacenza | Azienda USL di Piacenza-Ospedale Civile Piacenza | 1 |
| Parma | Azienda Ospedaliero-Universitaria di Parma | 13 |
| Reggio Emilia | Azienda USL IRCCS Reggio Emilia-Arcispedale Santa Maria Nuova | 10 |
| Modena | Azienda Ospedaliero-Universitaria Policlinico di Modena | 40 |
| Bologna | Università di Bologna-Ospedale S.Orsola-Malpighi | 35 |
| Ferrara | Azienda Ospedaliero-Universitaria di Ferrara | 0 |
| Ravenna | Azienda USL della Romagna-Ospedale di Ravenna | 5 |
| Forlì | Azienda USL della Romagna-Ospedale Morgagni Forlì | 6 |
| Cesena | Azienda USL della Romagna-Ospedale Bufalini di Cesena | 12 |
| Rimini | Azienda USL della Romagna-Ospedale di Rimini | 13 |
| Total number of cases, 2017-2020 | | 135 |

## *1.6 Diagnosis of TL*

Histological and molecular diagnosis were conducted on skin or mucosal biopsies. For histological examination, biopsies were formalin-fixed and paraffin-embedded (FFPE) routinely. Sections wide from five- to six-µm sections were obtained from the block and histochemical staining were performed with haematoxylin and eosin (HE) to observe leishmanial amastigotes. Slides observation was performed by objective magnification 10x, 40x or 60x and ocular magnification 10x, respectively. In case of necessity, a Giemsa stain was evaluated in addition to HE. Molecular diagnosis was performed on FFPE at RRL. DNA was extracted by using NucleoSpin DNA FFPE XS

kit (Macherey-Nagel, Duren, Germany). Real-time PCR was performed by using two in-house assays, one specific for a part of the small-subunit ribosomal RNA (rRNA) gene [215] and the other for the conserved region of minicircle kDNA [120] (Table 4). A real-time PCR assay amplifying human β2-microglobulin was simultaneously run as amplification control of the extracted DNA. PCR reactions were carried on by using a CFX real-time PCR detection system (Bio-Rad, Hercules, CA, USA) or Rotor-Q system (Qiagen, Hilden, Germany). Primers and probes sequences are described in Table 4; thermal conditions were as follows: 95 °C for 2 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 1 min and fluorescence acquisition.

*Table 4.* ***Primers and probes used to identify Leishmania DNA in TL specimens.*** *Leishmania minicircles kDNA and Leishmania small-subunit rRNA gene primers and probes were respectively designed according to Mary et al [120] and Wortmann et al [215].*

| Sequence target | Primer/Probe name | Primer/Probe sequence |
|---|---|---|
| Leishmania minicircles kDNA | Fluorogenic probe **LEINF** | 5'-TTTTCGCAGAACGCCCCTACCCGC-3' |
| | Primer LEINF RV1 | 5'-CTTTTCTGGTCCTCCGGGTAGG-3' |
| | Primer LEINF RV2 | 5'-CCACCCGGCCCTATTTTACACCAA-3' |
| Leishmania small-subunit rRNA gene | Fluorogenic **probe LEIS** | 5'-CGGTTCGGTGTGTGGCGCC-3' |
| | Primer **LEIS U1** | 5'-AAGTGCTTTCCCATCGCAACT-3' |
| | Primer **LEIS U2** | 5'-GACGCACTAAACCCCTCCAA-3' |
| Human β2 microglobulin | Fluorogenic **probe β2M** | 5'-CCATGTGACTTTGTCACAGCCCAAGATAGTT-3' |
| | Primer **β2F** | 5'-TGAGTATGCCTGCCGTGTGA-3' |
| | Primer **β2R** | 5'-ACTCATACACAACTTTCAGCAGCTTAC-3' |

## *1.7 Data collection*

We collected all confirmed cases of TL (both CL and ML) diagnosed in the RER area and which biopsy samples collection was performed between January 1, 2017, and December 31, 2020. Data were gathered by the national notification system (SMI) and by the retrieval activity of the Skin_Leish_RER network. Once collected information were organized in an electronic database and

shared with network members through a password-protected platform. Patient-sensitive information in this study was made anonymous by using an alphanumeric code. The study was conducted in compliance with the Declaration of Helsinki, and the research protocol received approval from the Ethics Committee of the Area Vasta Emilia Centro (study number: EM1008-2021_97/2017/O/Tess/AOUBo).

### *1.8 Statistical analysis*

Descriptive analysis of TL cases was conducted by considering factors as the year of diagnosis, notification to LHU, case origin (autochthonous or imported), type of leishmaniasis, gender, age, molecular confirmation carried out by RRL, clinical presentation, location of lesions, the number of lesions, and the presence of immunosuppressive conditions. For autochthonous cases, we also calculated the annual incidence rate. Additionally, we examined the seasonality of symptom onset and assessed diagnostic delays. All data analyses were conducted using Ms Excel 2017 and R 4.0.2 [216]. The spatial distribution of autochthonous cases was visualized using QGis (version 3.14.15-Pi).

# 2. Study II

## *2.1 Study design*

In study IIa we typed 103 TL cases diagnosed from 2014 to 2020 by sequencing 2 targets ITS1 and hsp70, both useful for typing of leishmania at species level. Hsp70 sequence results of autochthonous cases was then analyzed by BLAST search and then confirmed by in in-depth analysis of the same region from NGS sequences of 151 *L. donovani complex* strains obtained by Franssen et al in 2020.

## *2.2 Study area*

The area where study IIa was conducted coincides with the RER territories described in study I.

## *2.3 Sample collection*

A total number of 109 samples were collected between January 1, 2014, and December 31, 2020, were included in this study. These samples consisted of five to six μm-wide sections FFPE biopsies of mucosal or skin tissues obtained from 104 patients with confirmed diagnosis of TL. Samples were acquired as part of the diagnostic activities conducted by the RRL and stored at -20°C until the molecular analyses were performed. Demographic and clinical data of TL cases were collected through the Skin_Leish_RER network. The data were systematically organized in an electronic database shared with network members through a password-protected platform. Patient information in this study was anonymized using an alphanumerical code. The study adhered to the principles of the Declaration of Helsinki, and its protocol received approval from the Ethics Committee of the Area Vasta Emilia Centro (study number: EM1008-2021_97/2017/O/Tess/AOUBo).

## *2.4 Case definition*

TL case identification, definition of TL clinical form and severity of the disease used in study IIa coincides with the ones described in study I. Cases were considered potentially imported only when

presenting history of travel in countries that are endemic for leishmaniasis in the 12 months before lesions' onset, on contrary the case was considered as autochthonous.

### *2.5 DNA extraction, amplification and sequencing*

DNA was extracted from FFPE biopsies by using NucleoSpin ® DNA FFPE XS kit (Macherey-Nagel, Duren, Germany) or Maxwell "RSC/CSC Decktray" kit Maxwell® CSC DNA FFPE Kit, following to the manufacturer's instructions. Verification of Leishmania DNA presence in samples was performed by an in-house Real-Time PCR assay targeting two regions of the minicircle kDNA [120] and of small-subunit rRNA gene [215] as described in study I.

Amplification of the ITS1 fragment (320 bp) was performed in a PCR final volume of 30 µL using the primer pairs LITSR/L5.8S and the mastermix GoTaq® G2 Hot Start Colorless (Promega, Madison, Wisconsin, United States), following the amplification protocols as described by El Tai et al [217] with some modifications: 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, with a final elongation at 72 °C for 5 min. In the case of no amplification, a second ITS1 amplification round was performed using the same primer set as above. A no template control (NTC) was run along the amplification reaction to check the absence of carryover contamination. Primers used for ITS1 amplification are described in Table 5.

*Table 5. Sequences of primers that were used for amplification and sequencing of ITS1 fragments. Primers were described by El Tai et al [217].*

| Sequence target | Primer Name | Primer sequence | Amplicon size |
|---|---|---|---|
| ITS1 Fragment | LITSR | 5'_CTGGATCATTTTCCGATG_3' | 300-350 bp |
| | L5.8S | 5'_TGATACCACTTATCGCACTT_3' | |

hsp70 coding sequence fragment and PCR design was optimized for its application on FFPE sections. Thus was performed using primers to 3 fragment (N-, P- and Ps) described in Table 6 in a three-step nested-PCR (Figure 7). Amplification of N-fragment was previously described [152], while smaller

P and Ps primer sets were in house designed to increase PCR sensitivity also in FFPE samples often characterized by scarce or damaged parasitic DNA. The Ps smaller fragment sequence presents in their sequence yet described variable sites distinguishing among *L. infantum*, *L. donovani*, *L. major* and *L.tropica* complex. PCR reaction was performed by Thermocycler T-Gradient ThermoBlock (Biometra, Göttingen, Germany) and mix preparation by the HotStarTaq plus kit mix (Qiagen, Hilden, Germany) according to the manufacturer's condition and amplification protocols as described by [152]. In case no evident amplification for the N-fragment (593 bp) was observed, the N-fragment PCR product was purified with Exosap® (Thermofisher, Waltham, Massachusetts, USA) following the manufacturer's instructions and then used as a template for a nested PCR reaction by using P-fragment primers (Table 6; Figure 7) to obtain a shorter amplicon of 295 bp. If still no amplification was observed, the P-fragment PCR reaction was purified with Exosap® and run as template for the amplification of the internal "Ps-fragment" of 262 bp. To ensure absence of cross contamination for hsp70 double-nested PCR, an NTC sample was run alongside samples for every amplification reaction.

*Table 6. hsp70 primers used for amplification and sequencing of hsp70 fragments.*

| hsp70 fragments | Primer Name | Primer sequence | Position* | Amplicon Size |
|---|---|---|---|---|
| N-fragment | F25_var (Sense) | 5'_GGACGCCGGCACGATT**G**CT_3' | 480 – 498 | 593 bp |
| | R617_var (Antisense) | 5'_CGAAGAAGTCCGA**C**ACGAGGGA_3' | 1072 – 1051 | |
| P-fragment | F-paraf (Sense) | 5'_GGACTTCGACAACCGCCTC_3' | 699– 717 | 295 bp |
| | R-paraf (antisense) | 5'_CTTGTCCATCTTCGCGTCCT_3' | 993– 974 | |
| Ps-fragment | For-paraf-s (forward) | 5'_CGTCACGTTCTTCACCGAGGAGT_3' | 717- 739 | 262 bp |
| | Rev-paraf-s (reverse) | 5'_GTCCTGCAGCACGCGCTCCAC_3' | 978-958 | |

** Annealing position in GenBank accession number: XM_001470287.1_inf_LLM-877_Leishmania infantum JPCM5, according to [152]. N-fragment primers set were designed according to [152] with modification (in bold).The sets of primers in the grey shaded rows are new primers, designed using the Primer Express® software v.3.0 (Applied Biosystems, Foster City, CA, USA). In silico sensitivity and specificity were evaluated on a panel of Leishmania and other Trypanosomatidae hsp70 sequences available from GenBank.*

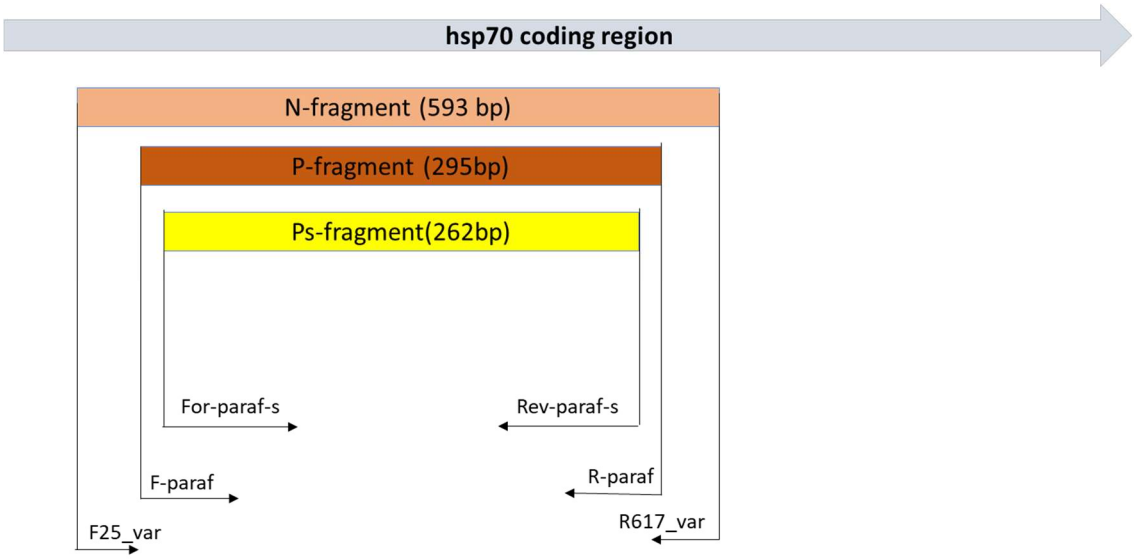


*Figure 7. Primers used and amplification products obtained and sequenced within the hsp70 coding region.*

Sequencing reaction, both for ITS1 and hsp70 amplicons (N-, P- or Ps-fragment) were purified with the Agencourt AMPureXP PCR Purification Kit (Beckman Coulter™, Indianapolis, IN, United States). Sanger sequencing reactions were performed in both 5' and 3' directions. This was performed

by using GenomeLab DTCS Quick Start Kit (Beckman Coulter) in a final volume of 20 µL following manufacturer's conditions. Sequencing primers used were the same amplified the analyzed amplicons (Table 6). Sanger reaction was performed with a Sciex GenomeLabTM GeXP sequencer (Beckman Coulter).

## *2.6 Whole genome sequenced (WGS)-based analysis of hsp70 gene*

NGS reads of 37 *L. infantum* isolates were obtained from Franssen et al. [27]. Sequence reads were trimmed with Trimmomatic (https://github.com/usadellab/Trimmomatic) [218], also paired-ends adaptors removal was performed by using Trimmomatic option: 'ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 TRAILING:25 MINLEN:60'. Trimmed reads were mapped confronting them with gDNA of the reference strain, JPCM5 (MCAN/ES/98/LLM-724) of *L. infantum* [184] (TriTrypDB v65 (https://tritrypdb.org) by BWA v0.7.17 (https://github.com/lh3/bwa) [219] using the bwa-mem aligner and default options. Single nucleotide polymorphisms (SNP) and InDel variants were called using BCFtools v1.16 utilities (https://github.com/samtools/bcftools) [220]. First vcf files were produced with bcftools mpileup, then variants calling was performed with bcftools call. InDels were normalized with bcftools norm (default options). A reference-based assembly with bcftools consensus and -H I option (use IUPAC code for all genotypes) was performed using all detected variants in the vcf format to JPCM5 LINF_280036000 hsp70 coding sequence (LinJ.28:1112868-1114832, TriTrypDB v65).

