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The conundrum of Human Visceral Leishmaniasis in Emilia-Romagna, Italy: Are wild and peridomestic animals potential reservoirs?

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Chapter 1

General Overview

1.1 Leishmaniasis in the Mediterranean area

Human leishmaniasis is a complex of diseases that constitute a major health burden, caused by protozoan of the genus *Leishmania*. The disease is endemic in nearly 100 countries localized in tropical, sub-tropical and temperate Regions (Gradoni, 2018) and it is estimated that 350 million people are at risk of infection (Bern *et al*., 2008) with 700,000 to 1 million new cases and 26,000 to 65,000 death per year (World Health Organization, https://www.who.int/leishmaniasis/en/).

Three main clinical forms of leishmaniasis are described (WHO, https://www.who.int/leishmaniasis/en/): (*i*) visceral (VL) (also known as kala-azar) with fatal outcome in 95% of cases if untreated, is considered the most severe form which causes irregular bouts of fever, weight loss, anemia and hepato-splenomegaly; (*ii*) cutaneous (CL), the most frequently observed, it is characterized by skin lesion, mainly ulcers resulting in life-long scars, disability or social stigma; (*iii*) mucocutaneous (MCL), mainly diagnosed in Southern America, leads to partial or total destruction of the nasal or oropharynx mucosa.

Leishmaniasis still ranks on top of neglected tropical diseases (NTDs), in particular it is the third tropical neglected disease for social burden caused by protozoans after malaria and infantile cryptosporidiosis (Fenwick, 2012). The reason beyond this is uncertain, some authors hypotize that leishmaniasis is a poverty-related disease - trait d'union of NTDs (Pace, 2014), others because of its epidemiological and medical complexity (Gradoni, 2018).

The link between leishmaniasis and poverty is strong as well as complex for its burden is surely on the poorest segments of global population (Alvar *et al*., 2006). Poverty not only increases the risk of infection because of poor housing conditions and subsequent lack of personal protective measures, as well as the poor health care and

diagnostic technique (Santos *et al*., 2005), but it also constitutes a potentiator of morbidity and mortality most pervasively through poor nutrition (Alvar *et al*., 2006).

Nevertheless, leishmaniasis is a multifaced disease for several factors. As early mentioned, the disease has a wide range of clinical manifestations, but also asymptomatic infection can occur along with acute or chronic forms that can be less common or even unrecognized (Gradoni, 2018). Furthermore, leishmaniasis is a vector-borne disease, and the transmission occurs almost exclusively through the bite of the phlebotomine vector (CDC, https://www.cdc.gov/parasites/leishmaniasis), therefore, even the interaction between vertebrate host, invertebrate host and environment is a crucial factor, and regional differences in the epidemiology of the disease should be considered (Fuehrer and Savić, 2017; Inceboz, 2019).

Leishmaniasis is endemic in the Mediterranean regions and *Leishmania infantum* is the main causative agent (Fig. 1) (ECDC, 2022), even though *Leishmania major* and *Leishmania tropica* are reported, as agents of the cutaneous form in Northern Africa (Amro *et al*., 2017) and, only the latter, in the island of Crete (Greece) (Christodoulou *et al*., 2012).

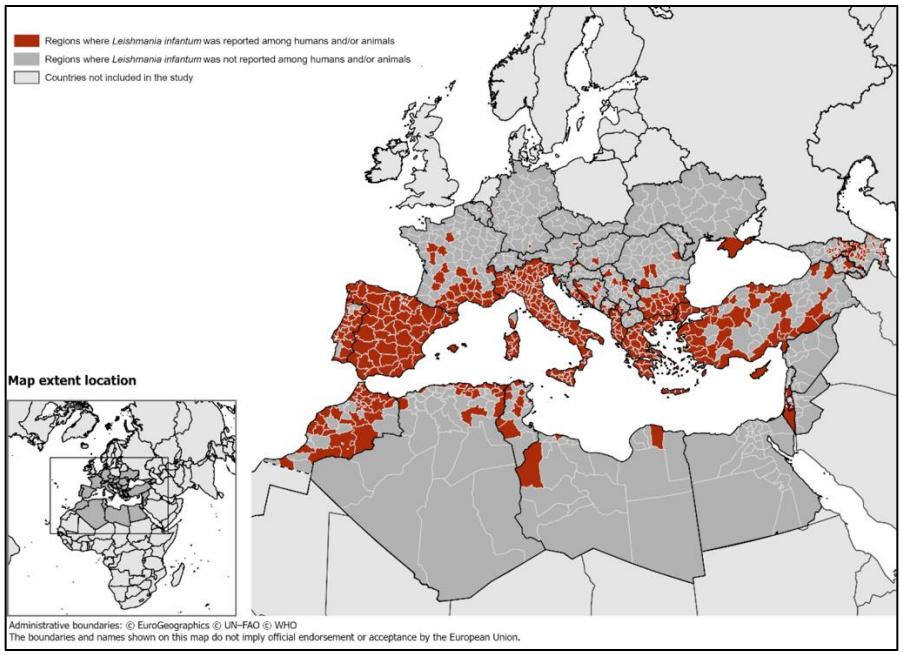

Figure 1. Map representative of Leishmania infantum *reports in human and animal patients (ECDC, 2022).*

L. infantum has a dixenic life cycle that includes a vertebrate host and an insect vector. To date sand flies are the only vectors recognized of the pathogenic *Leishmania* species (Van der Auwera *et al*., 2015). With reference to the Old World, *L. infantum* is spread mostly by female hematophagous sand flies of the genus *Phlebotomus* during the blood meal, while in the New World the role of vector is attributed to *Lutzomyia* spp. (Pace, 2014).

The main reservoir of *L. infantum* is the dog, in which the protozoa cause canine leishmaniasis (CanL). Conversely than human leishmaniasis that are differentiated in mainly three clinical presentations, in dogs CanL is a chronic, life-long infection that is not distinguished in different clinical forms but causes a reticuloendotheliosis with a wide range of symptoms (Alvar *et al*., 2004; Roura *et al*., 2021).

Risk factors for human leishmaniasis differ according to the countries. In eastern Europe is usually mostly reported in children and is associated with poor living conditions, while in western Europe is diagnosed as coinfection in HIV-patients (WHO, https://apps.who.int/gho/data/node.main.NTDLEISH?lang=en). At last, in southern Europe peri-urban residential areas have been associated to major risks of infection (WHO, https://apps.who.int/gho/data/node.main.NTDLEISH?lang=en). The notification of human leishmaniasis is compulsory in all the endemic countries except for France, Egypt, and Serbia, while the animal leishmaniasis is not notifiable in France, Turkey, Romania, Serbia, and Palestine (ECDC, 2022).

In Italy, leishmaniasis has been described since 1905 (Pampiglione, 1975), mostly in the Tyrrhenian coastal area, the low Adriatic coast and in the Islands. The disease is subjected to mandatory notification both for human and canine cases by Ministerial Decree 15/12/1990 and Italian presidential Decree n. 320, 08/02/1954, respectively. Phlebotomine sand flies and dogs are still recognized as vector and major reservoir, respectively.

However, the progressive increase in geographical distribution into previously non-endemic territories and the consistent growth of human cases made questionable the role of dog as the only reservoir (Gradoni *et al*., 2022). Focus has shifted in the

search of a potential sylvatic animal reservoir in the presence of conditions such as its close relationship with humans and relative abundance, further supported by the results of surveys carried out in Spain, Portugal and Greece which indicated the role of wild hare, mice, and rodents respectively, as reservoir of *L. infantum* (Molina *et al*., 2012; Helhazar *et al*., 2013; Tsakmakidis *et al*., 2017).

Dogs are still recognized as the major domestic reservoir, especially in Southern and Insular regions of Italy. In this epidemiological scenario, the Emilia-Romagna region (ER), located in north-eastern Italy, always had a distinct epidemiological situation. Indeed, CL was reported since 1934 (Monacelli) when it was first described, and in 1951 ER ranked on top three regions for CL cases (Pullè, 1951).

On the contrary, autochthonous cases of Visceral leishmaniasis were described starting from 1951 (Suzzi Valli and Dominci, 1953). Until the '70, only three other autochthonous cases were here reported (Fusaroli, 1952; Giungi, 1954; Artusi and Grossi, 1962), possibly owing to the massive use of insecticides in agriculture and holyday localities (Pampiglione, 1975). In late 1971 an outbreak of VL involved several municipalities from the province of Bologna: 60 cases were confirmed as autochthonous, and the lethality observed was 21.7% (Pampiglione, 1975). Besides the astonishing lethality, partially related to late diagnosis and onset of specific therapy, distinctive traits were characterized, such as specific geographical localization and clinical presentation of the cases. To note that Sanitary Authority performed an investigation on the canine population of the studied area which revealed a seroprevalence of 1.6% (Pampiglione *et al*., 1974). Over the years, the progressive increase in geographical distribution into previously non endemic territories and the consistent growth of human cases (Varani *et al*., 2013) questioned the role of dog as the only reservoir in ER, as previously suggested by Pampiglione (1975).

1.2 First description and taxonomy of *Leishmania infantum*

The first isolation of *Leishmania* spp. is dated in November 1900, when the Scottish pathologist William B. Leishman observed "ovoid bodies" in smears taken post-mortem from the spleen of a 23-year-old soldier died in Dum Dum (Calcutta, India). The soldier had passed away after 7 months of a very severe form of what was called Dum Dum fever, showing symptoms as emaciation and splenomegaly (Leishman, 1903). After experimental infection on a rat, he supposed that the bodies observed were degenerated forms of trypanosomes. Few weeks later, the Irish doctor Charles Donovan reported similar bodies from spleen samples from native Indian patients with similar clinical manifestation (Donovan, 1903). Not persuaded by the theory of Leishman, Donovan sent a splenic slice to Mesnil and Laveran who firstly described them as a new species of the genus *Piroplasma* (Laveran and Mensil, 1904). Shortly after, the physician Ronald Ross classified the "ovoid bodies" as a new genus and species and proposed the name *Leishmania donovani* (Ross, 1904). A related syndrome was also reported in Tunisia in children suffering from splenic anemia by the French bacteriologist Charles J.H. Nicolle, who erected the new species *L. infantum* (Nicolle, 1908).