## *2.7 Statistical analysis*

hsp70 and ITS1 sequence results were analyzed and assembled using the "Sequencing" and "Investigator" packages of GenomeLabTM System software, version 11.0.24. Obtained consensus sequences identity within *Leishmania* spp. was conducted by a Blast search in Gen Bank at the National Center for Biotechnology Information (NCBI). Statistical significance of differences

between categorical variables were analyzed using Chi-square test. Differences were considered significant for p-value $< 0.05$.

# 3. Study III

## *3.1 Study design*

In study III we performed an NGS sequencing by using Oxford nanopore technologies (ONT, Oxford, UK) *Leishmania* isolates from the center and north-east of Italy. Following a study conceptualized by Solana et al [39], NGS data was precisely identify phylogenetic position of the strains respect other *L. infantum* strains confronting maxicircles conserved region sequences. The study was performed in collaboration with the WHO Collaborating Centre for Leishmaniasis National Center of Microbiology Institute of Health Carlos III, Madrid, Spain.

## *3.2 Study area*

The two isolates were obtained from patients residing in different areas; the isolate MHOM/IT/2021/IZSLER-MO44 (MO44) was obtained from a patient residing in Bologna (RER, northeastern Italy, while the isolate MHOM/IT/2019/cur-1 (cur-1) was obtained from a patient residing in the Pesaro-Urbino (central Italy).

## *3.3 Sample and parasite's isolation and culture condition*

*Leishmania* parasites were isolated form biopic specimens of infected skin or mucosal tissues. Biopsied tissues were placed in sterile liquid Evans' Modified Tobie's Medium (EMTM) and disrupted by pipetting, then a 0.5 mL volume of tissue suspension was added to semisolid EMTM and incubated at 26 C°. After 5-7 days parasite isolation was verified by direct observation of the promastigotes by using inverted microscope at magnification x20.

Once parasites isolation was confirmed, 0.2 mL of promastigote suspension was transferred into a flask containing 5 mL of hemoflagellate-modified minimum essential medium (HOMEM, Gibco Thermo Fisher Scientific Inc., Waltham, USA) supplemented with 20% of Fetal Bovine Serum (FBS, EuroClone SpA, Milan, Italy) and 1% of penicillin streptomycin (EuroClone SpA) for parasite

cultivation. In such conditions promastigotes reach a stationary phase every 6-7 days, after that a small culture aliquot was transferred in fresh medium. Leishmania parasites used in the study were isolated by two TL cases occurred in the northeast of Italy.

The first, MO44, was isolated in February 2021 from a case of ML recurrence. The lesion origins from a primary ML lesion appeared in nose cavity in 2016 and apparently cured after multiple treatments described by Gaspari et al [90] (Figure 8a). The second, cur-1, was kindly provided by prof. Galluzzi (University of Urbino). This isolate was obtained from a case of CL that was resistant to treatment with antimonials. Accurate description of the strain is reported in Diotallevi et al [221] (Figure 8b).

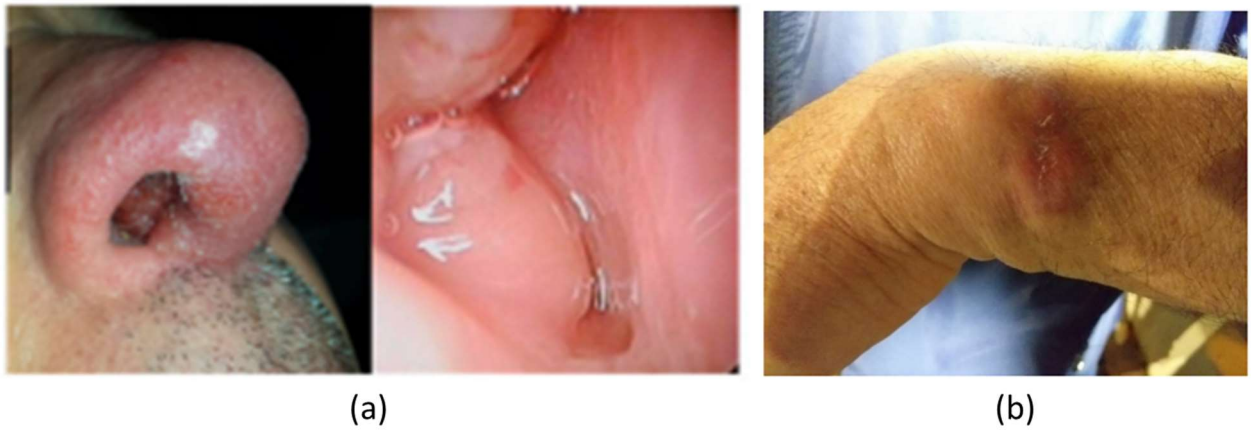


*Figure 8. Photograph of the TL lesions from which originated the two Leishmania isolates in the north-east of Italy.(a) Clinical and endoscopic appearance of the autochthonous ML case from the province of Bologna in 2016. MHOM/IT/2021/MO44 was isolated from recurrence of this lesion in 2021. Adapted from Gaspari et al 2020. (b) Dorsal forearm skin lesion on the patient with CL from the province of Pesaro-Urbino, twelve weeks after the beginning of treatment. MHOM/IT/2019/cur-1 was isolated from the presented lesion.*

## *3.4 DNA extraction and purification*

DNA extraction was performed on 5-20 x 10^8 promastigotes in late logarithmic growth phase. The correct number of harvested promastigotes for the two strains was 6.8 x $10^{8}$ and 18x10^8 respectively for cur-1 and Mo-44. Once harvested, the parasite suspension was pelleted, washed twice in PBS, and then resuspended in 0.5 mL of Lysis buffer (NaCl 0.15 M, EDTA 0.1M, 0.5% SDS and pH 8) and 0.1 mg/mL of proteinase K. Lysis suspension was incubated at 50 °C for 4 hours.

After lysis, DNA extraction was performed in three purification steps by using in this order: 1 vol phenol, ½ phenol ½ f chloroform/isoamyl alcohol (24:1) and 1 vol ice-cold chloroform/isoamyl alcohol (24:1).

Aqueous phase from last purification step was recovered and purified. Suspended DNA into aqueous phase was precipitated by adding 0.1 equivalent volume of Sodium Acetate 3 M pH 8 and 2.5 equivalent volumes of ice cold pure EtOH. Condensed visible DNA strands were taken up and washed in ice-cold 70% EtOH, then centrifuged and air dried. Once dried, genomic DNA (gDNA) was redissolved in 100 µL of $H_2O$. Purification quality and concentration was assumed by electrophoretic run-in gel agarose 0.8% and NanoDrop™ One (ThermoFischer Scientific, MA, USA) Quantus™ fluorometer (Promega, WI, USA). Good DNA concentration but RNA contamination was observed, so gDNA was redissolved in RNAse buffer (TrisHCl pH 7.5 + 15 mM NaCl, boiled 10 min at 100ºC to inactivate DNases) and incubated 65°C for 10 minutes for RNA denaturation. After that RNAse 20 µg/ml was added and solution was incubated 30 min at 37 °C. Purified gDNA was extracted by using phenol:chloroform:isoamylalcohol (25:24:1), precipitation and washing steps were performed as above. gDNA was finally dissolved in 100 µl $H_20$, quantified by NanoDrop™ and Quantus™ fluorometer, and quality checked by electrophoretic run on gel agarose/TAE 0.8%.

### *3.5 Oxford nanopore DNA library preparation and sequencing*

Library preparation was performed by using Nanopore Ligation Sequencing Kit (SQK-LSK110) from by Oxford Nanopore Technologies according to manufacturer instruction (ONT, Oxford, UK). Library preparation was performed by using 1 µg of post-RNAse gDNA for both the samples according to the manufacturer's condition. During ligation reaction step the short-fragment buffer (SFB) was used to retain DNA fragments of all sizes.

Once prepared, library was loaded onto a flow cell device (Figure 9a), previously prepared and checked as suggested by manufacturer advice, for sequencing using the Mk1c (MinION, ONT) (Figure 9b) and run for around 24 hours for MO44 and 48 hours for cur-1.

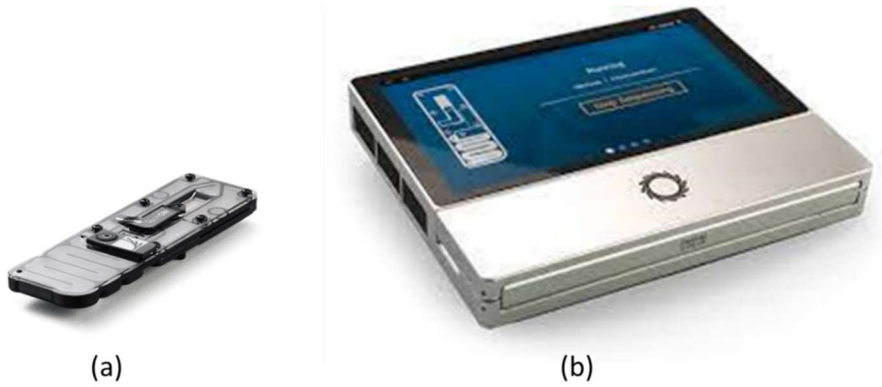


*Figure 9.* ***Oxford Nanopore Technologies devices used for NGS reaction****.(a) SpotON flow cell device (b) MinION sequencer.*

## *3.6 Maxicircles assembly*

Maxicircles assembly was performed following pipeline described by Chamacho E. et al [40] (Figure 10) with modifications. In brief, WGS data from sequenced *Leishmania spp.* strains were aligned against the reference nuclear genome using Bowtie2 to separate the non-aligned reads. *Leishmania* reference genomes assembly was retrieved in public databases: Trytrip [181] and Leish-ESP (http://leish-esp.cbm.uam.es/). Quality filtering/trimming of the so obtained unaligned reads (cut-off value, 20) and exclusion of fragments shorter than 60 nucleotides was performed by PrinseqQuality (http://prinseq.sourceforge.net/). In these conditions de novo assembly was performed by using the IDBA_UD (version 1.1.3) assembler [222], selecting parameters: --mink 20, --maxk 120, --min_support 1, --min_contig 500 and --pre_correction. Contigs of at least 500 nucleotides were then analyzed by NCBI-BLAST tool to obtain their sequence identity with sequences within maxicircle CR. The CR sequences were assessed by positions of the genes 12S rRNA (start) and ND5 (end), as reference we used the *L. infantum* maxicircle annotations from [40]. For cur-1 and Mo-44 reads, alignment against reference genome was performed using minimap2 (version 2.26), selecting

parameters specific for ONT long-reads [223]. Non-aligned reads were assembled using canu 2.0 [224] to obtain the maxicircle contigs.

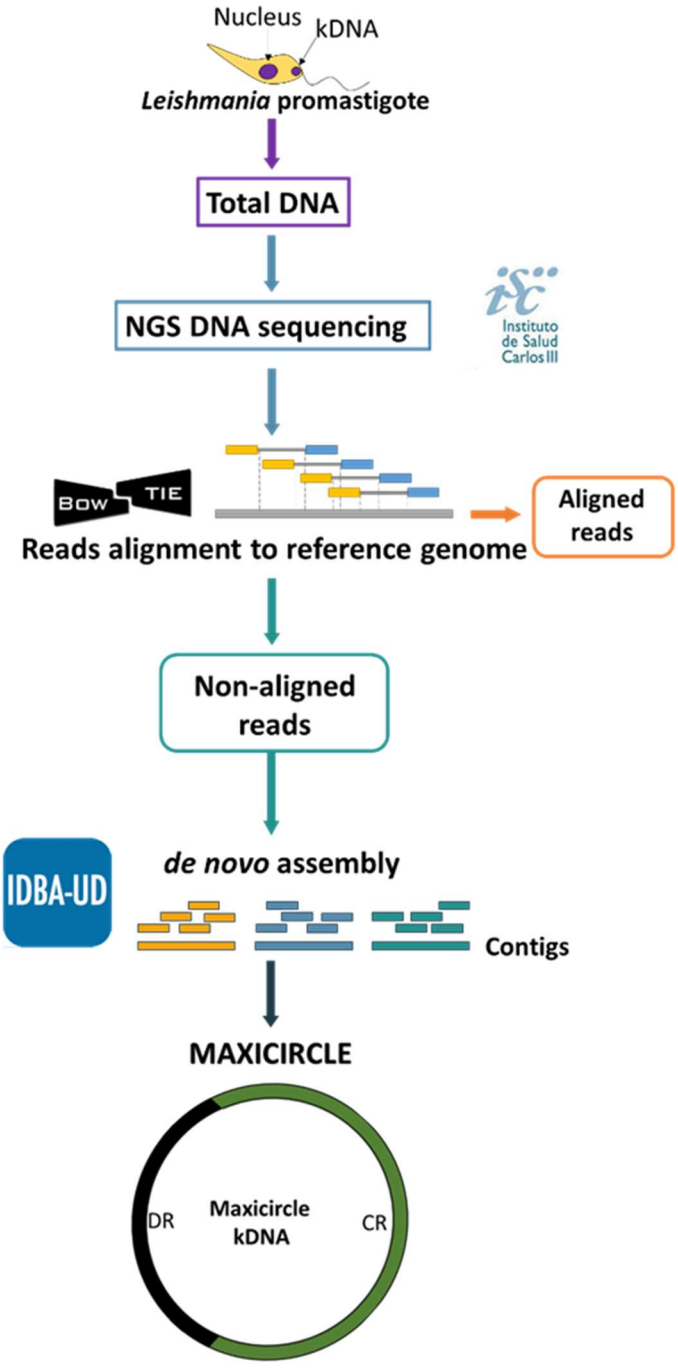


*Figure 10.* ***Pipeline diagram for the assembly of WGS data into maxicircle CR sequence****. Adapted from Camacho ester et al 2019 [40].*

## *3.7 Phylogenetic analysis*

Phylogenetic analysis of the studied strains was exclusively based on maxicircle CR sequence (Figure 11) and performed as indicated by Solana et al. [39]. Phylogenetic analysis and multiple alignment of sequences were performed by using MEGA11 and ClustalW 4 [225]. Bootstrap consensus tree and measurement of phylogenetic relationship of the examined taxa and number was performed by applying maximum likelihood method and Tamura-Nei model 5 [226], with 1000 replicates.

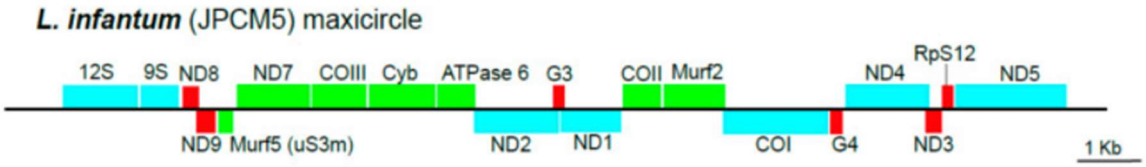


*Figure 11.* ***Schematic diagram of L. infantum (JPCM5) maxicircle conserved region (CR).*** *Adapted from Solana et al 2022 [39].*

# Results

## 1. Study I

The original article was published in Int J Environ Res Public Health. 2022 Nov 30;19(23):16047. doi: 10.3390/ijerph192316047 (PMID: 36498130; PMCID: PMC9740434.)

### *1.1 Case surveillance*

One-hundred thirty-five cases of TL diagnosed in the RER were identified between January 2017 and December 2020. Among them, 113 (84%) cases were notified to the LHU (Table 7).

*Table 7.* ***Tegumentary leishmaniasis cases that were identified in the Emilia-Romagna region2017-2020****. Adapted from Gaspari et al 2022 [117].*

| Year | TL cases (n.) | notified to LHU n. (%) | Autochthonous cases n. (%) | CL cases n. (%) | ML cases n. (%) |
|---|---|---|---|---|---|
| 2017 | 28 | 21 (75%) | 21 (75%) | 23 (82%) | 5 (18%) |
| 2018 | 39 | 36 (92%) | 34 (87%) | 38 (97%) | 1 (3%) |
| 2019 | 44 | 38 (86%) | 40 (91%) | 41 (93%) | 3 (7%) |
| 2020 | 24 | 18 (75%) | 18 (75%) | 22 (92%) | 2 (8%) |
| Total | 135 | 113 (84%) | 113 (84%) | 124 (92%) | 11* (8%) |

** 7 autochthonous cases and 4 cases in which the place of infection was defined outside RER, within Italy*

The research activities of the Skin_Leish_RER network contributed to the recovery of 22 TL cases (16%) that were not communicated to the public health authorities (Table 8).

*Table 8.Tegumentary leishmaniasis: case notification and/or molecular confirmation at the Regional Reference laboratory (RRL) of the Emilia-Romagna region. Adapted from Gaspari et al 2022 [117]*

| | | Case notification | |
|---|---|---|---|
| | | yes | no |
| PCR confirmation | yes | 92 (68%) | 11 (8%) |
| | no | 21 (15%) | 11 (8%) |
| **Total** | | 113 (84%) | 22 (16%) |

*In brackets: percentage are calculated based on total cases (n=135) of tegumentary leishmaniasis.*

Regarding the place of infection, 113 TL cases (84%) were identified as autochthonous (Table 6). Among the non-autochthonous cases (n=22), the infection was probably acquired in other Italian regions in 14 cases and outside Italy (Tunisia, Burkina Faso, and Morocco) in 5 cases. In the remaining three cases, we were not able to define the place of infection. By localizing probable infection sites for autochthonous TL cases, their distribution clustered in the foothill area of the RER (Figure 12).

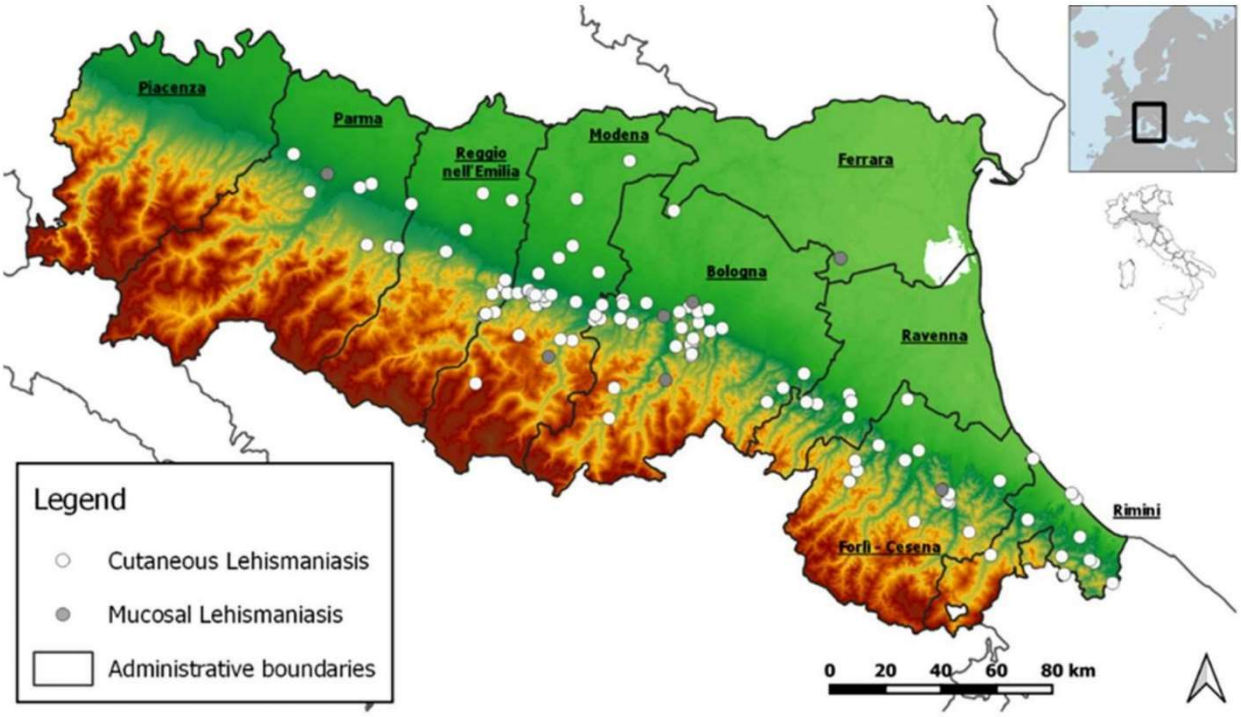


*Figure 12.* ***Distribution of autochthonous cases of tegumentary leishmaniasis within the Emilia-Romagna region, northeastern Italy.*** *Number of TL cases: 113. Adapted from Gaspari et al 2022 [117].*

Considering the different clinical forms of TL, the most frequent was CL (n = 124 cases, 92%), while ML was observed in 11 cases (8%) (Table 6). No cases of MCL were detected in our study group. TL cases were more frequent in males than females (n = 84, 62%). The median age of the infected patients was 57 years, spanning from 1 to 84 years; the majority of cases (75%) were older than 43 years (Figure 13).

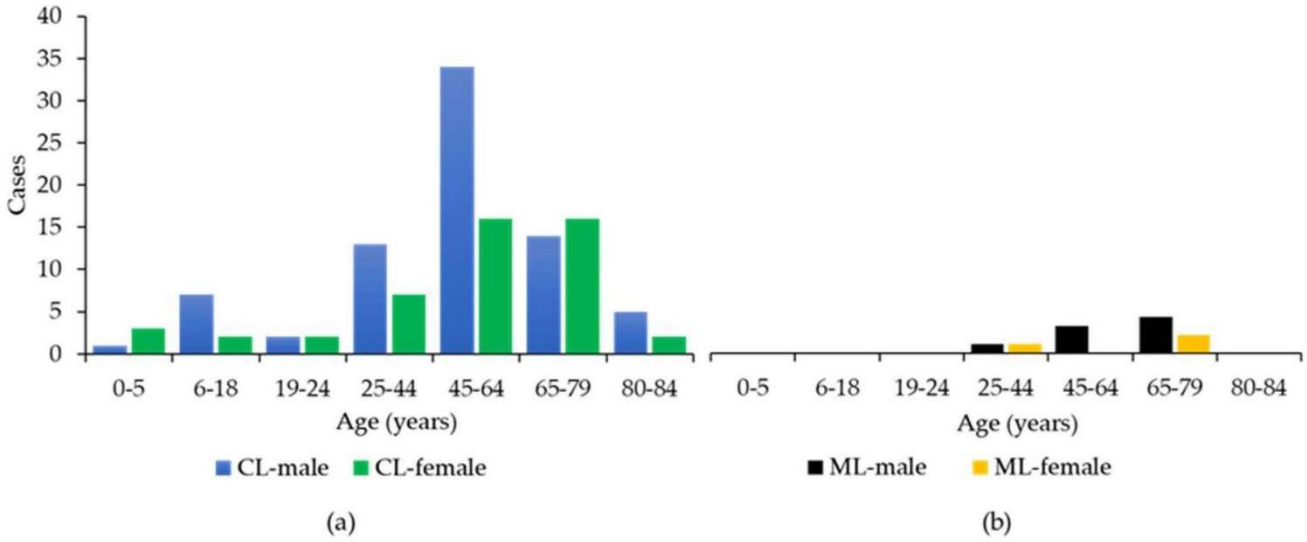


*Figure 13.* ***Age and sex distribution of cases of (a) cutaneous (CL) and (b) mucosal (ML) leishmaniasis.*** *Total case number was 135. Adapted from Gaspari et al 2022 [117].*

During the study period, TL incidence increased from 0.63 in 2017 to 0.98 in 2019 and then dropped to 0.54 in 2020 (Figure 14a). The highest incidence of TL was registered in patients between 65- and 79-years of age (Figure 14b).

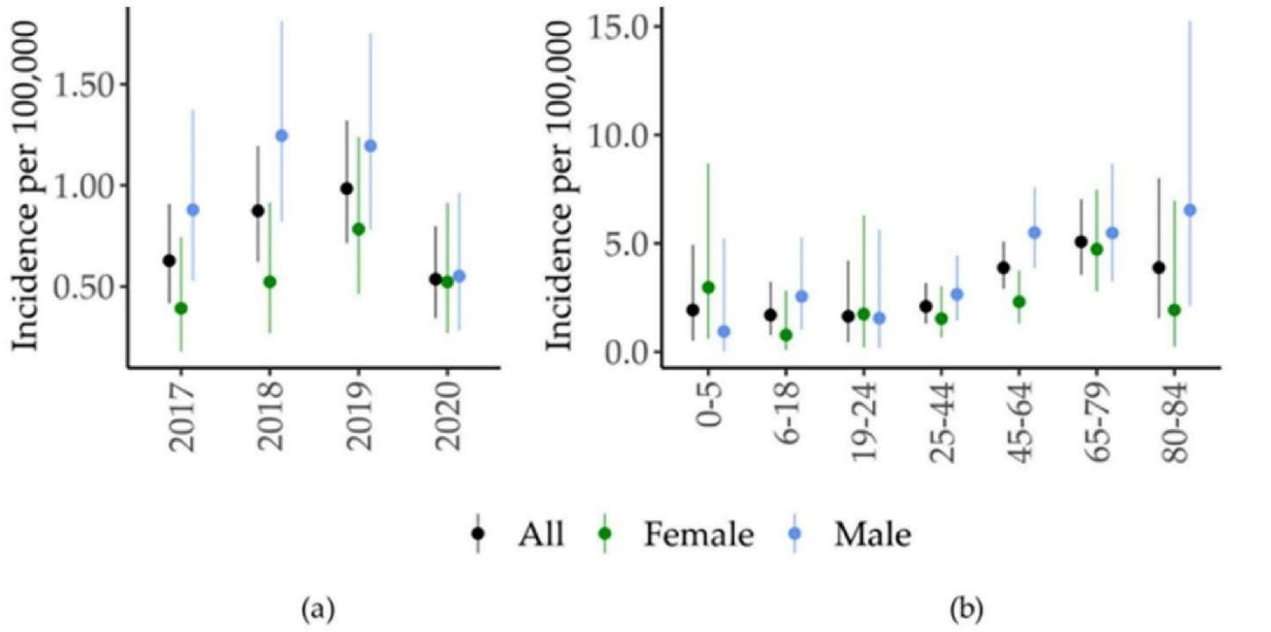


*Figure 14.* ***Incidence of tegumentary leishmaniasis cases was segregated by sex and year*** *(a) and by age (b). Total number of cases [117].*

No evident seasonality in lesion onset was observed in the study (Figure 15, Kruskal-Wallis test, p = 0.39).

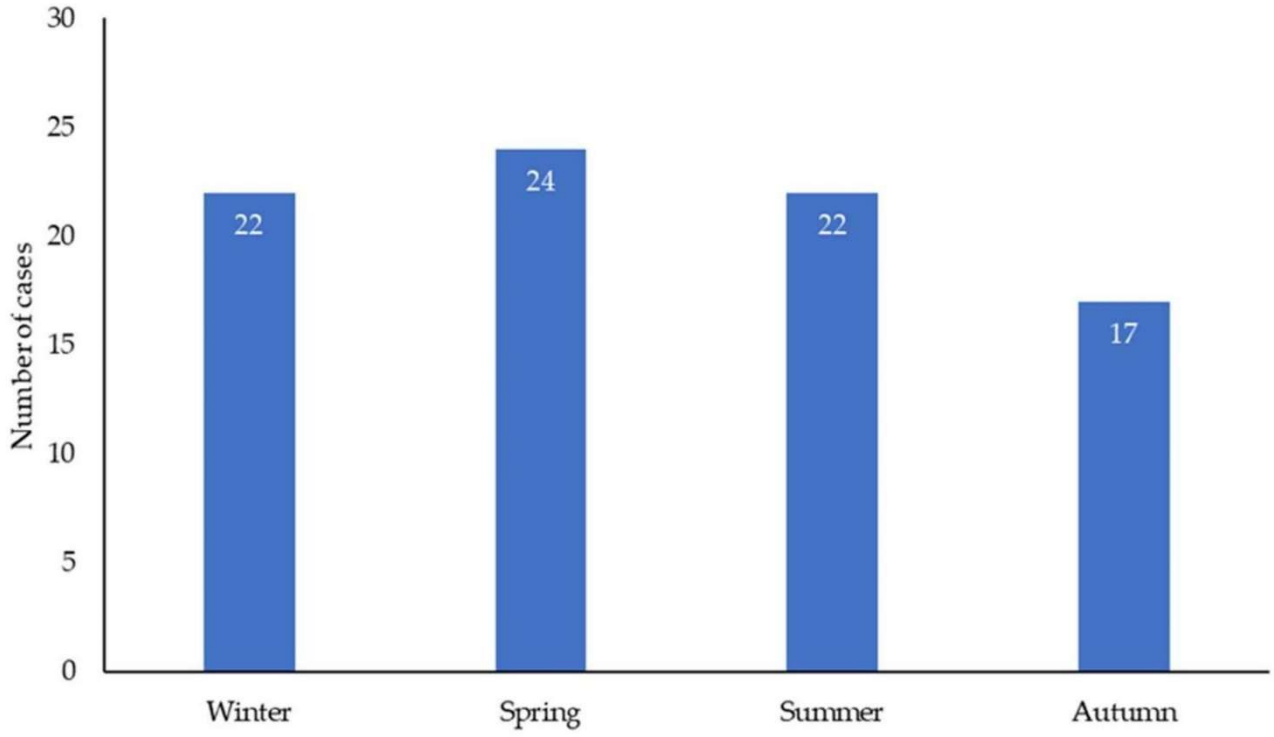


*Figure 15.* ***Seasonal distribution of tegumentary leishmaniasis based on lesions onset****. Data on lesion onset were available for n= 85 TL cases. Adapted from Gaspari et al 2022 [117].*

## *1.2 Clinical characteristics of the lesions*

Regarding CL, we observed that lesions were more often single (n= 88) than multiple (n= 19), 17 CL cases did not report data regarding the number of lesions. Concerning ML, 5 out of 11 (46%) cases presented multiple lesions, 5 cases (46%) showed only single lesion, while for one ML case we did not have information about number of lesions. Data regarding the type of lesion (Figure 16a) were obtained for 98 out of 135 cases (n= 90 CL and n= 8 ML). Among these, most represented lesion types in CL were non-ulcerated nodules (n= 35 cases, 39%), followed by non-ulcerated plaques (n= 18, 20%), ulcers (n= 12, 13%), and ulcerated nodules (n= 11, 12%). Other lesion types are rarely observed, i.e. ulcerated plaques and non-ulcerated papules. Regarding ML, most of the lesions were identified as mucosal ulcers (n= 4, 50%), semi-mucosal nodules (n= 2, 25%), and plaques (n= 2,

25%). Data on lesion type were not available for three ML cases. As shown in Figure 16b, the most common body locations for CL (124 cases could be examined) were the head and neck (n= 47, 38%), followed by the upper (n= 45, 36%), lower (n = 10, 8%) extremities, and trunk (n= 4, 3%). Distant body sites affected by multiple CL lesions were observed in 6 cases (5%). ML lesions were mainly reported affecting the head and neck (n= 9 out of 11, 82%). One HIV-coinfected patient presented multiple and distant lesions, while we had no data for one case. Among ML cases, two patients (18%) reported previous presence of CL lesions.