Leishmania infantum belongs to the phylum Euglenozoa, class Kinetoplastida, order Trypanosomatida, family Trypanosomatidae (Deplazes *et al*., 2016). The Trypanosomatidae family is comprehensive of eight monoxenous genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Sergeia*, *Wallaceomnas*, *Blachomonas* and *Jaeniomonas*; three dixenous genera, whose life cycle occur in two hosts (one invertebrate and a vertebrate or a plant): *Trypanosoma, Phytomonas* and *Leishmania;* and the free-living Strigomonadinae subfamily characterized by the presence of endosymbiontic bacteria with *Angomonas*, *Strigomonas* and *Kentomonas* (Akhoundi *et al*., 2016). The genus *Leishmania* is further divided in three subgenera: *Leishmania* comprehensive of mammalian pathogenic species, *Sauroleishmania*, including only the species *L. tarentolae*, and the subgenus *Viannia* only reported in Central and South America as agent of CL and MCL (Llanes *et al*., 2018; Maurício, 2018).

The taxonomy of *Leishmania* genus has always been subject of debates among parasitologist; in earlier times major critical factors were the lack of differences in morphology between species and the arduous cultivation. With the introduction of biochemical and molecular techniques WHO (1990) proposed a new taxonomic scheme still in use, excluding some subsequent redefinitions of species or species complex (Fig. 2) (Maurício *et al*., 2000; McMahon-Pratt and Alexander, 2004; Akhoundi *et al*., 2017).

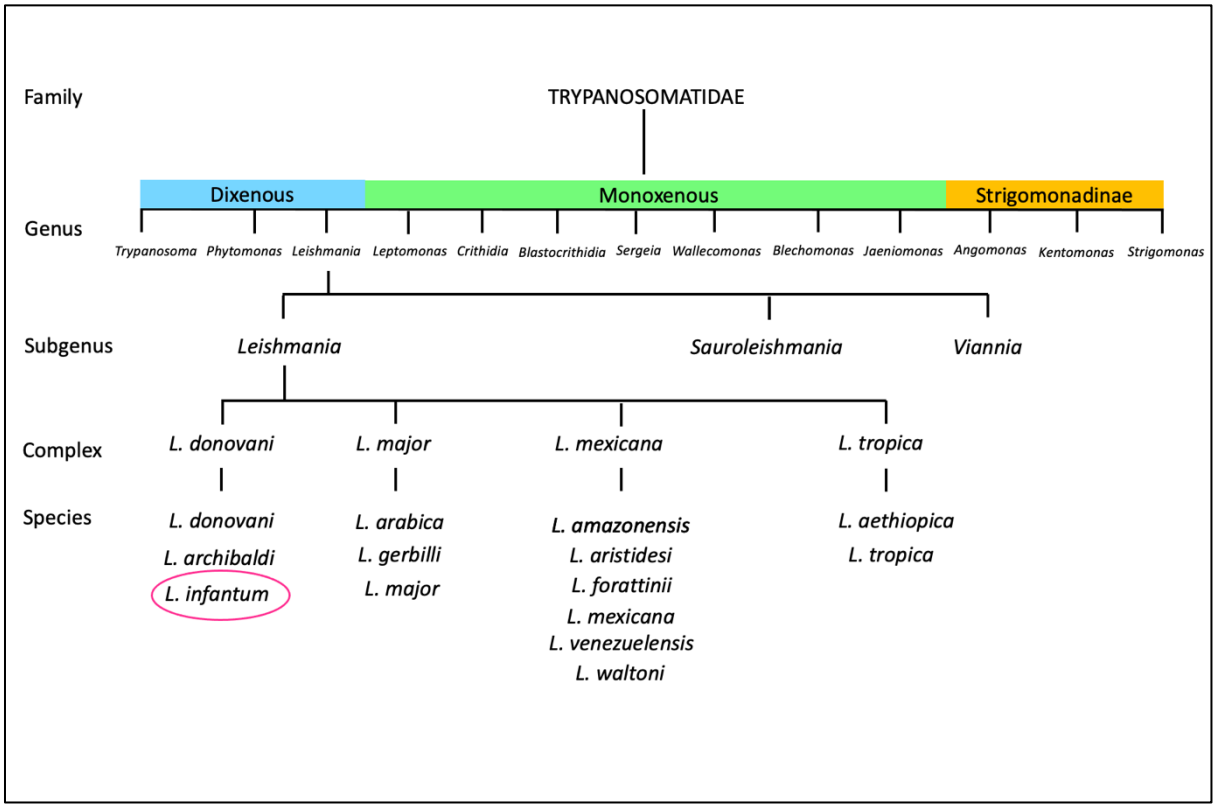

Figure 2. Taxonomic scheme of Leishmania *genus not comprehensive of synonymous species. Please note this figure is a visual representation of the current taxonomy of* Leishmania *genus, and not a phylogenetic tree.*

L. infantum belongs to the *L. donovani* complex which also includes the species *L. donovani*, *L. archibaldi*, and formerly *L. chagasi* (later recognized as a synonym of *L. infantum*) (Fernández-Arévalo *et al*., 2020), and is the only parasitic species circulating in Italy (Rugna *et al*., 2018)

Among *L. infantum* different strains have been described by the MultiLocus Enzyme Electrophoresis (MLEE), which to date is still considered the gold standard for *Leishmania* strain identification according to WHO 2010, whereas for diagnosis,

evidence of parasites is considered the gold standard (OIE, 2021). This technique differentiates *Leishmania* spp. into strains called zymodemes based on electrophoretic mobility of 15 enzymes (Pratlong *et al*., 2016). The reference laboratory that performs this analysis is the *Centre de Ressources Biologiques des Leishmania* (CRB-Leish) in Montpellier (France). Each strain is then defined by a CRB-Leish called "LEM" and WHO strain code which gives information on strain identification as well as host species, country, and year of isolation (Tab. 1) (WHO, 2010).

	VL	CL	Co-HIV
Zymodemes	MON-1* MON-72	MON-24* MON-78, MON-29, MON-11, MON-189	MON-136, MON-188, MON-190, MON-201, MON.228, MON 183 var. MDH100, MON-189 var. NH140

Table 1. L. infantum *zymodemes isolated in the Mediterranean basin. * = the most frequently isolated (Maroli* et al.*, 2007; Gramiccia* et al.*, 2013; Castelli* et al.*, 2020).*

However, the classification based on zymodemes has some limitation (Schönian *et al*., 2011): (*i*) the enzymatic panel used in the Old and New World are different, therefore strains cannot be compared directly; (*ii*) different genotypes can show the same enzymatic profile; (*iii*) different enzymatic profiles can be result of heterozygosity at single nucleotide positions; moreover, MLEE is culture dependent, implying that it cannot be applied to any kind of samples and can be performed only in few laboratories (Akhoundi *et al*., 2017). Nowadays, strains are often characterized at local level with different molecular techniques that will be later characterized in chapter 3.

1.3 Life cycle of *Leishmania infantum*

Leishmania infantum has an indirect life cycle and is transmitted to the mammalian host through the bite of phlebotomine sand flies. According to the host, the protozoa can be found in two different forms: intracellular amastigote in mammalians and promastigote in sand flies (Deplazes *et al*., 2016), thus is defined dimorphic (Fig. 3).

Amastigotes are ellipsoidal and have no visible flagellum, therefore are not-motile forms. Their body has a length of 2-5 μm and width of 2-3 μm; they are among the smallest nucleated cells known. A rudimental flagellum can be observed in certain preparations. In electron micrographs also a flagellar pocket and the kinetoplast can be observed (0.7 μm x 0.3 μm) (Marquardt *et al*., 2000). Kinetoplasts are peculiar kind of mitochondria composed of interlocked circular DNA that encompasses the Trypanosomatidae family (Cavalcanti and de Souza, 2018).

Promastigotes are elongated (10 μm) and with a flagellum (therefore motile) and can be found in sand flies. The kinetoplast is usually frontal to the nucleus, and in its proximity the flagellum emerges from the body. Different promastigote forms can be observed in sand flies according to the stage of invasion (see paragraph 1.3.1); the infective forms for mammalians are metacyclic promastigotes, which present a thin body with long flagellum and are characterized by intense motility (Gradoni and Gramiccia, 2004).