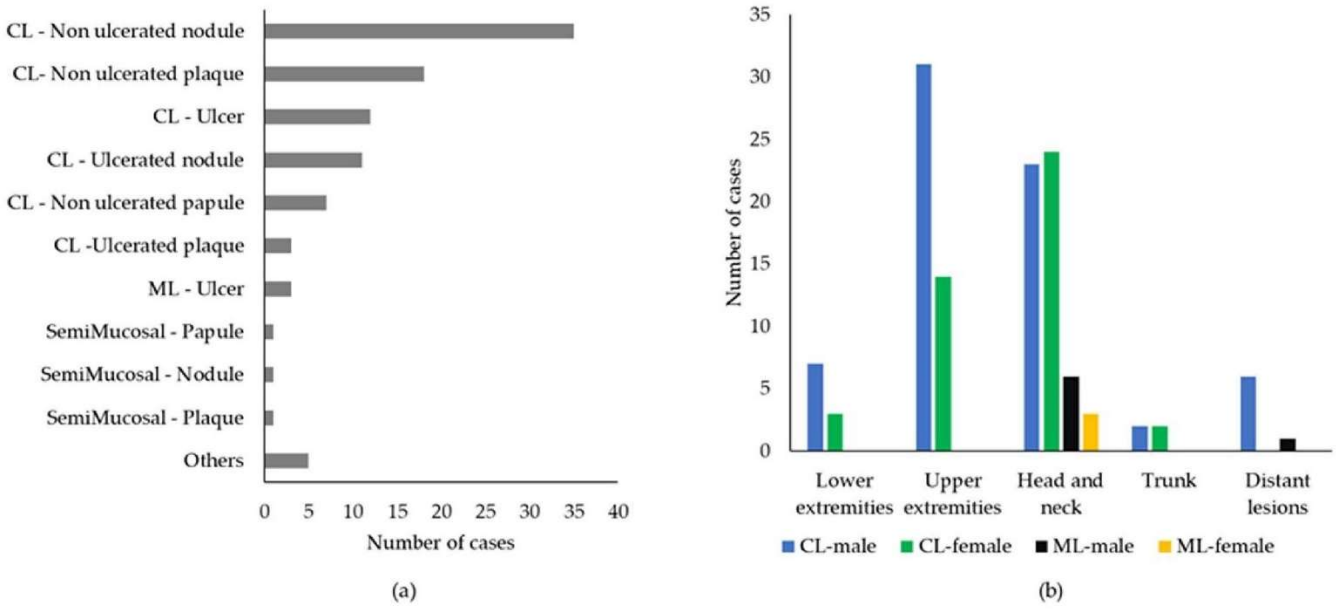


*Figure 16.* ***Clinical characteristics of tegumentary leishmaniasis cases.*** *(a) Lesions were segregated based on the body location, disease form and sex. Data were available for 124 cases of cutaneous leishmaniasis (CL) and 10 cases of mucosal leishmaniasis (ML); (b) frequency of different types of lesions. N= 90 CL cases and 8 ML cases. Adapted from Gaspari et al 2022 [117].*

We were able to collect data about immunosuppressive conditions for 102 cases of TL. Sixteen patients showed conditions associated with immune impairment, HIV-coinfection (n= 4), autoimmune pathology (n= 5), cancer (n= 5), and diabetes (n= 1), for one case the immunosuppressive condition was not defined. Among the 16 immunocompromised patients, we observed 10 severe TL

cases (63%), while among 86 immunocompetent individuals only 26 (30%) were affected by severe TL (Figure 17). Severe and mild cases of TL were defined in the Material and Methods.

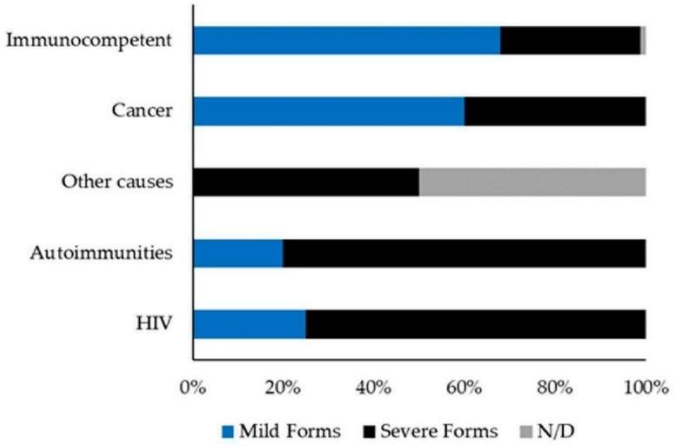


*Figure 17.* ***Severe and mild forms of TL segregated on the basis of patient's underlying diseases (n = 102)****. ND; not defined. Adapted from Gaspari et al 2022 [117].*

## *1.3 Diagnosis of tegumentary leishmaniasis*

Information about time to diagnosis were recorded for 92 patients. Among them, 36 patients (39%) received diagnosis of TL more than 6 months after the lesions' onset (Table 9).

*Table 9.* ***Time to diagnosis and notification status of tegumentary leishmaniasis cases(n=92).*** *Adapted from Gaspari et al 2022 [117].*

| **Lesion onset/diagnosis time gap** | **Public health notification** | | **Total cases (%)** |
|---|---|---|---|
| | **yes** | **no** | |
| ≥ 1 month | 9 | 0 | 9 (10%) |
| 1-3 months | 11 | 4 | 15 (16%) |
| 3-6 months | 26 | 6 | 32 (35%) |
| 6-12 months | 28 | 2 | 30 (33%) |
| 12 months | 6 | 0 | 6 (7%) |
| Total | 80 | 12 | 92 (100%) |

TL diagnosis was performed by histological and/or by molecular tools. Histological examinations were carried out in 118 out of 135 cases (Table 9), among these, infecting amastigote were directly

observed in 92 (78% of sensitivity). PCR was performed in 103 out of 135 cases; of them 100 cases tested positive for Leishmania DNA (97% of sensitivity). Also, we observed that 11 of the 12 non-notified cases (50%) were not sent to RLL for molecular confirmation of the histological diagnosis.

*Table 10.* ***Diagnostic methods for tegumentary leishmaniasis, including histology (n=118) and by PCR (n=103).*** *Adapted from Gaspari et al 2022 [117].*

| | | Histology | | | |
|---|---|---|---|---|---|
| | | **Amastigotes +** | **Amastigotes -** | **Not done** | **Total** |
| **Molecular diagnosis** | PCR + | 57 | 26 | 17* | 100 |
| | PCR - | 3 | 0 | 0 | 3 |
| | Not done | 32 | 0 | 0 | 32 |
| | Total | 92 | 26 | 17 | |

**11 out of 17 cases were not reported to the public health service*

## *1.4 Treatment strategies and outcome*

Data regarding TL treatment (Figure 18) were recorded in 90 cases out of 135 (67%). Eleven different treatment strategies were carried out, including (i) intralesional pentavalent antimonial (Glucantime) (n= 27, 30%), (ii) topical cream containing paromomycin (n= 16, 18%), (iii) surgical excision (n= 13, 14%), (iv) cryotherapy alone (n= 2, 2%) or associated with intralesional Glucantime (n= 11, 12%), (v) systemic liposomal amphotericin-B (n= 11, 12%), (vi) other treatments (n= 10, 11%). "Other treatments" group includes oral fluconazole (n= 2), oral miltefosine (n= 1), surgical excision associated with fluconazole (n= 1), intralesional Glucantime associated with miltefosine (n= 2), intralesional Glucantime associated with liposomal amphotericin-B (n= 2), or intralesional Glucantime associated with cryotherapy, and intramuscular pentamidine (n= 2).

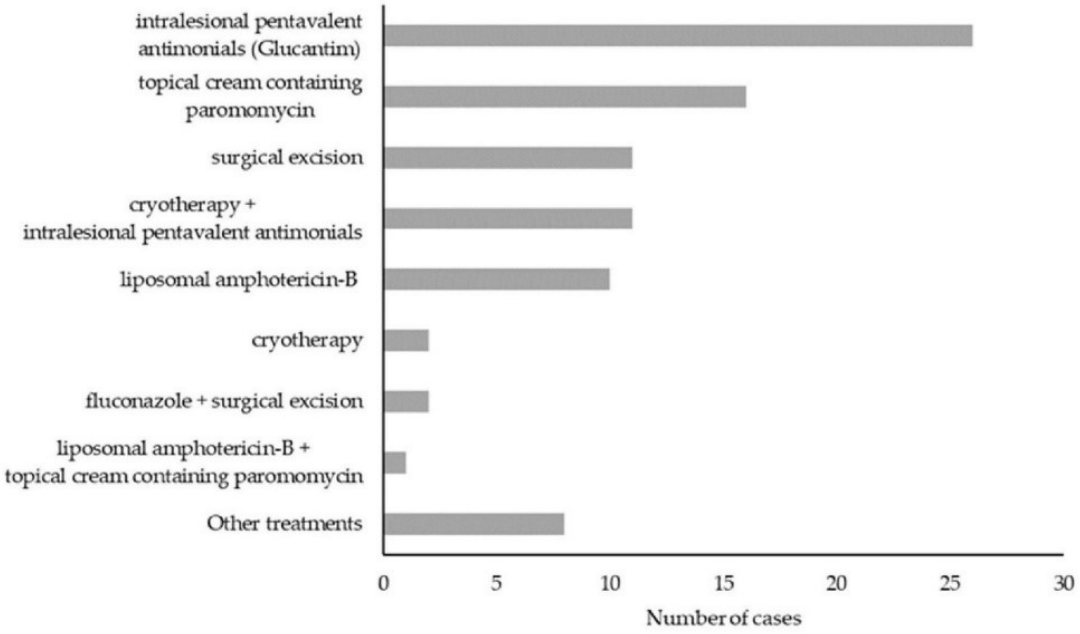


*Figure 18.* ***Treatment strategies employed for tegumentary leishmaniasis , n= 90****. Adapted from Gaspari et al 2022 [117].*

Data about treatment outcome were collected for 84 out of 135 cases (62%, Figure 19). Among these, events of treatment failure were observed in 18 TL cases (14 CL and 4 ML, 21%). By considering only TL cases, for which treatment outcome was available, the rate of treatment failure observed within various therapeutic approaches was as follows: 7 cases out of 11 for liposomal amphotericin-B alone (64%), 4 cases out of 11 for surgical excisions (36%), 3 cases out of 14 for topical cream containing paromomycin (21%), and 1 case out of 26 for intralesional Glucantime (4%). Among the cases of treatment failure, 5 (28%) were observed in patients with immune suppressing conditions (2 HIV-coinfected individuals and 3 oncological patients), 6 (33%) in immunocompetent individuals, while in 7 patients (39%) the immune status was not defined.

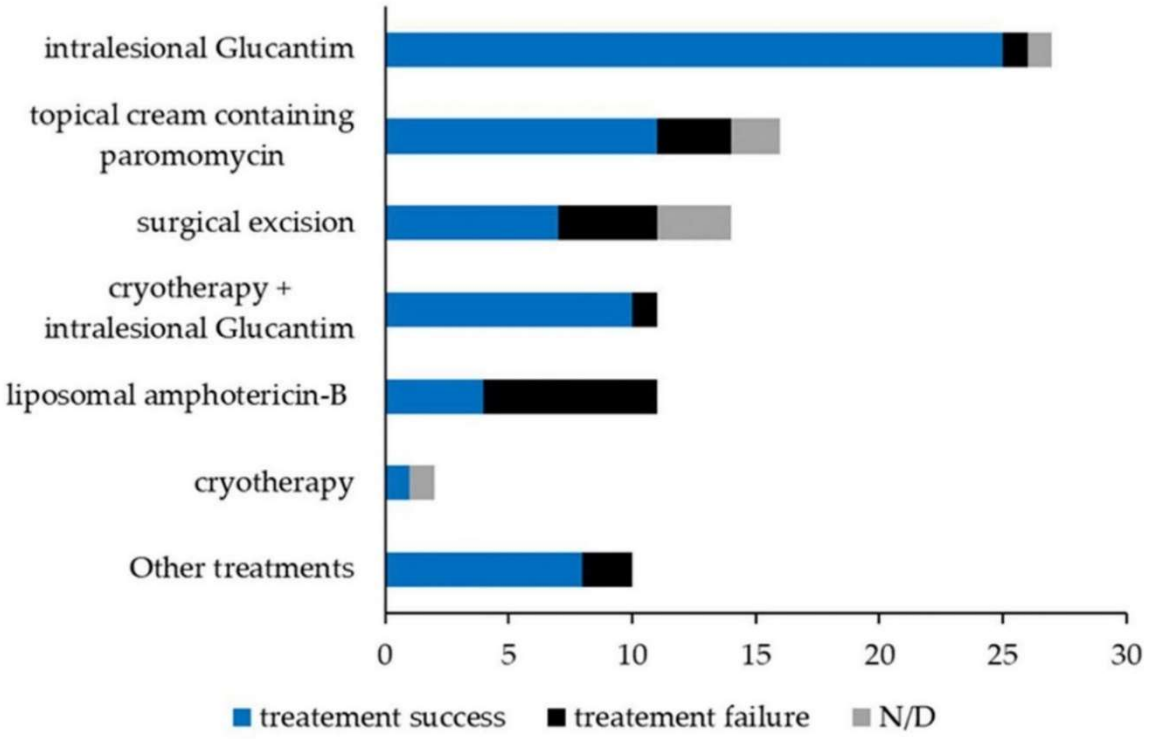


*Figure 19.* ***Cases of treatment failure were segregated on the basis of the therapeutic approach.*** *N=84 . Adapted from Gaspari et al 2022 [117].*

# 2. Study II

## *2.1 ITS1 and hsp70 typing results*

This study was performed in 109 sections of FFPE biopsies that were collected from skin or mucosal lesions, in which diagnosis of TL was carried out between 2014 and 2020 at the RRL, Unit of Microbiology, Bologna University Hospital.