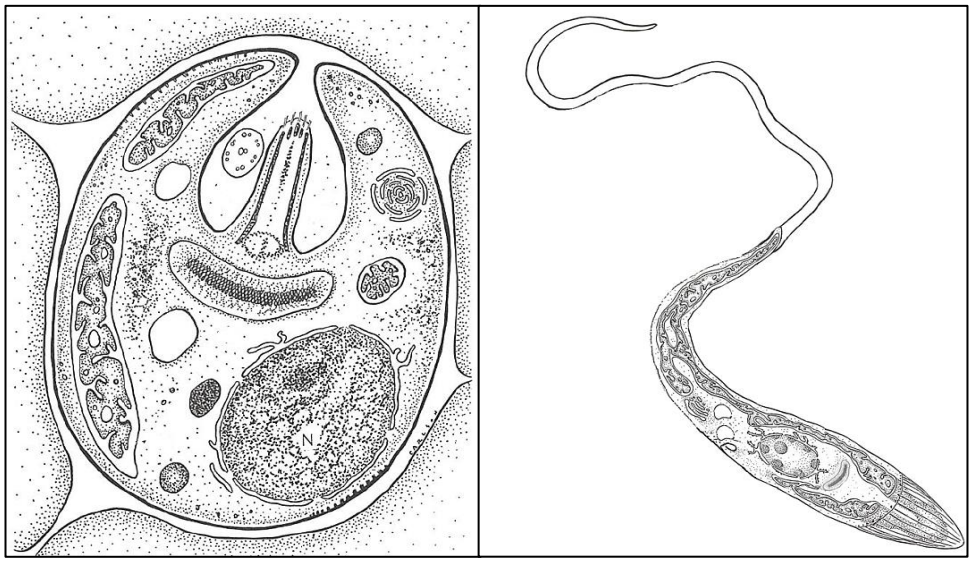

Figure 3. Different forms of Leishmania*: amastigote on the left, promastigote on the right (adapted from Marquardt et al., 2000).*

1.3.1 *The Phlebotomine vector*

Phlebotomine sand flies are holometabolic insects considered of great medical and veterinary importance as they are proven vectors in the transmission of Oroya fever as well as Sand flies Fever Virus (Ratcliffe *et al*., 2022; Socha *et al*., 2022), as well as of *Leishmania* spp. Sand flies are small, usually with a body length of 3 mm; color may vary from white to black according to the species (Fig 4). They can be distinguished for three main characteristics (Killick-Kendrick, 1999): (*i*) when resting, wings are held at angle above the abdomen; (*ii*) they are hairy; (*iii)* before engorging, females hop around the host before biting.

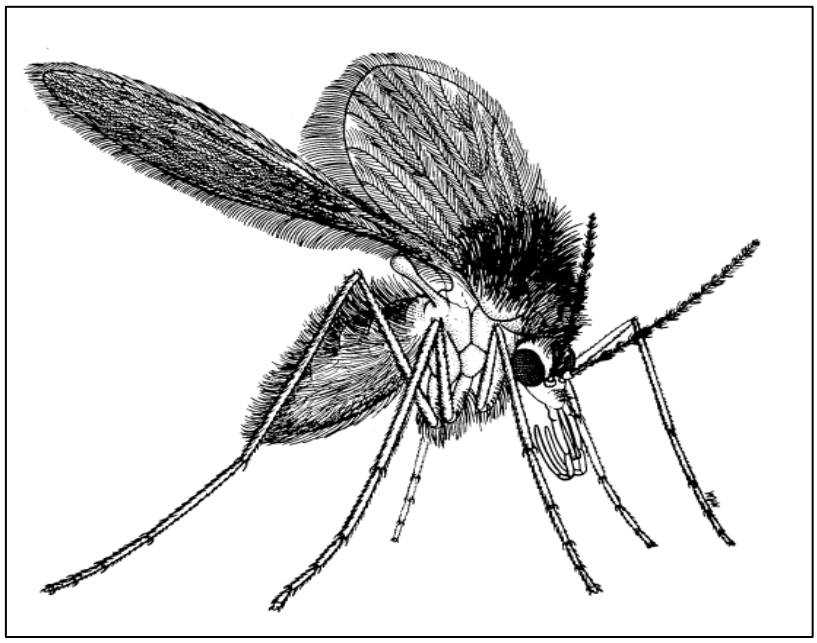

Figure 4. Female sand fly (Killick-Kendrick, 1999).

Their life cycle is fully terrestrial, comprehensive of four different larval developmental stages and a pupal period after which an adult emerges (Dvorak *et al*., 2018). Breeding sites can be domestic, peridomestic and sylvatic but most frequently eggs are laid in animal's shelters, stone walls, and organic debris (Feliciangeli, 2004). Sand flies are active only during sunset and night while they hide in dark places in the daytime. Adults usually feed on plant sap, nectar, or honeydew; however, females require one or more blood meal to reproduce (Killick-Kendrick, 1999).

With reference to Italy two different species are recognized as vector of *L. infantum*: *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*, the first widely distributed in

Tyrrhenian and Southern regions, the latter in Central Italy (Michelutti *et al*., 2021). In two distinct areas of the Country a third species has been described: *Phlebotomus neglectus*, who is confined to the regions of Friuli-Venezia Giulia, Veneto and Piedmont in northern Italy and Calabria, Apulia, and Sicily in southern Italy (Maroli *et al*., 2002).

1.3.2 *Transmission cycle of* Leishmania infantum*: Through the vector*

In the sand fly, *L. infantum* develops in the digestive tract and more precisely in the midgut (Fig. 5). The female sand fly acquires the protozoa from the mammalian host during blood meal. In the midgut, after exposure to the peritrophic matrix of the digestive tract, the amastigotes transform in procyclic promastigotes which proliferate. After the destruction of the peritrophic matrix (a membrane that surrounds food protecting the midgut), *L. infantum* assumes a long form called nectomonads, followed by the shorter leptomonads. These forms are attached to the midgut epithelium and here proliferate. In the last stage of the sand fly infection, the leptomonads migrate in the thoracic midgut where they produce the promastigote secretory gel and transform into the (infective) metacyclic form, then attach to the stomodeal valve ready to be transmitted to a mammalian host (Omondu *et al*., 2022).

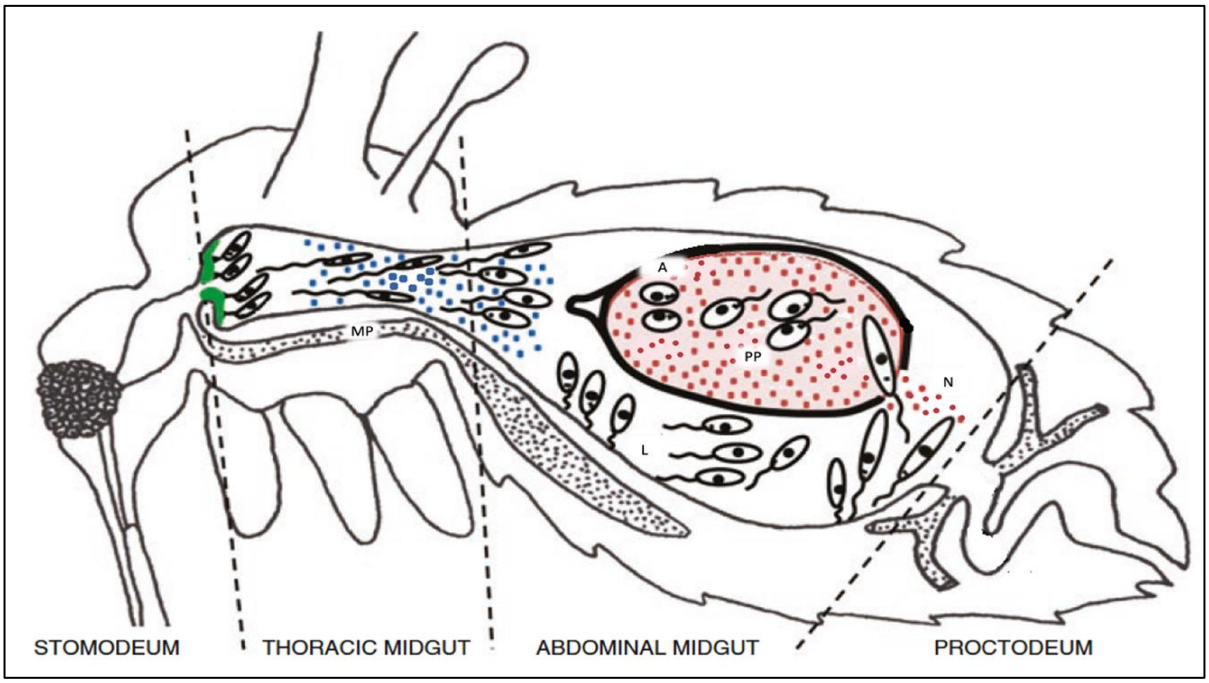

Figure 5. Development of L. infantum *in female sand fly (adapted from Dvorak* et al.*, 2018).*
A: amastigotes; PP: prometacyclic promastigotes; N: nectomonads; L: leptomondas; MP: metacyclic promastigotes.

As final note on the interaction between the protozoa and its phlebotomine vector, several studies have shown that different *Leishmania* species, including *L. infantum*, in the vector may actuate genetic exchanges also referred as sexual cycle (Volf *et al*., 2007; Rogers *et al*., 2014; Romano *et al*., 2014). This hybridization may result in increased fitness of the parasite, different clinical presentation in the mammalian host, ability to colonize new sand fly vector or emergence of new foci (Rougeron *et al*., 2010).

1.3.3 *Of dogs and men: what happens in the mammalian hosts?*

Dog is considered the main host of *L. infantum*, while human patients are usually accidental hosts. After inoculation by a sand fly, *L. infantum* acts as an obligate intracellular parasite, therefore needs to rapidly locate in the host cells. Phagocytosis is stimulated from the promastigotes themselves by activation of complement system. This mechanism is regulated by surface proteins of the protozoa like glyco-protein 63 (gp63), lipophosphoglycan (LPG) and fibronectin (FN) (Brittingham *et al*., 1995; Vannier-Santos *et al*., 2002; Späth *et al*., 2003). In mammalians, *L. infantum* resides mainly in macrophages where parasites differentiate from promastigotes to amastigotes and then survive in the hostile environment of phago-lysosome-like organelles (Rossi and Fasel, 2017). Here the LPG inactivates the hydrolases, and the protozoa releases antioxidant agents which inhibits the development of intermediate oxygen metabolites (Rossi and Fasel, 2017). In the phago-lysosome the amastigotes undergo massive reproduction until disruption of the macrophage and then invade other macrophages. In this stage *Leishmania* invades the hemolympathic system and can be detected in several organs like spleen, liver, bone marrow, skin, and lymph nodes (Piergili Fioretti and Moretti, 2020). The cycle is completed when amastigotes are ingested by a competent insect vector (Fig 6).

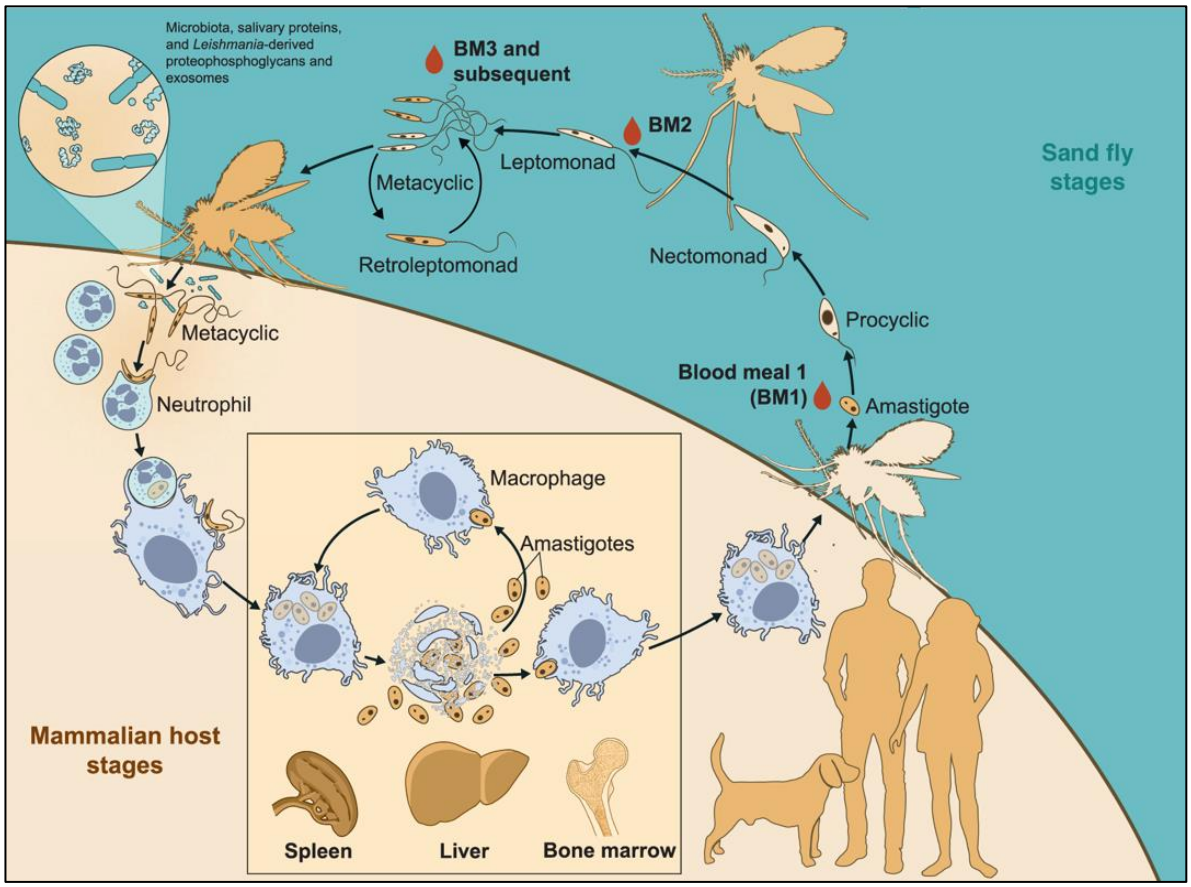

Figure 6. Life cycle of Leishmania infantum *(adapted from Serafim* et al. *2020).*

In dogs, the pathogenesis of leishmaniasis (CanL) varies according to the immune response. When cellular response is activated the recruitment of lymphocytes T-helper 1 (Th-1) mediates the production of interleukins (IL-12 and IL-2), γ-interferon (γ-IF) and tumor-necrosis factor (TNFα) which enable macrophage to eliminate the parasite. In case the humoral immune response is activated Th-2 are recruited, there is the production of IL-4 and IL-5 which induce the production of immunoglobulins. Only the Th-1 is protective against leishmaniasis (Figure 7). The reasons beyond the development of a protective or not-protective immune response are not fully understood, but genetic host factors have been hypotized, in fact Ibizan hounds are reported as more resistant than other canine breeds, while boxers, cocker spaniels, rottweilers and German shepherd dogs are considered as more susceptible (Burnham *et al*., 2020).

When the humoral response is activated, amastigotes replicate in the macrophagic phagolysosomes thanks to their ability to neutralize host cells pH and detoxify oxygen

metabolites (Rossi and Fasel, 2017). With macrophages rupture, parasites disseminate from the inoculation site throughout the body of their host via the hemolymphatic system and infect other monocytes and macrophages in the reticulo-endothelial system. T-lymphocytes undergo depletion in the lymphoid tissues where mainly B-cell, histiocytes and macrophages proliferate; this contributes to cause enlargement of lymph nodes and spleen, and hypergammaglobulinemia. Moreover, the higher concentration of antibodies and the large amount of *Leishmania*-antigens can give rise to circulating immune complexes (CICs). CICs determine vasculitis and activate the complement cascade ending with dermal, visceral, ocular, and renal lesions, because of the reduced activity of scavenger macrophages induced by CICs themselves (Gizzarelli *et al*., 2020).

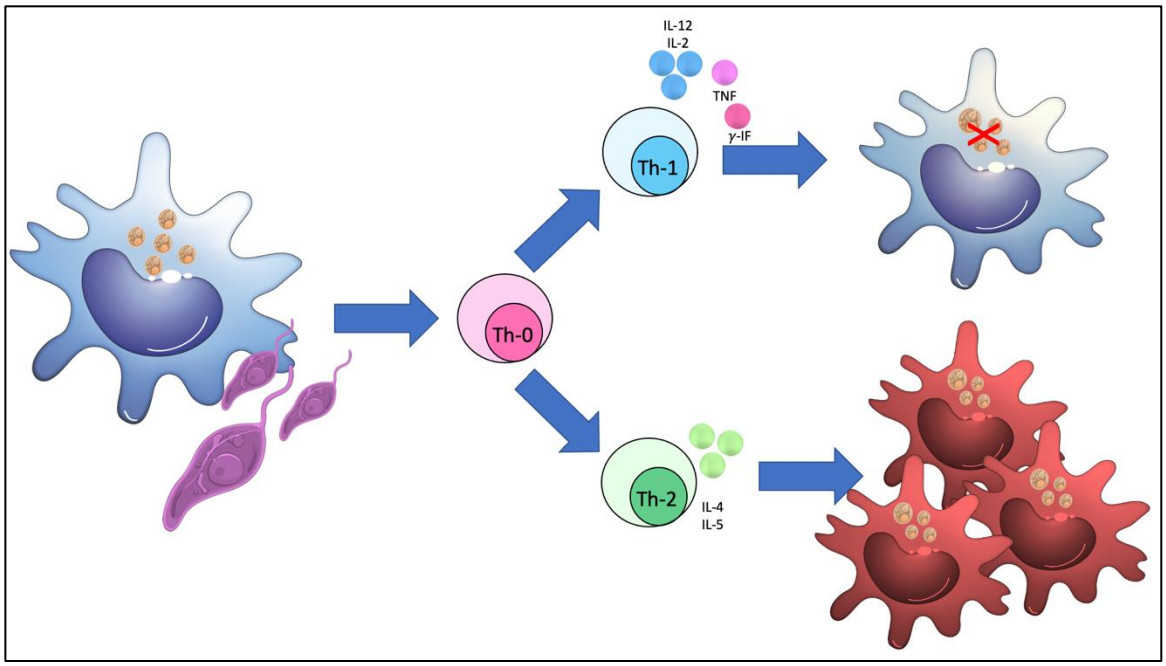

Figure 7. Possible canine immune response after infection with Leishmania infantum.

CanL is usually a chronic multisystemic disease with different clinical manifestations that usually appear after an incubation period ranging from three months to seven years after infection (Silvestrini, 2021). In dogs, clinical manifestations are mainly due to inflammatory infiltrates. CanL is not distinguished in three different forms, as in human leishmaniasis, but presentation is miscellaneous. At clinical examination dogs present poor body condition, muscular atrophy,

lymphadenomegaly and abnormal scaling skin (Bañuls *et al*., 2007). Frequent clinical presentation may also include pallor of mucosae, splenomegaly, cachexia, fever, epistaxis and onychogryphosis (Baneth *et al*., 2008). Skin diseases are reported in 81-89% of symptomatic dogs and includes exfoliative, ulcerative, nodular, and pustular dermatitis with frequent localization at periorbital level (Baneth *et al*., 2008).

Ocular diseases occur in 16-80% of dogs affected and are mostly caused by inflammatory infiltrates. Anterior uveitis was described as the most frequently observed sign, as well as blepharitis, keratoconjunctivitis (or combinations) (di Pietro *et al*., 2016). Inflammatory infiltrates are also responsible for kidneys, joints, and bones lesions in CanL (Soares *et al*., 2005; Silva *et al*., 2021). Interstitial nephritis is also often reported, and it can progress asymptomatically to nephrotic syndrome or to chronic renal failure (Ribeiro *et al*., 2018). Diagnosis of CanL is not synonym of renal diseases however, practitioners suggest routine monitoring of renal functions (Roura *et al*., 2021).

Currently, survival and progression of CanL are influenced by early diagnosis and adequate therapy (Roura *et al*., 2021).