Molecular typing was performed for two markers, ie ITS1 and hsp70. Amplification and sequencing reaction for at least one target (ITS1 and/or hsp70) was successfully carried out for 88 samples (80.7%) [Appendix A]. These 88 sequenced samples derived from 83 TL cases (n= 77 CL cases and n= 6 ML cases); 8 samples were obtained from 4 patients undergoing relapse, while 2 samples were obtained from one patient with multiple lesions. All typing results regarding samples deriving from the same patient were concordant. Among the 88 typed samples, 79 were obtained from 75 patients whose infection probably occurred inside Italy (autochthonous cases) while 9 samples were obtained from 8 cases, for whom the place of infection was probably located in countries outside Italy and known to be endemic for leishmaniasis.

ITS1 successful typing was obtained for 75 samples out of 109 (68.8 %) (Accession Number (A.N.) are reported in Appendix A); they derived from 70 TL cases as ten samples were obtained from five patients exhibiting relapses or multiple lesions (4 from *L. infantum* and 1 from *L. major*). 65 TL cases (92.8 %) were identified as *L. infantum*, 3 (4.3 %) as *L. major*, 2 (2.9%) as *L. tropica* (Table 11). All cases whose ITS1 sequence identity correspond to *L. infantum* were precedent classified as autochthonous, on the other hands the other 5 cases, whose lesions were typed as *L. major* and *L. tropica*, reported travels to Tunisia (n=2, *L. major*), Burkina Faso (n=1, *L. major*) or Morocco (n=2, *L. tropica*).

*Table 11.* ***Comparison of ITS1 and hsp70 typing results*** *(n=83 cases of tegumentary leishmaniasis).*

| | | hsp70 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L. donovani complex | | | | | | | | L. major | L. tropica | NA | Total |
| | | A | B | C | D | E | F | G | H | | | | |
| ITS1 | L. infantum | 25 | 1 | 4 | 1 | 6 | 1 | 28 | 1 | 0 | 0 | 0 | 67 |
| | L. major | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4(4) | 0 | 0 | 4(4) |
| | L. tropica | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2(2) | 0 | 2(2) |
| | NA | 4 | 2(1) | 1 | 0 | 1 | 0 | 1 | 0 | 1(1) | 0 | 0 | 10(2) |
| | Total | 29 | 3(1) | 5 | 1 | 7 | 1 | 29 | 1 | 5(5) | 2(2) | 0 | 83(8) |

*NA; not amplified.*

*In brackets; number of imported cases*

Among the 109 total samples, hsp70 fragments were successfully typed for 87 of them (79.8%) obtained from 83 TL cases (see A.N. in Table S1). For all 87 samples, consensus sequence obtained was 218 bp long, at least. Analysis of the consensus sequences showed that 76 out of 83 cases (91.5 %) clustered within the *L. donovani* complex. Regarding the rest of the cases, five (6.0 %) were typed as *L. major* and two (2.4 %) as *L. tropica* (Table 11).

All cases whose hsp70 sequence was identified within the *L. donovani complex* were previously classified as autochthonous, except for one (exhibiting hsp70 sequence variant B) that reported traveling to Bangladesh. All cases typed as *L. major* and *L. tropica* by hsp70 reported travels in countries considered endemic for these species, respectively Tunisia (n=3, *L. major*), Burkina Faso (n=1, *L. major*), Middle East (n=1, *L. major*) and Morocco (n=2, *L. tropica*).

Among 76 cases whose hsp70 sequence clustered within the *L. donovani complex* , 65 were typed also by ITS1 that identified them as *L. infantum*. The remaining 11 cases were not typed with ITS1. ITS1 and hsp70 typing results completely overlapped for *L. major* (n= 3 cases) and *L. tropica* (n= 2 cases), nevertheless two samples identified as *L. major* by hsp70 could not be typed with ITS1 (Table 11).

## *2.2 hsp70 sequence variants within TL autochthonous cases*

The eight hsp70 sequence variants clustering within the *L. donovani* complex and observed within autochthonous cases were further investigated (Figure 20); among them we observed 8 sequence variants (named from A to H) in respect to the hsp70 sequence of the reference strain of *L. infantum JPCM5* (A.N.: XM_001470287.1_inf_LLM-877). hsp70 variant A consensus sequence was observed in 29 cases (38.7 % of total cases within the *L. donovani* complex); this sequence was identical to the *L. infantum* JPCM5 reference strain (A.N.: XM_001470287.1_inf_LLM-877). Variants B-E (n= 15 cases, 20.0%) and variants G-H (n= 31 cases, 41.3%) showed the presence SNPs in respect to the hsp70 sequence of the JPCM5 reference strain. SNPs observed in these sequences were in positions 764 and/or 868, both within the Ps generated amplicons. In position 764, we observed an A → G substitution in C and E sequence variants or the presence of the corresponding degenerated base R, if both nucleotides were present in the same position, as for sequence variants D and G. The T → C substitution was observed in position 868 for sequence variants B-D, and the corresponding degenerated nucleotide Y in E-H. In addition, in sequence variants F, which was detected in one sample, a degenerated nucleotide Y was observed in position 768.

| *Position* | 760 | . . . | 764 | I | . . . . | 770 | - | I | . | . | 868 | . |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **XM_001470287.1_inf_LLM-877_*Leishmania infantum* JPCM5** | A | AGA | A | CC | T | G | G | C | - | G | C | G | T | T |
| **HAPLOTYPE (A)** | . | . . . | . | . . | . | . | . | . | - | . | . | . | . | . |
| **FN395028_don_MHOM/IN/00/DEVI** | . | . . . | . | . . | . | . | . | . | - | . | . | . | C | . |
| **HAPLOTYPE (B)** | . | . . . | . | . . | . | . | . | . | - | . | . | . | C | . |
| **MZ362375.1 *L. donovani* MHOM/AF/2016/ITM16052211** | . | . . . | G | . . | . | . | . | . | - | . | . | . | C | . |
| **HAPLOTYPE (C)** | . | . . . | G | . . | . | . | . | . | - | . | . | . | C | . |
| **MZ362312.1 *L. donovani* complex MHOM/TR/2019/ITM19061037** | . | . . . | R | . . | . | . | . | . | - | . | . | . | C | . |
| **HAPLOTYPE (D)** | . | . . . | R | . . | . | . | . | . | - | . | . | . | C | . |
| **HAPLOTYPE (E)** | . | . . . | G | . . | . | . | . | . | - | . | . | . | Y | . |
| **HAPLOTYPE (F)** | . | . . . | . | . . | . | R | . | . | - | . | . | . | Y | . |
| **MW658450.1 *L. infantum* isolate L111** | . | . . . | R | . . | . | . | . | . | - | . | . | . | Y | . |
| **HAPLOTYPE (G)** | . | . . . | R | . . | . | . | . | . | - | . | . | . | Y | . |
| **HAPLOTYPE (H)** | . | . . . | . | . . | . | . | . | . | - | . | . | . | Y | . |

*Figure 20.* ***Representation of partial sequences of the hsp70 sequence variants obtained in the present study.*** *The hsp70 partial sequences of the sequence variants, were aligned with sequences available in NCBI Gene Bank by Clustal W, as implemented in BioEdit v.7.0.8.0. [227]. N=75 sequences obtained from autochthonous cases of TL were analyzed.*

As shown in Figure 20, a BLAST search of the B-H sequence variants returned the best score with sequences corresponding to *L. donovani* strains from India (FN395028_don_MHOM/IN/00/DEVI) and from Afghanistan (MHOM/AF/2016/ITM16052211) for sequence variants B and C, respectively. For sequence variant G, the best score was obtained with sequence of *L. infantum*, place of infection unknown (L111, A.N.:MW658450.1), while for sequence variant D the best alignment was obtained with a strain from Turkey belonging to the *L. donovani* complex (MHOM/TR/2019/ITM19061037). Sequence variants E, F and H have not yet been reported.

## *2.3 WGS-based analysis of hsp70 gene*

One main limitation of the BLAST search is that this tool generates identity scores without considering ambiguous positions (as in variants D-E-F-G-H).

To assess if SNPs that we found in the hsp70 Ps fragments of TL samples from northeastern Italy were can be found in *L. infantum* we used NGS data of 37 *L. infantum* strains isolated from various

mediterranean countries and performed an in-depth analysis of their hsp70 Ps fragments. WGS reads accession number and variation in sequences are collected in Appendix B. We observed that the alignment of reads to the reference strain JPCM5 showed no variations or polymorphic sites in the N, P or Ps sequence of hsp70 (coverage ranges from 50 to 200X, aprox).

## *2.4 hsp70 variants and potential correlation with disease severity*

On the basis of the hsp70 in depth analysis results, we segregated autochthonous cases in two groups, ie those expressing the hsp70 sequence variant A (identical to *L. infantum* strain JPCM5 and other *L. infantum* strains from southwestern Europe) from the other variants B-H (all sequences that show at least one SNP variation).

Among the 75 autochthonous cases of TL typed by hsp70, 29 clustered within hsp70 variant A group (38.7%) while 46 cases exhibited the hsp70 variants B-H (61.3%). The two populations were compared considering variation of clinical features, ie mild or severe disease. Severe and mild TL were defined in the Material and Methods.

As shown in Figure 21a, we observed a slightly higher frequency of severe disease in the group expressing the B-H variants (n= 27 severe cases, 58.7%) when compared with the group exhibiting variant A (n=11 severe cases) (p= 0.077, Chi-square test). By considering only patients for whom the immunocompetent status was known (n=61, Figure 21b), 25 cases clustered within group A and 36 within B-H group. In immunocompetent patients, the proportion of TL cases with severe disease was significantly higher in hsp70 variant B-H, with a frequency of 58.3 % (n=21) over the total cases as compared to 32.0% (n=8) for hsp70 variant A (p=0.011, Fisher's test) (p=0.042, Chi-square test).

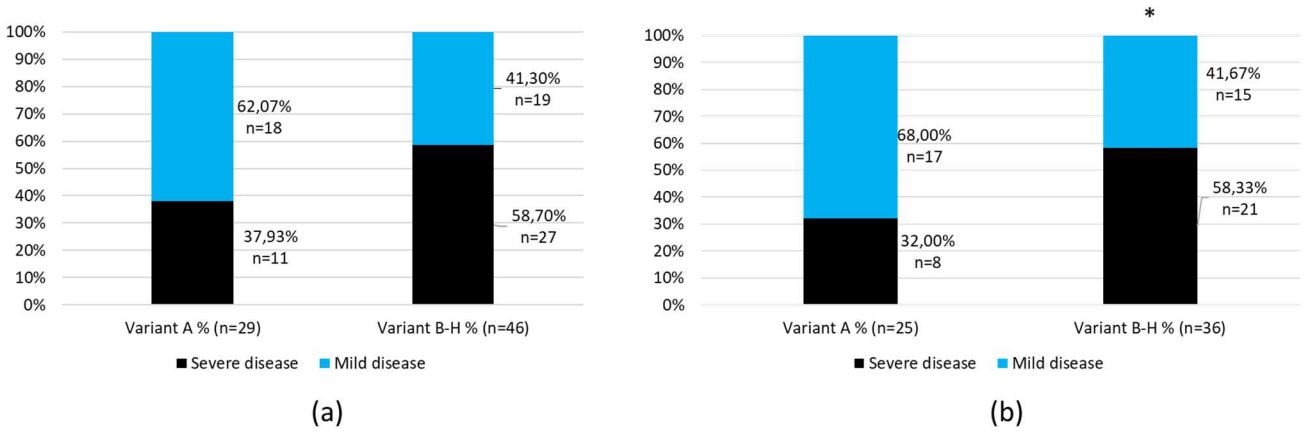


*Figure 21.* ***Segregation of disease severity based on hsp70 genotypes in tegumentary leishmaniasis (TL).*** *Distribution of mild and severe forms of TL according to the hsp70 typing in (a) all autochthonous cases (n=75, p-value=0.077); (b) restricted to the immunocompetent TL patients (n=61, , p-value=0.077). *: p<0.05*

# 3. Study III

## *3.1 ONT sequencing results and Maxicircles assembly*

Genomic DNA from the two dermotropic isolates from northeastern and central Italy (MO44 and cur-1) was extracted and purified as described in Materials and Methods. The quality of the pre- and post-RNAse gDNA was assessed by electrophoretic run (Figure 22) and dsDNA concentration was measured (Table 12). MO44 and cur-1 isolates were sequenced obtaining respectively 5.39 Gb sequenced and 39.7 x 10^5 of reads for MO44 (average coverage 127.274) and 1.3 Gb sequenced and 1.5 x 10^5 reads for cur-1 (average coverage 29.4127). Total WGS reads from the two strains were used to de-novo assemble maxicircle CR sequences obtaining a consensus sequence 16169 bp for MO44 and one 16142 bp long for cur-1. MO44 and cur-1 complete CR consensus sequence can be found in Appendix C. The two novel CR sequences were then aligned with available sequences, in details: 1) 61 strain of *L. infantum* and one strain of *L. donovani* HU3 that were assembled by Solana et al. [39]; 2) 14 strain of *L. infantum* obtained from ILLUMIA NGS data by Franssen et al [27][6]; 3) one reference strain, i.e. IPT1, that was obtained from ONT NGS data (A.N. NCBI-NIH SRA SRR21601459).[183] [7]. The total analysis included 77 *L. infantum* and 1 *L. donovani* CR sequences [Appendix D].