In human patients the pathogenesis of leishmaniasis is still uncertain. After infection the patient may develop the disease according to the activation of cellular or humoral response (Machado *et al*., 2019). What precisely determines the development of clinical forms remains unclear; as for canine patients, the strain of *L. infantum* or the genetics of the host might be involved (Kumar and Nylén, 2012). As already mentioned, contrarywise than CanL, human leishmaniasis is differentiated in three clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). CL is considered the least severe form of the disease; it appears with a single ulcerative or nodular lesion usually found in uncovered areas of the body like face, forearms, or lower legs (McGwire and Satoskar, 2014). MCL is a manifestation frequently observed as secondary form of CL which can present even years after resolution of the first lesion (David and Craft, 2009). The third form of leishmaniasis is VL, clinically considered the most severe form. The disease is

the result of the proliferation of *L. infantum* in the cells of the reticuloendothelial system, progressively the parasite invades liver, spleen, and bone marrow. Unless treated, patients develop pancytopenia and immunosuppression (Chakravarty *et al*., 2019). In case of coinfection with HIV, clinical presentation may vary from the typical ones (Lindoso *et al*., 2018).

1.4 Treatment and prophylaxis of canine leishmaniasis

With few exceptions, dogs are currently considered the main reservoir of leishmaniasis in the Mediterranean basin (Molina *et al*., 2012). Therefore, preventive measures against the bite of sand flies are crucial to protect dogs from infection and to avoid further spread of *L. infantum* especially in endemic areas. On infected dog, also treatment may have the same effect, since it reduces parasitemia and consequently the possibility to transmission to sand flies (Maroli *et al*., 2009; Simonato *et al*., 2020).

Treatment of patients with CanL is a clinical challenge, and if authors have recently reported that life spawn and life quality of patients is comparable to other dogs (Roure *et al.* 2021), it is also true that early diagnosis and onset of a treatment are crucial factor for the survival of the patients (Silvestrini, 2021). Complete parasites clearance doesn't occur; therefore, owners must understand that therapy has only the purpose of remission of symptoms, clinicopathological normalization and antibody levels negative or below test's cut-off, and that relapses are frequent (Silvestrini, 2021). To date there is not a unique standard protocol for CanL treatment; drugs of first choice are meglumine antimoniate, miltefosine that can be combined with allopurinol. Even the use of domperidone, an immune stimulator, has been described since it can prevent overt disease (shifting the immune response against a protective one) or improve the clinical condition of infected dogs. To date, the use of amphotericine B in the veterinary practice is not allowed to limit drug resistance phenomena since is a first-choice drug for human patients. Most of these compounds are nephrotoxic or may cause galactorrhea (domperidone) or digestive disorders (miltefosine and domperidone) hence patients must be regularly monitored (Reguera *et al*., 2016).

Nevertheless, treatment of infected dog is important, not only for patient welfare but also to reduce the prevalence and incidence of the disease (Reguera *et al*., 2016).

Prophylactic measures against CanL should also be adopted because they not only prevent the infection, but further contribute to limit the spread of the disease (Maroli *et al*., 2009; Simonato *et al*., 2020). Prophylaxis should be approached from two sides: (*i*) protection of dogs against the bite of sand flies, and (*ii*) use of vaccines; most frequently a combined approach is the most effective (ESCCAP, 2016; Simonato *et al*., 2020).

Preventive measures against canine infection based on the protection of dogs against the bite of sand flies are quite basilar as keeping dogs in shelters with tight mesh during night or sunset, when phlebotomine are active; but mostly on the use of synthetic pyrethroids which have an anti-feeding effect. These measures should be applied even if the dog is infected (symptomatic or not) because they prevent further spread of *L. infantum*, to dogs or humans as well (Maroli *et al*., 2009).

In Europe there are two vaccines commercially available for dogs against *L. infantum*. The first one is an inactivated vaccine with adjuvant containing excreted secreted proteins (ESP) of *L. infantum*. The vaccination protocol consists of one vaccine dose administered to dogs over 6 months of age, every 21 days for a total of three doses followed by a single booster dose administered yearly (CaniLeish © - EMA, www.ema.europa.eu). However, several studies demonstrated that the use of this vaccine can interfere with serological diagnosis (Sagols *et al*., 2013; Ceccarelli *et al*., 2016; Velez and Gállego, 2020) The second vaccine, commercially available from 2019, is based on a recombinant protein (protein Q) created with five antigenic fragments from different *L. infantum* proteins, without adjuvant. This vaccine has no reported adverse effects and vaccination does not seem to elicit false-positive results in serological diagnostic tests (Velez and Gállego, 2020). The first dose can be injected from an age of 6 months and the booster dose should be administered once a year (LetiFend © - EMA, www.ema.europa.eu).

Chapter 2

Chronicle of a neglected pathogen: The forgotten history of *Leishmania infantum* in the Emilia-Romagna region (Italy)

2.1 Visceral leishmaniasis in Italy

Leishmaniasis has long been recognized endemic in the Italian peninsula. As early mentioned, the only species currently circulating in the territory is *L. infantum* (Rugna *et al*., 2018), even though other *Leishmania* species have been isolated from international travelers infected abroad (di Muccio *et al*., 2015), i.e., *L. major*, *L. tropica*, *L. braziliensis*, *L. panamensis*, *L. mexicana*, *L. aethiopica* and *L. donovani*. With reference to Italy, both the visceral and the cutaneous form are described, and in the last years also the muco-cutaneous form was reported, but not exclusively, in HIV positive patients (Casolari *et al*., 2005; Madeddu *et al*., 2014).

The disease was known since the XIX century in the province of Naples and in the island of Ischia (Campania) as lienal leukemia or infantum splenic anemia. At first, VL was considered a pediatric disease with poor prognosis, until the development of a suitable therapy by di Cristina and Caronia (1915) (Sirrotti, 1954).

The first parasitological finding is attributed to Gabbi in Messina (Sicily) in 1908, and in the following years several cases were reported from all Italian regions in patients of all ages (Fusaroli, 1952). Because of that, for several years VL was distinguished in two different forms: pediatric Mediterranean leishmaniasis and Indian Kala-azar; such differentiation was lately abandoned since the etiological agent was recognized as the same (Girolami, 1948).

Up to the 1931, VL was so widespread that Italy was recognized as the most critical country in the Mediterranean basin in terms of number of cases. Three main foci were identified in the provinces of Catania (Sicily, 150-200VL cases/year), Palermo (Sicily, 70 cases/years) and Naples (Campania, 70 cases/years) (Pampiglione, 1975).

From the first parasitological finding in 1908, to 1942 more than 90 VL outbreaks/cases were described (Fusaroli *et al.*, 1952), from all over the Peninsula but

mostly along the Tyrrhenian and the low Adriatic coasts and on the islands involving children but also adults which were the majority of cases reported (Pampiglione, 1975). Patients were mostly male living in coastal areas, in the countryside, or in isolated villages close to water stream (Bevere and Tobia, 1947). Agricultural workers were the most involved professionals, followed by workers, artisans, and transport workers. Dogs were considered the main reservoir of the parasite, but especially in the rural environment the presence of other animal reservoirs was also hypothesized (Bevere and Tobia, 1947).

Starting from 1948, the VL reports fainted, due to the massive use of Dichlorodiphenyltrichloroethane (DDT) and later of pesticides in agriculture and in holiday facilities (Corradetti, 1960; Pampiglione, 1975) so that in the '70s VL was described as sporadic. In subsequent years, reports progressively increased, especially after a dramatic outbreak occurred in 1971-1972 in the Bologna province (Emilia-Romagna, ER) with a lethality of 21.7% (Pampiglione, 1975). Thanks to the increased awareness of the spread of VL, reports escalated in Italy so that the geographical distribution of *L. infantum* appeared wider than the initial one. In the Peninsula, the number of VL cases remained stable during the '80, when the annual cases ranged from 10 to 30 cases/year, to increase in the following years with a peak in 2000 and 2004 with more than 200 cases/year (Gramiccia *et al*., 2013). Several reports came even from regions or provinces that were considered not endemic, including all the provinces of ER (except Ferrara), Piedmont, Lombardy and, with a single case, in Aosta Valley. From 1990 to 2005 in all northern Italy the VL cases represented the 10.9% of the Italian cases with an average of 14 cases/year (Maroli *et al*., 2008). These findings encouraged to considered new territories as endemic, further supported from the increased diagnosis of CanL (Capelli *et al*., 2004; Baldelli *et al*., 2011), and the

northward spread of sand flies, reinforcing the endemicity of *L. infantum* into previously free territory (Fig. 8) (Salvatore *et al.*, 2013; Mendoza-Roldan, 2020).

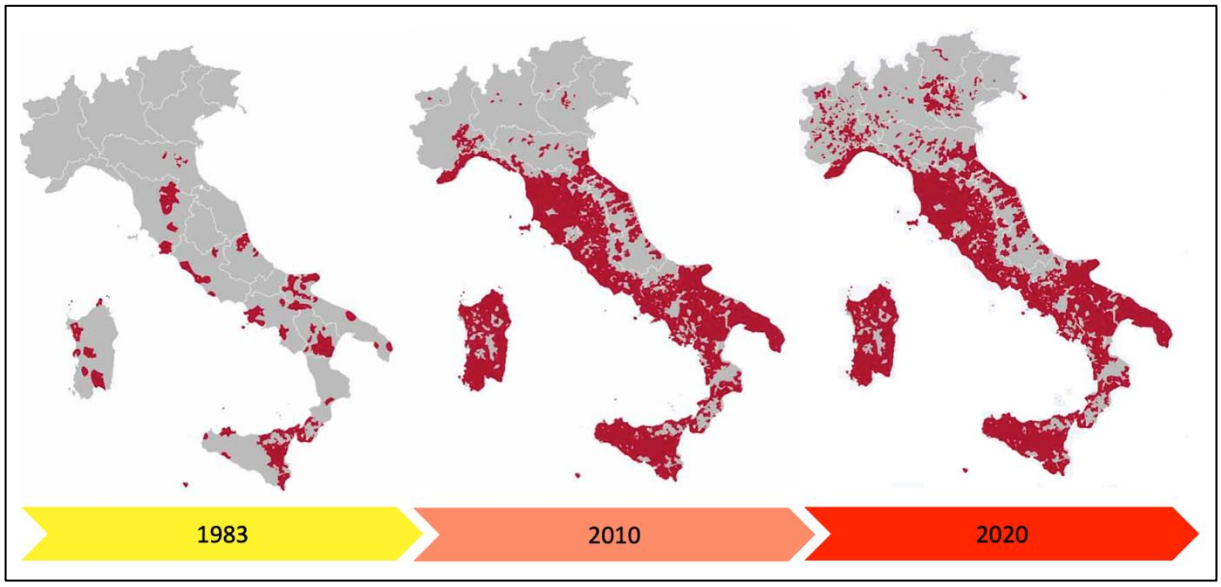

Figure 8. Distribution of L. infantum *in years 1983, 2010, 2020.*
The progressive increase in geographical distribution is highlighted in red (adapted from BAYER, 2020).

2.2 The strange case of visceral leishmaniasis in Emilia-Romagna

The Emilia-Romagna region is in north-eastern Italy, its territory is partially hilly in the south-west zone and plain in the north-eastern one, divided by an ancient roman road called via Aemilia (Fig 9).

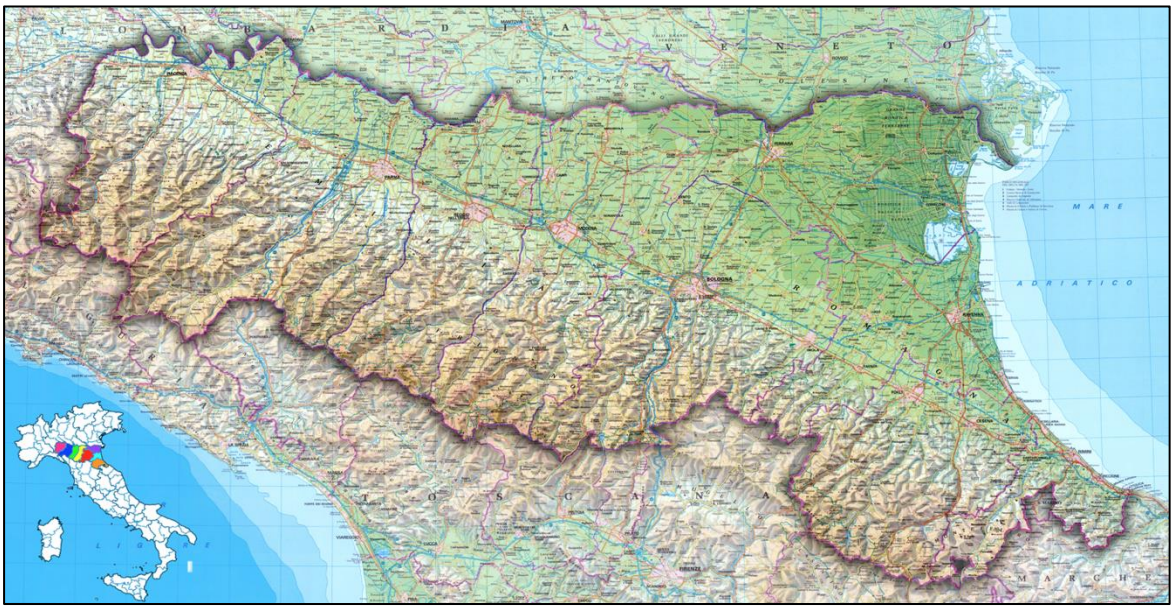

Figure 9. Map of Emilia-Romagna.
Provinces are marked in different colors: Piacenza in pink, Parma in blue, Reggio Emilia in green, Modena in yellow, Bologna in red, Ferrara in purple, Ravenna in light-blue, Forlì-Cesena in orange (adapted from Geoportale Emilia-Romagna).

In this region, human leishmaniasis has long been described mostly as CL (in 1948 ranked on top three Regions for number of cases) (Pullè, 1951), VL instead was not notified until 1950 (Suzzi Valli e Dominici, 1953). During the '50 only four ascertained VL cases were here reported: the first was described by Suzzi Valli and Dominici in 1950, in the province of Rimini involving a 44-year-old farmer; the second by Fusaroli (1952) in a 20-year-old farmer woman from the province of Forlì, with fatal outcome. Few years later a third case was reported in the province of Bologna by Giungi (1954): a 62-year-old day laborer from the foothill side of Bologna province. At last, Artusi and Grossi (1962) notified the case of a 22-year-old man from the province of Forlì who worked in Switzerland for a tanning industry and got infected in a short trip back to Forlì.

All patients had no recent travel history in endemic areas and three of them were agricultural workers. By that time, in other Italian regions it had been noticed a correlation between the geographical distribution of leishmaniasis (both visceral and cutaneous) and canine leishmaniasis. Hence, for the cases described by Suzzi Valli and Dominici (1953) and Fusaroli (1953), the dogs belonging to the patients (or of their neighbors) were tested for *Leishmania* sp. but with negative results. As earlier noticed (Girolami, 1948), Authors recognized that dog was indeed an important species in the transmission of leishmaniasis but suggested that in the ER region other animal species should be investigated as reservoir. Moreover, since the cases were observed at approximately 60 km from each other and in an area where CL was widely distributed, it was questioned whether cutaneous and visceral leishmaniasis were caused by two distinct strains circulating in the same area or by different immune response of the patient (Suzzi Valli and Dominici 1953; Fusaroli, 1953; Giungi, 1954). Also, in the province of Modena 10 cases were notified in a 10-years period from 1943 to 1954: three of them were surely autochthonous. All patients lived in a foothill side area of the province characterized by grey calanques and close to the river Panaro (Sirrotti, 1954). In the following years, reports of both CL and VL notably decreased, probably owing

to the massive use of pesticides in agriculture and in holiday localities that heavily reduced the presence of sand flies (Corradetti, 1960; Pampiglione, 1975).

The first VL outbreak in ER was described in 1971-1972 involving several municipalities of the province of Bologna (Fig. 10). In October 1971 several patients were hospitalized with an acute infective syndrome characterized by a severe fever that differed from those usually reported within the region (like typhus fever or brucellosis). The syndrome was recognized as VL only in May 1972. Thanks to the cooperation with the National Communicable Disease Center of Atlanta (U.S.A.) 60 cases were confirmed as VL, mostly from a restricted area of 80 x 20 km located on the foothill side southern to the via Aemilia characterized by fallow fields, water streams, wooded mountains and grey calanques, likewise the cases previously reported in Modena (Sirrotti, 1954). Lethality was 21.7%, mostly due to late diagnosis and specific treatment (all deceased patients were adult males) (Pampiglione, 1975). Other traits of the outbreak were considered anomalous, i.e.: majority of the patients were adults (42/60 mostly men fond with hunt), fever was high, remittent, or irregular, often preceded by rigor, and the presence of hepatic granulomatous lesions never observed in the Mediterranean areas before (Pampiglione *et al*., 1974).

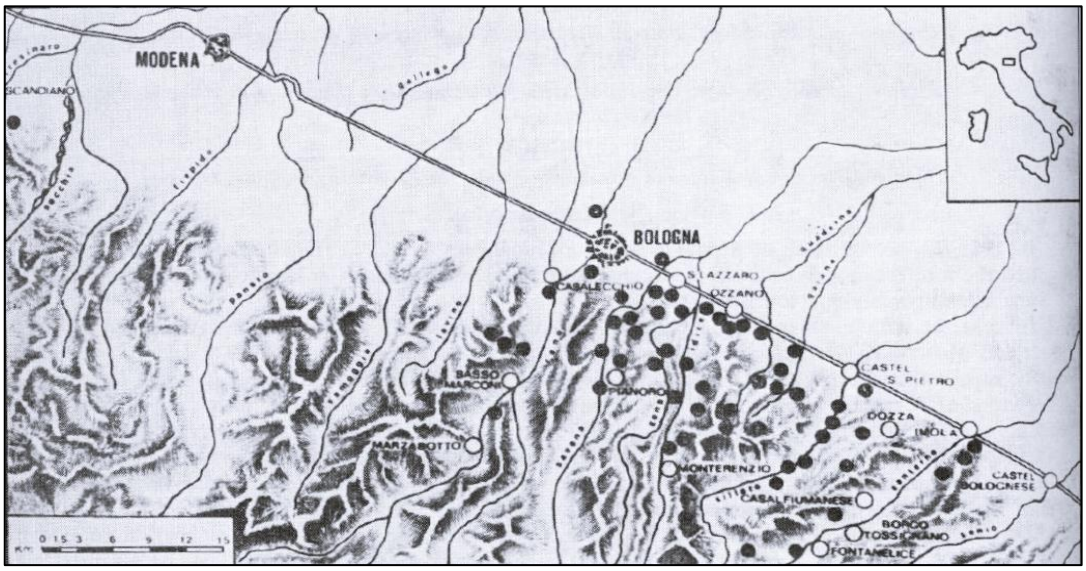

Figure 10. Geographical distribution of the cases in the Bologna outbreak 1971-1972. Municipalities are represented with white dots, VL cases in black. (Pampiglione et al*., 1974).*

Concerning findings of CanL, no dogs were reported as symptomatic in the same area of the outbreak. A mass serological control was then performed via Complement

Fixation Test on 8454 serum samples from dogs of that area: 132 (1.6%) tested positive with titer of 1:10 or 1:20 with no clinical manifestations (Pampiglione *et al*., 1974). Three different hypotheses were formulated on the development of such extraordinary outbreak (Pampiglione, 1975): (*i*) the sudden migration of infected dogs from endemic regions; (*ii*) the arrival of a wild animal reservoir not yet identified; (*iii*) the re-emerge of a cryptic VL already present in the territory, with an increase of cases due to the extreme dry weather of summer 1971.