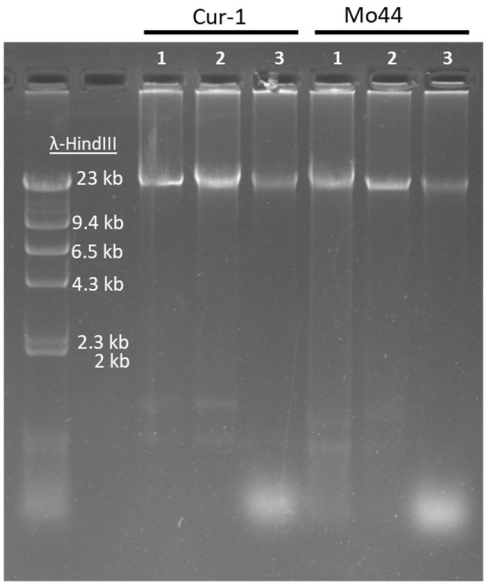


*Figure 22.* ***Agarose gel visualization of high molecular weight Leishmania gDNA after extraction and RNAse purification.*** *1- extracted gDNA post-RNAse purification (undiluted); 2- extracted gDNA post-RNAse purification (diluted 1/10); 3- extracted gDNA pre-RNAse purification.*

*Table 12. Concentration of Leishmania gDNA after phenol-chloroform extraction and RNAse treatment.*

| Sample | Nanodrop (ng/µL) | QuantusFluorimeter (ng/µL) |
|---|---|---|
| cur-1 (pre-RNase) | 210 | 37 |
| cur-1 (post-RNase) | 563 | 160 |
| cur-1 (post-RNase) 1/10 | 72 | 12 |
| MO44 (pre-RNase) | 178 | 15 |
| MO44 (post-RNase) | 291 | 105 |
| MO44 (post-RNase) 1/10 | 81 | 9.6 |

## *3.2 Phylogenetic analysis*

By aligning the 78 above mentioned CR sequences a phylogenetic tree was built (Figure 23). By this analysis we observed that the dermotropic isolates from northeastern and central Italy (MO44 and cur-1) grouped outside the European JPCM-5 like clade and the South American group and were located close to an outgroup of *L. infantum* strains from Cyprus, Spain, and Turkey. This observation

is supported by strong bootstrap values observed in the nodes that generated the clades including the northeastern and central Italian strains and the outgroup of *L. infantum* strains from Cyprus, Spain, and Turkey. On the other hand, we observed that these out grouping clades are phylogenetically closer to the remaining *L. infantum* strains than to the *L. donovani HU3* strain that was used as control.

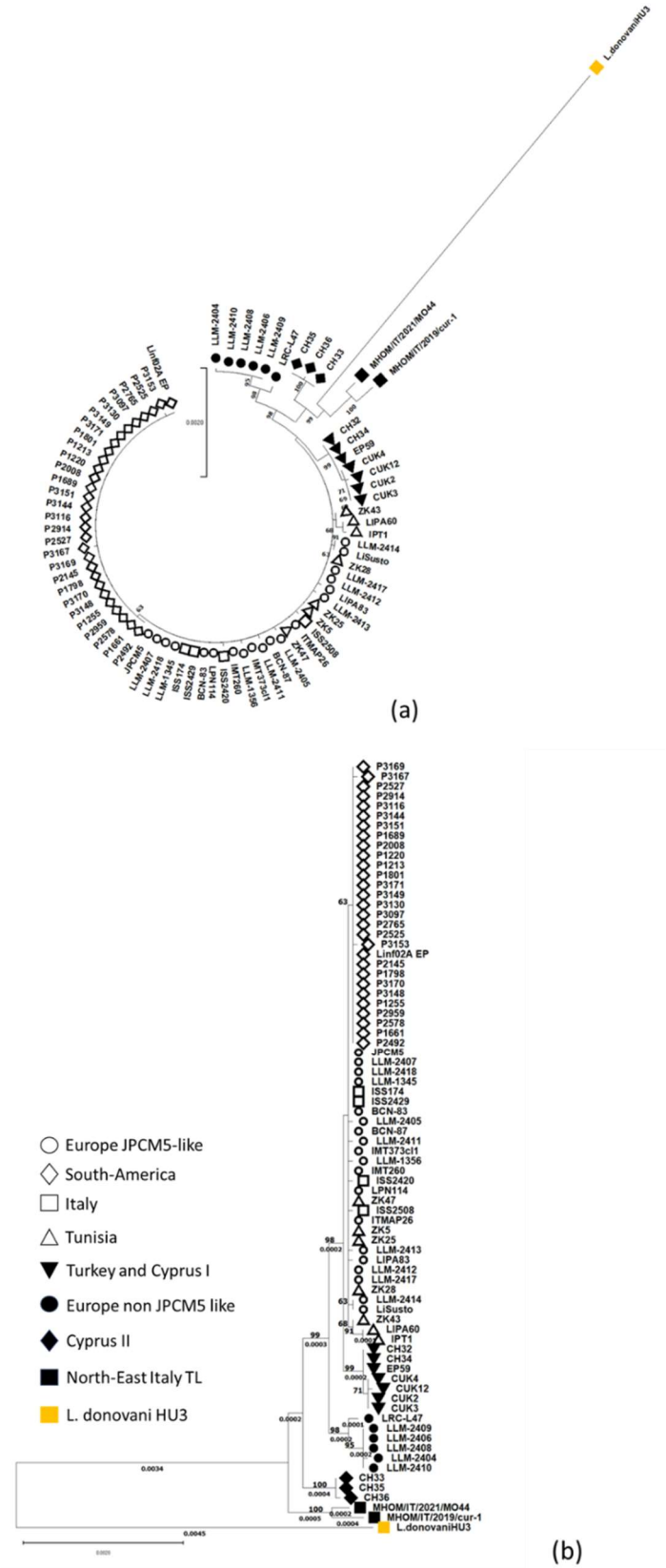


***Figure 23. Phylogenic relationship among Mediterranean and South American L. infantum isolates.*** *Phylogenetic trees were depicted as Circular (a) and as Rectangular with length value at the bottom of the branches(b). The evolutionary history was inferred*

*by using the Maximum Likelihood method and Tamura-Nei model [226]. The tree with the highest log likelihood (-21311.86) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). This analysis involved 78 nucleotide sequences. There were a total of 16286 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [225]. Europe JPCM5-like ; Italy ; Tunisia ; South America ; Turkey and Cyprus I ; Europe non JPCM5-like ; Cyprus II ; northeastern and central Italy ; L. donovani HU3.*

The observations reported by the phylogenetic tree were then confirmed by the calculation of the "pairwise phylogenetic distance" and the number of variable sites that were found between the reference strain of *L. infantum* JPCM5 and the various clades observed in the tree (Table 13); the two index isolates (MO44 and cur-1) showed a marked sequence divergence from the reference strain JPCM5, differently from the other Italian isolates.

***Table 13. Phylogenetic distances among maxicircle CR sequences obtained from L.infantum strains.*** *Phylogenetic distance values between reference L. infantum strain JPCM5 and clades emerged in phylogenetic tree in Figure 23. Pairwise distance was calculated by using "Distance" functionality, "compute pairwise distance" , "maximum likelihood method" and Tamura-Nei mode on MEGA11. L.donovani HU3 strain was used as control.*

| Clade (example strain) | Average pairwise distance (to JPCM5) | Average number of substitutions (to JPCM5) |
|---|---|---|
| Europe JPCM5-like (LLM1356) | 0.000024 | 0.4 |
| Italy (ISS2420) | 0.000031 | 0.5 |
| Tunisia A (ZK28) | 0.000000 | 0 |
| Tunisia B (IPT-1) | 0.000164 | 2.7 |
| South America (P1220) | 0.000066 | 1.1 |
| Turkey and Cyprus I | 0.003513 | 5.7 |
| Europe non JPCM5-like | 0.000677 | 11.0 |
| Cyprus II | 0.001066 | 16.4 |
| MHOM/IT/2021/MO44 | 0.001363 | 22.2 |
| MHOM/IT/2019/cur-1 | 0.001641 | 26.3 |
| L. donovani HU3 | 0.008462 | 137.8 |

# Discussion and Conclusion

Leishmaniasis is an emerging disease in Europe, which raise is driven by climate change and other environmental conditions. Historically countries of southern Europe (Italy, Spain, Portugal, southern France, Balkans and Greece) have been considered endemic for this disease, caused by *L. infantum [5]*. However, recent years evidenced that the epidemiological situation is rapidly changing with the detection of new epidemiological foci of infection [113,115,118,228], the introduction of *L. tropica* in Greece [229], the emergence of *Leishmania* hybrids [27,230,231] and new finding of both the vector and the parasite in non-endemic countries of continental Europe [104,105].

In Italy, the northward spread of leishmaniasis is well represented by the epidemiological dynamic in the northern regions, where the first cases of CanL were observed twenty years ago, followed by a multiannual outbreak of human leishmaniasis in RER [98,112,113,115,118]. In this context, TL is often overlooked despite being more frequent than VL; the lack of awareness about this infectious disease by dermatologists, otorhinolaryngologists, and other clinicians often results in misdiagnosis. Even when a proper diagnosis of TL is performed, case notification to the public health services is frequently lacking.

The retrospective epidemiological Study I examined TL cases that were identified within RER between 2017 and 2020 and implemented the data collected by the establishment of a multidisciplinary network of medical specialists (Skin_Leish_RER network). The creation of the network allowed to recover 22 unnotified cases among the total 135 confirmed cases of TL in the 4-years study period. Yearly incidence observed ranged from 0.54 to 0.98 cases per 100,000 inhabitants raising from 2017 to 2019 just to drop in 2020 (0.54 cases/100,000 inhabitants), likely because of the disruption of the health system due to the COVID pandemic [232].

Considering the total diagnosed TL cases, most of them were autochthonous (84%) and distributed around the foothills areas of the region, overlapping with the distribution of VL cases

[115,116,209,233] and with the areas that are colonized by *Phlebotomus (Ph). perfiliewi*, a sand fly species able to maintain the circulation of *L. infantum* [234-236].

Similar to previously reported data [7,14], the most common infected subjects were males older than 43 years and the most common disease form observed were skin nodules on the head and neck. Furthermore, we observed that 8% of the total cases were ML, thus confirming a pronounced tendency of *L. infantum* to invade mucosal tissues as described in a recent study [7].

From the point of view of clinical management of TL, the study underlined two important aspects; concerning TL diagnosis, PCR guaranteed better sensitivity than histological examination (97% and 78%, respectively) showing the importance of performing molecular tests to avoid misdiagnosis, as widely demonstrated by other studies [95,237]. Furthermore, we observed that physicians belonging to the Skin_Leish_RER network used a wide variety of clinical approaches to treat TL, including pharmacological therapies, cryotherapy, or surgical excision that is not recommended for CL treatment but that was sometimes performed in the suspicion of skin cancer. Taken together, the abovementioned observations suggest that standardization of TL diagnosis and treatment is needed, including the development of national guidelines, as done in other European countries [238].

Despite study I showed limitations due to its retrospective nature, it revealed an evolving and active epidemiological pattern of TL cases in RER and highlighted the need to better characterize the dermotropic *Leishmania* populations causing the disease in the selected area. For these reasons, we performed a study of molecular typing (Study II of this thesis).

Studies of molecular surveillance in TL endemic areas are necessary because different *Leishmania* species cannot be recognized by direct clinical or microscopic observation, but they may necessitate different treatment regimens [10,14,239]. In addition, it is important to monitor possible introduction of allochthonous *Leishmania* species/strains that may be potentially transmitted by local sand flies in endemic areas, causing unpredictable consequences. In southern Europe, particular attention should

be paid for the potential establishment of imported anthroponotic species, such as *L. tropica* and *L. donovani*, while the risk of introduction of exotic zoonotic species is considered low [123].

Study II was based on the combined sequencing results of two *Leishmania* genomic targets (i.e. ITS1 [134] and hsp70 [156]) to characterize the *Leishmania* populations at strain and species level.

We succeeded in typing parasites in skin and mucosal biopsies from 83 TL cases diagnosed in the RER between 2014 and 2020. All the cases were amplified and successfully sequenced by at least one of the two typing targets. The combined results from the two markers as well as the improvement of PCR tools for hsp70 amplification allowed to obtain species identification in 80% of the FFPE samples.

Sequencing results of ITS1 and hsp70 showed complete concordance for the identification of *Leishmania* spp. causing imported cases of TL; three and two imported cases that were caused respectively by *L. major* and *L. tropica* were typed by both the targets, and two additional *L. major* cases were typed by hsp70 sequencing only. Our findings about patient's travel history and the parasitic species identified in the imported cases were in line with previous studies describing geographic species distribution of imported leishmaniasis into Europe; *L. major* and *L. tropica* are well known cause of CL in North Africa and the Middle East [240]; the first has been detected also in Burkina Faso [241]. Among the potential imported cases, one CL case with travel history to Bangladesh was identified by hsp70 sequence as caused by *L. donovani*. CL caused by *L. donovani* have been observed in South Asia, namely Sri Lanka [12,242], but no reports from Bangladesh are available so far. Nevertheless, an autochthonous origin cannot be excluded for this case.

All the autochthonous TL cases were identified as belonging to the *L. donovani* complex. Nevertheless, while ITS1 sequences were homogeneous and completely overlapping with the one from *L. infantum* reference strain [243], hsp70 sequences obtained from the same samples showed high SNP profile variability. Besides less represented sequence variants, autochthonous cases were

mainly split into two groups corresponding to variant A and variant G, with the former overlapping with the sequence of *L. infantum* reference strains from the Mediterranean basin (JPCM5), while variant G was not previously associated with *L. infantum* strains cases from the same area.

By a BLAST search, we also observed that seven autochthonous TL cases, exhibiting B and C sequence variants, completely overlapped with hsp70 sequences obtained from *L. donovani* strains [244], while four of the same TL cases were typed as *L. infantum* by ITS1.

As BLAST search results suffer of limitations in case of sequences with ambiguous SNP positions, we performed an in-depth analysis of hsp70 fragments by using NGS data of 151 strains belonging to the *L. donovani* complex. This analysis showed that only the hsp70 sequence of variant A was previously observed in *L. infantum* strains from the Mediterranean area with no variability in the analyzed sequence.

Following the findings observed with the in-depth analysis, we divided the 75 autochthonous cases in two groups; group 1 included TL cases that possessed the variant A and group 2, which included cases exhibiting the other variants (B-H group). We observed a higher frequency of severe disease in TL cases from the group 2; this difference was significant only when we analyzed immunocompetent individuals, to exclude the immunosuppression as potential contributor of disease severity. This difference suggests that the *L. infantum* strains presenting a peculiar hsp70 sequence could be characterized by different clinical/pathological features. However, the limited number of cases, the multifactorial nature of the disease and the shortness of the typed hsp70 sequence indicate that further studies are required with a bigger sample size to obtain conclusions about the potential increased virulence of the peculiar *L. infantum* population that is causing TL in the RER.