From this peculiar outbreak, the spread of VL was carefully monitored during the following years and Bologna province was included in the endemic territories. The re-emergence of VL in Italy in the 1990-2000 again involved ER, initially affecting HIV or immunocompromised patients (Varani *et al*., 2013, Franceschini *et al*., 2016). What caused this re-emergence was not clear; various factors were hypothesized such as environmental changes which allowed vector activity in areas that were previously not suitable; wider circulation of HIV; or infected human and canine migration (di Muccio *et al*., 2015).

In consideration of that, in ER region from 2007 leishmaniasis was included in a Surveillance Plan (PG/2007/108853) aimed to monitor the spread of vector-borne diseases (VBD) (Venturi *et al*., 2009). Regarding leishmaniasis, the plan has been focusing on three activities: (*i*) to perform serological tests on every dog entering a public kennel or housing facilities; (*ii*) to register any pet positive for CanL; (*iii*) to support the cooperation between veterinary practitioners and physicians by investigating the spread of vectors and of CanL once an autochthonous human case is confirmed.

Currently two foci are active in two municipalities from the province of Bologna, both located in the foothill side southern to the via Aemilia (Varani *et al.*, 2013; Ortalli *et al.*, 2020). One of them (Pianoro) was involved even in the '70s outbreak. In addition, several single cases have been reported from all over the region (Fig 11) (Santi *et al.*, 2022).

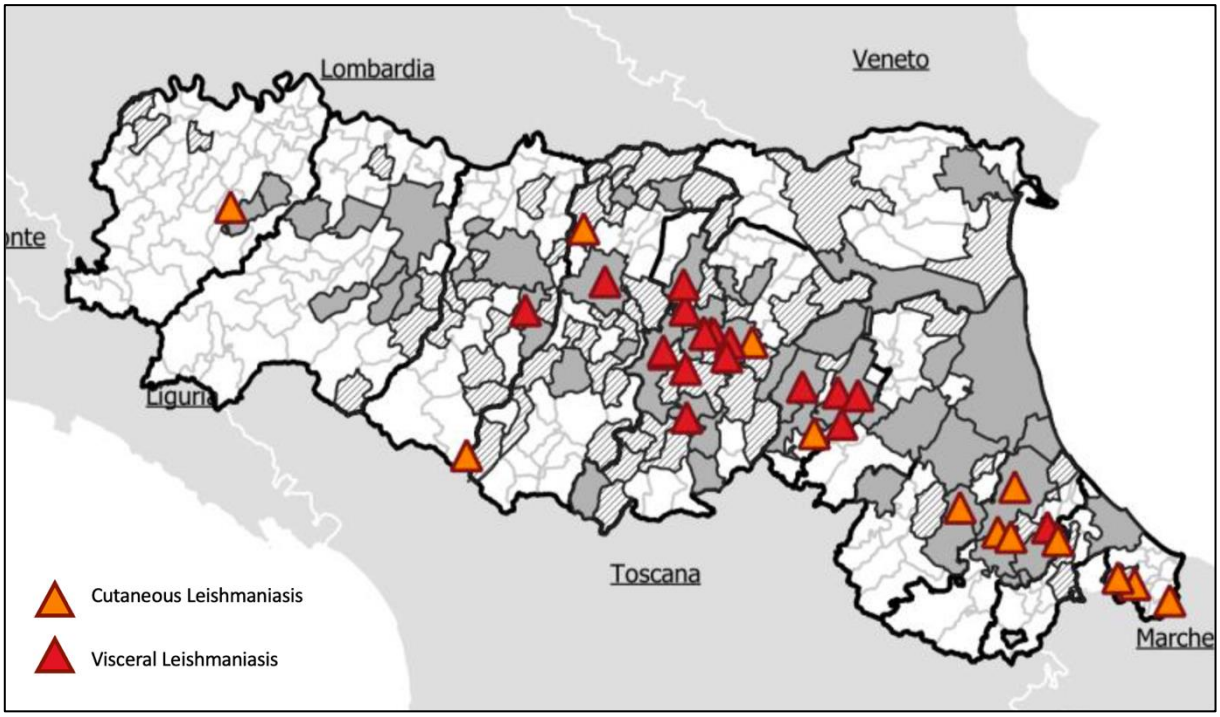

Figure 11. Distribution of CL and VL cases occurred in 2021 in Emilia-Romagna (adapted from Santi et al.*, 2022).*

Molecular characterization of *L. infantum* strains circulating within the ER region was performed by Rugna *et al.* (2017). To this purpose, 28 *L. infantum* isolates from dogs from ER, 12 isolates from humans (including 3 from ER), and 31 from sand fly pools from ER were examined targeting different DNA regions. The results revealed the presence of two different strains circulating in the ER: one circulating in dogs, and a distinct one in sand flies and humans. These data further confirmed (Rugna *et al.*, 2018), indicated the role of dog as reservoir of *L. infantum* in ER questionable.

2.3 Cutaneous leishmaniasis in Emilia-Romagna: Let's twist again

Cutaneous Leishmaniasis is considered by many authors the less severe form of leishmaniasis that usually comprises in its manifestation crusted ulcerated nodules or plaques. Contrarywise to VL, CL was commonly reported in Italy since the very first notification of leishmaniasis by Gabbi, in Sicily in 1911 (Gabbi, 1911). After that, reports quickly came from all over the Italian peninsula, so that immediately it appeared clear that no Italian regions were risk-free from CL. From 1927 to 1947, despite the two World Wars, 3,525 cases were reported all over the Country (Pullè, 1951). Regarding these reports its worth to notice that two regions (Trentino- Alto Adige and Aosta Valley) are excluded because they were annexed to Italy only from 1948; furthermore, notification of cases became compulsory only in 1940 and CL was often misdiagnosed with other dermatoses or undiagnosed due to the social conditions of that period: the first reliable prevalence is dated in 1950 (Piredda and Gasparri).

The first report of CL in ER is dated 1915 by Mantovani (1915). The patient was a 66-year-old man, market gardener from the province of Ravenna with no travel history. For four years the patient was unsuccessfully treated for a mycetoma-like lesion under the left foot; unfortunately, even if physicians eventually came to a diagnosis, the increasing fever peaks and the serious risk of infection made it necessary to amputate the foot of the patients as life-saving procedure (Mantovani, 1915). The second single report was notified in 1934 (Monacelli, 1934): it was a case of a 75-year-old woman living in the province of Forlì with no recent travel history. Before the diagnosis, the patient had for two years a lesion on the left cheek. The lesion was hitchy, swollen and its dimensions progressively increased (Monacelli, 1934). From this case report, in a period of 4 years, the first outbreak of CL was reported in the same province. In 1938 an impressive amount of diagnoses of *lupus*-like syndrome were notified in the province of Forlì (Poggi and Monti, 1939). Sanitary Authorities raised suspicions on the etiology of the syndrome for three main reasons: (*i*) the frequency of diagnosis of *lupus* was uncommon given time and little territory

involved; (*ii*) the syndrome had a favorable course, unlike *lupus*; (*iii*) CL outbreaks were notified in areas comparable with the province examined considering climatic and hydrographical factors; (*iv*) like other CL outbreaks, most of the patients lived in the countryside. Patients still hospitalized were tested for *Leishmania* spp., and cultures were successfully established confirming the diagnosis of CL. More than 100 cases were confirmed, and Authors dated the beginning of the outbreak to 1925 (Poggi and Monti, 1939). The initial identification of the parasite was *L. tropica* in all the afore mentioned cases because of the similarities with the CL cases reported from southern America; the parasite was identified as *L. infantum* only in the '50 after strong debates among parasitologists (Martinotti, 1952).

The number of cases kept on rising and in 1951 ER was the third Italian region for CL prevalence, after Sicily and Abruzzo, and Forlì was the fourth province most affected of Italy (Pullè, 1951). Even if CL was still considered a minor disorder when compared to the wide spectrum of other dermatoses, the progressive increase of the number of cases made necessary to create two anti-leishmaniasis centers in the municipalities of Cesena and Riccione. These dispensaries were active from 1950 to 1958; in this period 2670 cases were diagnosed and treated.

Concerning the overall geographical distribution, higher prevalence was observed close to the four rivers of the Forlì province, namely Savio, Rubicone, Marecchia and Conchia, or to small water streams (Fig 12); morbidity was higher at 100 m asl. Women resulted slightly more affected than men; most of the patients were aged between 10 and 30 years and, like VL, morbidity was higher in agricultural workers and, to a lesser extent, artisans. During the years of activity, the dispensaries also made huge efforts in teaching how to prevent the infection, with subsequent reduction of the incidence of CL in the province of Forlì (Piredda and Gasparri, 1961).