Considering the peculiarity of the *Leishmania* strains causing TL In RER, we decided to perform a deeper phylogenetic analysis by using NGS sequencing on two dermotropic *Leishmania* isolates obtained from an autochthonous ML case that occurred in the same area (MHOM/IT/2021/MO44)

and from a CL case resistant to antimonial treatment that occurred in the province of Pesaro/Urbino, which is located close to the RER (MHOM/IT/2019/cur-1).

In Study III, we used Oxford Nanopore technologies NGS data from these strains to assemble and analyze the complete sequence of the conserved region of the kinetoplast maxicircles, this tool was proven to be particularly precise to understand phylogenetic position of *L. infantum* strains as showed by Solana et al [39]. Results from this phylogenetic analysis revealed that both the tested isolates belong to a clade that is clearly separated from the classical *L. infantum* strains observed in southern Europe and in the American region, while clustering closer to other peculiar *L. infantum* strains observed in Spain [39] and Cyprus, the latter described as *L. infantum/L. donovani* hybrid strain by the WGS study performed by Franssen et al [27].

Altogether, findings presented in Study II and Study III evidenced that a peculiar dermotropic strain of *L. infantum* is circulating in the northeastern (and central) Italy; this strain exhibits some phylogenetic characteristics resembling *L. donovani* and could be the cause of the TL upsurge in the RER. These findings are in line with the hypothesis that *L. infantum* and *L. donovani* species form a continuum and are sometimes intermingled groups, probably as a consequence of progressive genetic differentiation [245]. In addition, considering the peculiarity of hsp70 sequence variants B-H in strains that were typed as *L.infantum* by ITS1 as well as the maxicircle phylogenetic analysis results, we cannot exclude the possibility of past hybridization events between *L. infantum* and *L. donovani* in these Italian strains.

In line with this hypothesis, recent MLMT studies described the presence of a peculiar *L. infantum* strain, causing VL in RER, highly divergent from the *L. infantum* population commonly causing leishmaniasis in humans and dogs in the Mediterranean basin [233,246] and WGS studies unrevealed a common origin of this *Leishmania* population with the *L. infantum/L. donovani* putative hybrids from Cyprus [231]. In addition, comparable findings were observed in Spain with the description of

*L. infantum* strains phylogenetically different from the ones classically circulating in southern Europe and south America [39,172].

In conclusion, this thesis work revealed that northeastern Italy is a currently active epidemiological focus of human TL that is characterized by raising incidence and relevant number of ML cases. Moreover, results of molecular studies evidenced the emergence of a new dermotropic strain of *L. infantum* that is characterized by peculiar phylogenetic characteristics and phenotypic features, potentially causing increased disease severity. All these findings suggest the need to raise awareness about TL among Italian clinicians and pathologists, to encourage the establishment of molecular diagnosis and surveillance networks for this neglected disease and to continue the characterization studies of strains circulating in the Italian peninsula.

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# Appendix

# Appendix A

| Patient ID | Sample ID | Sample collecion date | Authoctonous [Yes/No (Importing country)] | CL/ML | Severe/Mild Disease | ITS1 typing result | Accession Number ITS1 sequence | hsp70 typing result | *L. donovani* complex sequenc variant hsp70 | Accession Number hsp70 sequence |
|---|---|---|---|---|---|---|---|---|---|---|
| BO02 | BO02 | 03/04/2014 | Yes | CL | Severe | L. infantum | MN783222 | L. donovani complex | G | MN938328 |
| BO03 | BO03 | 02/12/2014 | Yes | CL | Mild | L. infantum | MN783223 | L. donovani complex | G | MN938329 |
| BO04 | BO04 | 15/07/2014 | Yes | CL | Severe | L. infantum | MN783224 | L. donovani complex | F | MN938330 |
| BO05 | BO05 | 30/09/2014 | Yes | CL | Severe | L. infantum | MN783225 | L. donovani complex | G | MN938331 |
| BO06 | BO06 | 24/04/2014 | Yes | CL | Severe | L. infantum | MN783226 | L. infantum | A | MN938341 |
| BO07 | BO07 | 15/12/2014 | Yes | CL | Mild | NA | / | L. infantum | A | MN938342 |
| BO08 | BO08 | 25/11/2014 | Yes | CL | Severe | L. infantum | MN783227 | L. donovani complex | G | MN938332 |
| BO09 | BO09 | 07/01/2015 | Yes | CL | Severe | L. infantum | MN783228 | L. infantum | A | MN938343 |
| BO10 | BO10 | 09/06/2015 | Yes | CL | Mild | L. infantum | MN783229 | L. infantum | A | MN938344 |
| BO101 | BO101 | 20/02/2019 | Yes | CL | Severe | L. infantum | MT013005 | L. infantum | A | OR682832 |
| BO102 | BO102 | 04/04/2019 | Yes | CL | Severe | L. infantum | MT013006 | L. infantum | A | OR682833 |
| BO11 | BO11 | 09/06/2015 | Yes | CL | Severe | NA | / | L. infantum | A | MN938345 |
| BO110 | BO110 | 16/06/2014 | Yes | CL | Severe | L. infantum | OR676879 | L. donovani complex | B | OR682834 |
| BO111 | BO111 | 22/11/2014 | Yes | CL | Severe | NA | / | L. donovani complex | B | OR682835 |
| BO112 | BO112 | 04/11/2015 | No (Bangladesh) | CL | Severe | NA | / | L. donovani complex | B | OR682836 |
| BO113 | BO113 | 14/03/2019 | Yes | CL | Severe | L. infantum | OR676880 | L. infantum | A | OR682837 |
| BO114 | BO114 | 23/06/2020 | Yes | CL | Mild | L. infantum | OR676881 | L. infantum | A | OR682838 |
| BO115 | BO115 | 30/10/2020 | Yes | CL | Mild | L. infantum | OR676882 | L. donovani complex | C | OR682839 |

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|---|---|---|---|---|---|---|---|---|---|---|
| BO116 | BO116 | 29/12/2020 | Yes | CL | Mild | L. infantum | OR676883 | L. donovani complex | G | OR682840 |
| BO117 | BO117 | 30/01/2020 | Yes | ML | Severe | L. infantum | OR676884 | L. donovani complex | G | OR682841 |
| BO118 | BO118 | 28/10/2019 | Yes | CL | Mild | NA | / | L. donovani complex | C | OR682842 |
| BO119 | BO119 | 09/01/2019 | Yes | CL | Mild | NA | / | L. donovani complex | E | OR682843 |
| BO12 | BO12 | 02/09/2015 | Yes | CL | Mild | L. infantum | MN783230 | L. infantum | A | MN938346 |
| BO120 | BO120 | 30/03/2020 | Yes | CL | Mild | L. infantum | OR676885 | L. donovani complex | E | OR682844 |
| BO121 | BO121 | 21/08/2020 | Yes | CL | Severe | L. infantum | OR676886 | L. donovani complex | G | OR682845 |
| BO122 | BO122 | 26/05/2020 | Yes | CL | Severe | L. infantum | OR676887 | L. donovani complex | E | OR682846 |
| BO123 | BO123 | 04/11/2019 | Yes | CL | Mild | NA | / | L. infantum | A | OR682847 |
| BO124 | BO124 | 17/03/2020 | Yes | CL | Mild | L. infantum | OR676888 | L. infantum | A | OR682848 |
| BO125 | BO125 | 07/08/2020 | Yes | CL | Mild | L. infantum | OR676889 | L. donovani complex | G | OR682849 |
| BO126 | BO126 | 01/12/2018 | Yes | CL | Mild | NA | / | L. donovani complex | G | OR682850 |
| BO127 | BO127 | 12/12/2020 | Yes | CL | Mild | L. infantum | OR676890 | L. donovani complex | G | OR682851 |
| BO128 | BO128 | 11/10/2017 | Yes | CL | Mild | L. infantum | OR676891 | L. donovani complex | G | OR682852 |
| BO129 | BO129 (a) | 04/12/2019 | Yes | CL | Mild | L. infantum | OR676892 | L. donovani complex | G | OR682853 |
| BO129 | BO129 (b) | 21/07/2020 | Yes | CL | Mild | L. infantum | OR676893 | L. donovani complex | G | OR682854 |
| BO13 | BO13 | 24/11/2015 | Yes | CL | Severe | L. infantum | MN783231 | L. donovani complex | G | MN938333 |
| BO130 | BO130 | 02/09/2020 | Yes | CL | Mild | L. infantum | OR676894 | L. infantum | A | OR682855 |
| BO131 | BO131 | 20/07/2020 | Yes | CL | Mild | L. infantum | OR676895 | L. infantum | A | OR682856 |
| BO132 | BO132 | 20/06/2020 | Yes | CL | Mild | L. infantum | OR676896 | L. donovani complex | G | OR682857 |
| BO133 | BO133 | 09/12/2019 | Yes | CL | Severe | L. infantum | OR676897 | L. infantum | H | OR682858 |
| BO134 | BO134 | 22/12/2020 | Yes | CL | Mild | L. infantum | OR676898 | L. infantum | A | OR682859 |

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|---|---|---|---|---|---|---|---|---|---|---|
| BO135 | BO135 | 10/09/2020 | Yes | CL | Mild | L. infantum | OR676899 | L. infantum | A | OR682860 |
| BO136 | BO136 (a) | 24/07/2020 | Yes | ML | Severe | L. infantum | OR676900 | L. donovani complex | G | OR682861 |
| BO136 | BO136 (b) | 24/07/2020 | Yes | ML | Severe | L. infantum | OR676901 | L. donovani complex | G | OR682862 |
| BO137 | BO137 (a) | 13/09/2017 | No (Burkina Faso) | CL | Mild | L. major | OR671123 | L. major | / | OR682802 |
| BO137 | BO137 (b) | 27/07/2017 | No (Burkina Faso) | CL | Mild | L. major | OR671122 | L. major | / | OR682803 |
| BO138 | BO138 | 01/07/2019 | No (Morocco) | CL | Severe | L. tropica | OR671704 | L. tropica | / | OR682805 |
| BO139 | BO139 | 15/05/2019 | No (Morocco) | CL | Mild | L. tropica | OR671705 | L. tropica | / | OR682806 |
| BO14 | BO14 | 21/04/2015 | Yes | CL | Severe | L. infantum | MN783232 | L. donovani complex | G | MN938334 |
| BO140 | BO140 | 28/01/2020 | No (Tunisia) | CL | Mild | L. major | OR671124 | L. major | / | OR682804 |
| BO15 | BO15 | 28/11/2018 | No (Tunisia) | CL | Severe | L. major | MT015655 | L. major | / | OR682801 |
| BO16 | BO16 | 03/03/2016 | Yes | CL | Severe | NA | / | L. infantum | A | MN938347 |
| BO17 | BO17 | 13/01/2016 | No (Middle East) | CL | Mild | NA | / | L. major | / | MN938352 |
| BO18 | BO18 | 16/01/2016 | No (Tunisia) | CL | Severe | L. major | MN783287 | L. major | / | MN938353 |
| BO19 | BO19 | 06/05/2016 | Yes | CL | Mild | L. infantum | MN783233 | L. infantum | A | MN938348 |
| BO20 | BO20 | 15/12/2016 | Yes | CL | Severe | L. infantum | MN783234 | L. donovani complex | G | MN938335 |
| BO21 | BO21 | 02/08/2016 | Yes | CL | Mild | L. infantum | MN783235 | L. infantum | A | MN938349 |
| BO22 | BO22 | 17/05/2016 | Yes | ML | Severe | L. infantum | MN783236 | L. infantum | A | MN938350 |
| BO23 | BO23 | 17/01/2016 | Yes | ML | Severe | L. infantum | MN783237 | L. donovani complex | G | MN938336 |
| BO24 | BO24 | 14/09/2016 | Yes | CL | Severe | L. infantum | MN783238 | L. donovani complex | G | MN938337 |
| BO25 | BO25 | 20/07/2016 | Yes | CL | Mild | L. infantum | MN783239 | L. donovani complex | G | MN938338 |
| BO26 | BO26 | 23/11/2016 | Yes | CL | Severe | L. infantum | MN783240 | L. donovani complex | C | MN938339 |
| BO27 | BO27 | 10/01/2017 | Yes | CL | Severe | L. infantum | MN783241 | L. donovani complex | C | MN938340 |
| BO41 | BO41 | 24/02/2017 | Yes | CL | Mild | L. infantum | MN783255 | L. donovani complex | G | OR682807 |
| BO42 | BO42 | 07/03/2017 | Yes | CL | Mild | L. infantum | MN783256 | L. infantum | A | OR682808 |

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|---|---|---|---|---|---|---|---|---|---|---|
| BO47 | BO47 | 13/11/2017 | Yes | ML | Severe | L. infantum | MN783261 | L. donovani complex | G | OR682809 |
| BO67 | BO67 | 10/12/2018 | Yes | CL | Severe | L. infantum | MN783280 | L. donovani complex | E | OR682810 |
| BO68 | BO68 | 05/04/2018 | Yes | CL | Mild | L. infantum | MN783281 | L. donovani complex | C | OR682811 |
| BO69 | BO69 | 26/07/2018 | Yes | CL | Severe | L. infantum | MN783282 | L. donovani complex | G | OR682812 |
| BO73 | BO73 | 03/12/2018 | Yes | CL | Severe | L. infantum | MN783286 | L. donovani complex | G | OR682813 |
| BO75 | BO75 | 20/03/2019 | Yes | CL | Mild | L. infantum | MN872286 | L. donovani complex | E | OR682814 |
| BO77 | BO77 | 14/06/2018 | Yes | CL | Mild | L. infantum | MT005077 | L. infantum | A | OR682815 |
| BO78 | BO78 | 29/06/2018 | Yes | CL | Mild | L. infantum | MT005078 | L. infantum | A | OR682816 |
| BO82 | BO82 (a) | 16/10/2018 | Yes | CL | Severe | L. infantum | MT005082 | NA | NA | / |
| BO82 | BO82 (b) | 01/03/2019 | Yes | CL | Severe | L. infantum | OR676902 | L. donovani complex | D | OR682817 |
| BO83 | BO83 | 10/10/2018 | Yes | CL | Mild | L. infantum | MT005083 | L. donovani complex | G | OR682818 |
| BO85 | BO85 | 12/10/2018 | Yes | CL | Mild | L. infantum | MT005085 | L. infantum | A | OR682819 |
| BO86 | BO86 | 14/12/2017 | Yes | CL | Mild | L. infantum | MT005086 | L. donovani complex | E | OR682820 |
| BO87 | BO87 | 06/02/2018 | Yes | CL | Severe | L. infantum | MT005087 | L. donovani complex | G | OR682821 |
| BO89 | BO89 | 10/01/2019 | Yes | CL | Severe | L. infantum | MT012994 | L. infantum | A | OR682822 |
| BO90 | BO90 (a) | 26/03/2019 | Yes | ML | Severe | NA | / | L. infantum | A | OR682823 |
| BO90 | BO90 (b) | 26/03/2019 | Yes | ML | Severe | L. infantum | MT012995 | L. infantum | A | OR682824 |
| BO92 | BO92 | 21/05/2019 | Yes | CL | Mild | L. infantum | MT012997 | L. infantum | A | OR682825 |
| BO94 | BO94 | 26/05/2019 | Yes | CL | Mild | L. infantum | MT012999 | L. donovani complex | E | OR682826 |
| BO95 | BO95 | 09/09/2019 | Yes | CL | Severe | L. infantum | MT013000 | L. donovani complex | G | OR682827 |
| BO96 | BO96 | 19/09/2019 | Yes | CL | Severe | L. infantum | MT013001 | L. donovani complex | G | OR682828 |
| BO97 | BO97 | 26/09/2019 | Yes | CL | Severe | L. infantum | MT013002 | L. donovani complex | G | OR682829 |
| BO98 | BO98 | 26/09/2019 | Yes | CL | Severe | L. infantum | MT013003 | L. infantum | A | OR682830 |

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|---|---|---|---|---|---|---|---|---|---|---|
| BO99 | BO99 | 24/02/2019 | Yes | CL | Mild | L. infantum | MT013004 | L. infantum | A | OR682831 |

# Appendix B

| Country | WHO code | Leish_group (Franssen et al 2020) | Accession number (ENA or SRA) | LinJ.28:1.114.069(-) (position 764) | LinJ.28:1.113.965(-) (position 868) |
|---|---|---|---|---|---|
| Spain | MCAN/ES/98/LLM-877 (JPCM5) | Linf1 | ERS001832 | A | T |
| Turkey | ITOB/TR/2007/CUK10 | CUK_Linf | ERS026264 | - | - |
| Turkey | ITOB/TR/2007/CUK11 | CUK_Linf | ERS026265 | - | - |
| Turkey | ITOB/TR/2007/CUK12 | CUK_Linf | ERS026266 | - | - |
| Turkey | ITOB/TR/2005/CUK2 | CUK_Linf | ERS026256 | - | - |
| Turkey | ITOB/TR/2005/CUK3 | CUK_Linf | ERS026257 | - | - |
| Turkey | ITOB/TR/2006/CUK4 | CUK_Linf | ERS026258 | A:36;C:0;G:0;T:1 | - |
| Turkey | ITOB/TR/2006/CUK5 | CUK_Linf | ERS026259 | - | - |
| Turkey | ITOB/TR/2006/CUK6 | CUK_Linf | ERS026260 | - | - |
| Turkey | ITOB/TR/2006/CUK7 | CUK_Linf | ERS026261 | - | - |
| Turkey | ITOB/TR/2006/CUK8 | CUK_Linf | ERS026262 | - | - |
| Turkey | ITOB/TR/2007/CUK9 | CUK_Linf | ERS026263 | A:16;C:1;G:0;T:0 | - |
| Cyprus | MHOM/CY/2006/CH32 | CH_Linf | ERS082780 | - | - |
| Cyprus | MHOM/CY/2006/CH33 | CH_Linf | ERS097150 | - | - |
| Cyprus | MHOM/CY/2006/CH34 | CH_Linf | ERS097157 | A:30;C:1;G:0;T:0 | - |
| Cyprus | MHOM/CY/2006/CH35 | CH_Linf | ERS082781 | - | A:0;C:0;G:1;T:54 |
| Cyprus | MHOM/CY/2006/CH36 | CH_Linf | ERS097158 | - | - |
| Turkey | MHOM/TR/??/EP59 | other_Linf | ERS082784 | - | - |
| Turkey | MCAN/TR/2000/EP50 | Linf1 | ERS3773247 | - | - |
| Italy | IPRF/IT/85/ISS174 | Linf1 | ERS097154 | - | - |
| Italy | MCAN/IT/02/ISS2420 | Linf1 | ERS104327 | - | - |
| Italy | MHOM/IT/02/ISS2426 | Linf1 | ERS104322 | A:33;C:0;G:0;T:1 | - |
| Italy | MHOM/IT/02/ISS2429 | Linf1 | ERS104329 | - | A:0;C:1;G:1;T:56 |
| Italy | MHOM/IT/02/ISS2508 | Linf1 | ERS104330 | - | - |
| Spain | MCAN/ES/92/BCN-83 | Linf1 | ERS177293 | - | - |
| Spain | MCAN/ES/92/BCN-87 | Linf1 | ERS177294 | - | - |
| Spain | MHOM/ES/1992/LLM373 | Linf1 | ERS3773245 | A:45;C:2;G:0;T:0 | - |
| France | MHOM/FR/90/LPN66 | Linf1 | ERS104316 | A:45;C:0;G:1;T:0 | - |
| France | MHOM/FR/96/LPM161 | Linf1 | ERS104318 | - | - |

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|---|---|---|---|---|---|
| France | MCAN/FR/87/RM1 | Linf1 | ERS097142 | - | - |
| Spain | MHOM/ES/1987/Lombardi | Linf1 | ERS3773249 | - | A:0;C:0;G:1;T:27 |
| France | MHOM/FR/62/LRC-L47 | Linf1 | ERS066261 | A:38;C:1;G:0;T:0 | - |
| Portugal | MCAN/PT/05/IMT373cl1 | Linf1 | ERS100733 | - | - |
| Tunisia | MHOM/TN/1980/IPT1 | Linf1 | ERS3773255 | - | - |
| Portugal | MHOM/PT/00/IMT260 | Linf1 | ERS066264 | - | A:0;C:0;G:1;T:58 |
| France | MHOM/FR/1996/LEM3249 | Linf1 | ERS3773261 | - | - |
| France | MHOM/FR/95/LPN114 | Linf1 | ERS066263 | - | - |
| Morocco | MHOM/MA/67/ITMAP26 | Linf1 | ERS407440 | - | - |

# Appendix C

## >MHOM/IT/2021/MO44

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TGATATCATTAAGTTTTATATATTATGGTTGTATAATATTTTATTTTTTAATATTGATTGTATTATGTTTTTT

GAAGAATATTTTTATTTGTAATGATTGGTTTTATTTTTTATATTTTCGATTTGATATTTTATTTGTTTTTATA

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# Appendix D

| WHO_code | Name in the tree | ENA/GenBank | Study accession | Reference |
|---|---|---|---|---|
| Leishmania infantum CH32 strain MHOM/CY/2006/CH32 | CH32 | ERR205748 | 10.17632/nnf649r9kf.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CH33 strain MHOM/CY/2006/CH33 | CH33 | ERR205749 | 10.17632/nnf649r9kf.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CH34 strain MHOM/CY/2006/CH34 | CH34 | ERR205780 | 10.17632/nnf649r9kf.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CH35 strain MHOM/CY/2006/CH35 | CH35 | ERR205781 | 10.17632/nnf649r9kf.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CH36 strain MHOM/CY/2006/CH36 | CH36 | ERR205782 | 10.17632/nnf649r9kf.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CUK1 strain MHOM/TR/2005/HUM1 | CUK1 | ERR328067 | 10.17632/vh55y46yr4.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CUK12 strain ITOB/TR/2007/TOB12 | CUK12 | ERR328078 | 10.17632/vh55y46yr4.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum CUK4 strain ITOB/TR/2006/TOB4 | CUK4 | ERR328070 | 10.17632/vh55y46yr4.1 | doi: 10.3390/genes13061070 |
| Leishmania donovani HU3 MHOM/ET/67/HU3 | L. donovaniHU3 | ERR2191875 | 10.17632/74d3b3pnvt.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum isolate 02A_EP | Linf02A_EP | SRR6369656 | 10.17632/twkkb6bmdv.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum isolate LIPA60 | LIPA60 | MT762281.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum isolate LIPA83 | LIPA83 | MT762282.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum isolate Susto | LiSusto | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-1345 strain MCAN/ES/2004/LLM-1345 | LLM-1345 | SRR6369628 | 10.17632/twkkb6bmdv.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum LLM-1356 strain MCAN/ES/2004/LLM-1356 | LLM-1356 | SRR6369636 | 10.17632/twkkb6bmdv.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum LLM-2404 strain MHOM/ES/2018/LLM-2404 | LLM-2404 | / | 10.17632/w65x97kng9.1 | doi: 10.1128/mBio.01399-18. |
| Leishmania infantum LLM-2405 strain MHOM/ES/2018/LLM-2405 | LLM-2405 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |

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| Leishmania infantum LLM-2406 strain MHOM/ES/2018/LLM-2406 | LLM-2406 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2407 strain MHOM/ES/2018/LLM-2407 | LLM-2407 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2408 strain MHOM/ES/2018/LLM-2408 | LLM-2408 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2409 strain MHOM/ES/2018/LLM-2409 | LLM-2409 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2410 strain MHOM/ES/2018/LLM-2410 | LLM-2410 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2411 strain MHOM/ES/2018/LLM-2411 | LLM-2411 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2412 strain MHOM/ES/2018/LLM-2412 | LLM-2412 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2413 strain MHOM/ES/2018/LLM-2413 | LLM-2413 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2414 strain MHOM/ES/2018/LLM-2414 | LLM-2414 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2417 strain MHOM/ES/2018/LLM-2417 | LLM-2417 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum LLM-2418 strain MHOM/ES/2018/LLM-2418 | LLM-2418 | / | 10.17632/w65x97kng9.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1213 strain MHOM/BR/PI/2013/1213 | P1213 | SRR10478769 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1220 strain MHOM/BR/MA/2013/1220 | P1220 | SRR10478768 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1255 strain MHOM/BR/MA/2016/1255 | P1255 | SRR10478787 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1661 strain MHOM/BR/PI/2016/1661 | P1661 | SRR10478775 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1689 strain MHOM/BR/MA/2016/1689 | P1689 | SRR10478774 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1798 strain MHOM/BR/PI/2016/1798 | P1798 | SRR10478773 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P1801 strain MHOM/BR/MA/2016/1801 | P1801 | SRR10478772 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2008 strain MHOM/BR/PI/2016/2008 | P2008 | SRR10478771 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |

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| Leishmania infantum P2145 strain MHOM/BR/PI/2016/2145 | P2145 | SRR10478770 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2492 strain MHOM/BR/MA/2016/2492 | P2492 | SRR10478767 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2525 strain MHOM/BR/MA/2016/2525 | P2525 | SRR10478796 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2527 strain MHOM/BR/PI/2016/2527 | P2527 | SRR10478795 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2578 strain MHOM/BR/PI/2013/2578 | P2578 | SRR10478794 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2765 strain MHOM/BR/PI/2016/2765 | P2765 | SRR10478793 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2914 strain MHOM/BR/MA/2016/2914 | P2914 | SRR10478792 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P2959 strain MHOM/BR/PI/2016/2959 | P2959 | SRR10478791 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3097 strain MHOM/BR/PI/2016/3097 | P3097 | SRR10478790 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3116 strain MHOM/BR/MA/2013/3116 | P3116 | SRR10478788 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3130 strain MHOM/BR/PI/2016/3130 | P3130 | SRR10478786 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3144 strain MHOM/BR/PI/2016/3144 | P3144 | SRR10478785 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3148 strain MHOM/BR/MA/2016/3148 | P3148 | SRR10478784 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3149 strain MHOM/BR/MA/2016/3149 | P3149 | SRR10478783 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3151 strain MHOM/BR/PI/2016/3151 | P3151 | SRR10478782 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3153 strain MHOM/BR/PI/2016/3153 | P3153 | SRR10478781 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3167 strain MHOM/BR/PI/2016/3167 | P3167 | SRR10478780 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3169 strain MHOM/BR/PI/2016/3169 | P3169 | SRR10478779 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum P3170 strain MHOM/BR/MA/2016/3170 | P3170 | SRR10478778 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |

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| Leishmania infantum P3171 strain MHOM/BR/PI/2016/3171 | P3171 | SRR10478777 | 10.17632/rmjc4wmr4x.1 | doi: 10.3390/genes13061070 |
| Leishmania infantum isolate ZK25 | ZK25 | MT762283.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1099/mgen.0.000444. |
| Leishmania infantum isolate ZK28 | ZK28 | MT762284.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1099/mgen.0.000444. |
| Leishmania infantum isolate ZK43 | ZK43 | MT762285.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1099/mgen.0.000444. |
| Leishmania infantum isolate ZK47 | ZK47 | MT762287.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1099/mgen.0.000444. |
| Leishmania infantum isolate ZK5 | ZK5 | MT762286.1 | 10.17632/nwm8dw97sg.1 | doi: 10.1099/mgen.0.000444. |
| MCAN/ES/92/BCN-83 | BCN-83 | ERS177293 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MCAN/ES/92/BCN-87 | BCN-87 | ERS177294 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| ITOB/TR/2005/CUK2 | CUK2 | ERS026256 | PRJEB2473 | doi: 10.7554/eLife.51243. |
| ITOB/TR/2005/CUK3 | CUK3 | ERS026257 | PRJEB2473 | doi: 10.7554/eLife.51243. |
| MHOM/TR/??/EP59 | EP59 | ERS082784 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MHOM/PT/00/IMT260 | IMT260 | ERS066264 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MCAN/PT/05/IMT373cl1 | IMT373cl1 | ERS100733 | PRJEB2724 | doi: 10.7554/eLife.51243. |
| MHOM/TN/1980/IPT1 | IPT1 | SRR21601459 | PRJNA881045 | doi: 10.3390/microorganisms10112256. |
| IPRF/IT/85/ISS174 | ISS174 | ERS097154 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MCAN/IT/02/ISS2420 | ISS2420 | ERS104327 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MHOM/IT/02/ISS2429 | ISS2429 | ERS104329 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MHOM/IT/02/ISS2508 | ISS2508 | ERS104330 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MHOM/TN/1980/IPT1 | ITMAP26 | ERS407440 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| Leishmania infantum JPCM5 MCAN/ES/98/LLM-724 | JPCM5 | LR697137 | 10.17632/nszm7rb8y7.1 | doi: 10.3390/genes10100758. |
| MHOM/FR/95/LPN114 | LPN114 | ERS066263 | PRJEB2600 | doi: 10.7554/eLife.51243. |
| MHOM/FR/62/LRC-L47 | LRC-L47 | ERS066261 | PRJEB2600 | doi: 10.7554/eLife.51243. |