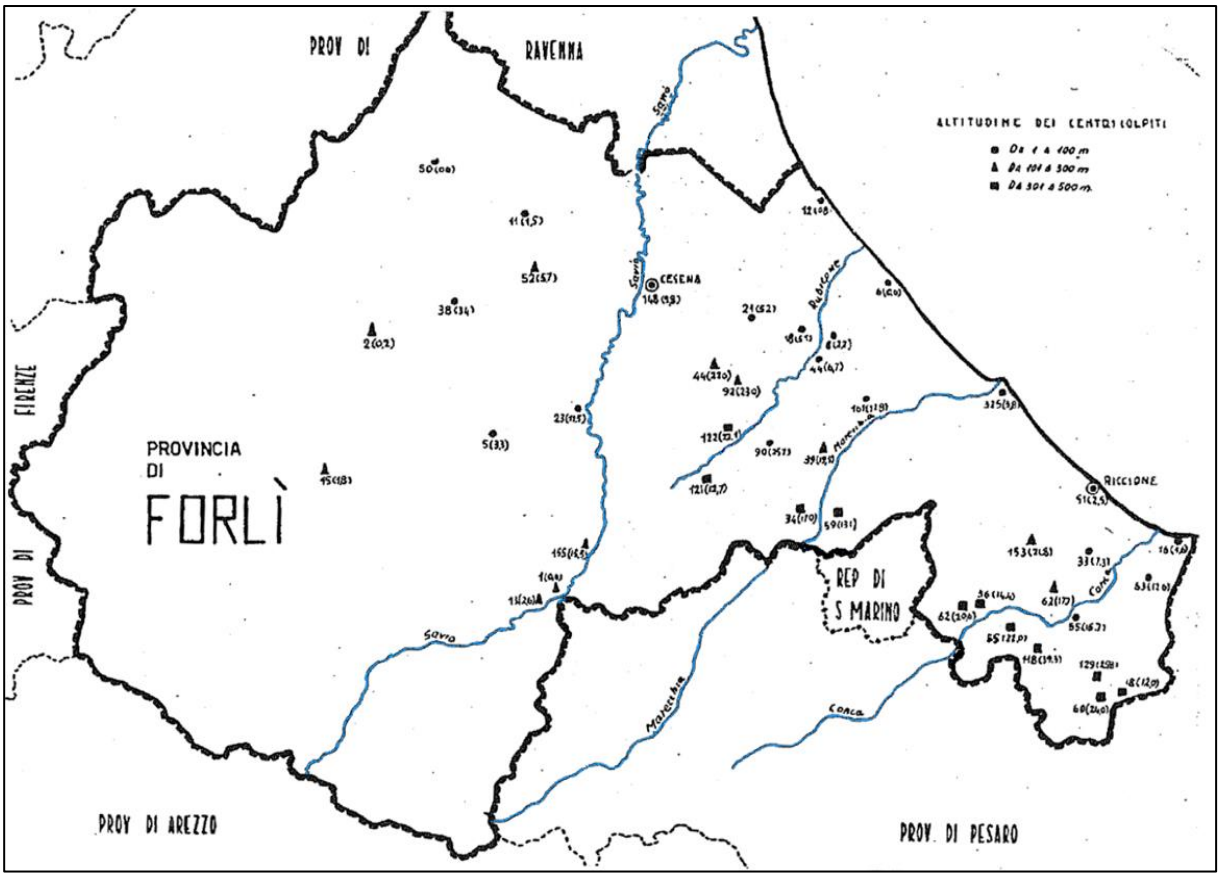

Figure 12. Geographical distribution of CL cases diagnosed in the province of Forlì from 1950 to 1958. Rivers are highlighted in blue (adapted from Piredda and Gasparri, 1961).

In the following years reports of CL were scant and mostly diagnosed in immunocompromised patients (Agostinoni *et al.*, 1998), until a new outbreak occurred in the province of Modena (Cesinario *et al*., 2017). From 1997 to 2016, 35 CL cases were diagnosed, of which 21 from 2014 to 2016. Except for two patients, none had travel history in endemic countries and most of the patients lived in the foothill side of the province (Fig X). According to the regional control plan, cases of CanL in this province were progressively decreasing, thus Authors suggested that other animal reservoirs could be involved in the transmission of the parasite (Cesinaro *et al*., 2017). A second outbreak was reported in the province of Bologna, where 30 cases were diagnosed during 2013-2015. The municipalities involved in the outbreak were located south of the via Aemilia on the foothill side, similarly to VL (Fig 13). To note that one of the municipalities involved was Valsamoggia, where a VL outbreak later occurred (Gasparri *et al*., 2017).

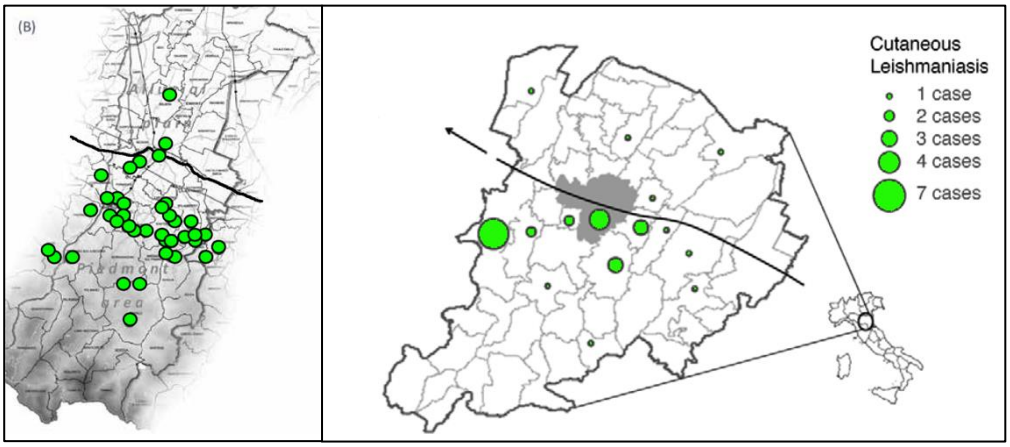

Figure 13. Geographical distribution of the CL cases occurred in the outbreaks of Modena, on the left, and Bologna, on the right (adapted from Cesinaro et al.*, 2017; Gasparri* et al.*, 2017).*

2.4 The wild side: Are dogs really the only possible reservoir of Leishmania infantum *in the Emilia-Romagna region?*

In the Mediterranean area and in Italy the role of reservoir of *L. infantum* has always been attributed to dog. This is related to different reasons (Dantas-Torres, 2007):

- although some dog breeds are reported to be more resistant, dogs are usually susceptible to *L. infantum* infection;
- the prevalence of infection in dogs is higher where human leishmaniasis is endemic;
- dogs often present an intense cutaneous parasitism, and can be source of infection for phlebotomine sand flies;
- dogs and humans have a close relationship that favor the transmission;
- infection in the dog often occurs asymptomatically;
- the zymodeme MON-1, commonly isolated in cases of VL is also frequently isolated in CanL.

However, the emergence of new foci in non-endemic areas and the findings of different strains in canine and human patients made the role of dog as the only reservoir of *L. infantum* questionable (Molina *et al.*, 2012; Rugna *et al.*, 2017). The identification of a proper animal reservoir is of great importance to design an adequate control program (Cardoso *et al.*, 2021).

While it may seem easy, defining what a reservoir is, is not an easy task. Several attempts have been made focusing of different characteristics (Ashford, 1996; Haydon *et al*., 2002; Silva *et al*., 2005). In this thesis, the term "reservoir" will be used to identify a population or a species in which a pathogen can be maintained and that can be a source of infection for susceptible hosts in a specific area (Becker *et al*., 2020). Furthermore, as a requirement to define a species as a reservoir, the prevalence of infection should be 20% or higher (Woolhouse *et al*., 1997).

In view of this, different animal species have been suspected to be a reservoir of *L. infantum*. Recently, attention has shifted to wild and synanthropic animals because of progressive urbanization, landscape and climate changes that affect the interface of vectors, wildlife, and human population (Tomassone *et al*., 2018; Messner *et al*., 2019).

In Europe, the role of wildlife as reservoir of *L. infantum* has been proven in Spain, precisely in Madrid for hares (*Lepus granatensis*), that were involved in an outbreak of human VL occurred in 2010 (Molina *et al*., 2012), and in Portugal and Greece for mice (Helhazar *et al*., 2013; Tsakmakidis *et al*., 2017). Considering these findings, several researches have been aimed to assess the prevalence of *L. infantum* in various wild and synanthropic species.

Firstly, attention was addressed to wild canids such as foxes (*Vulpes vulpes*) and wolfs (*Canis lupus lupus*), and later to mustelids, lagomorphs, rodents, and birds. Furthermore, also other domestic animals were tested, such as cats, ruminants, and horses (Cardoso *et al*., 2021).

With reference to Italy, several findings have been reported from different regions (Tab. 2). Interestingly, on Montecristo Island - in absence of wild and domestic carnivores - the presence of *L. infantum* was detected in black rats (*Rattus rattus*) with a prevalence of 15.1% (Zanet *et al*., 2014). However, for further evidence on the role that the sylvatic fauna may play in the transmission of *L. infantum*, prevalence should be combined with studies on the ability to transmit the pathogen to the vector, and the possible host-preference of sand flies.

Host	Region	Technique	Prevalence %	References
Red fox (*Vulpes vulpes*)	Tuscany	VI & M	6.2 (1/35)	Bettini *et al*., 1980
	Campania	PCR	40 (20/50)	Dipineto *et al*., 2007
	Tuscany	PCR	52.2 (48/92)	Verin *et al*., 2010
	Sicily	PCR	28.6 (2/7)	Abbate *et al*., 2019
	Piedmont	PCR	12.26 (19/157)	Battisti *et al*., 2020
Wolf (*Canis lupus lupus*)	Piedmont	PCR	25.71 (7/33)	Battisti *et al*., 2020
European badger (*Meles meles*)	Piedmont	PCR	53.33 (24/45)	Battisti *et al*., 2020
	Emilia-Romagna	PCR	25 (1/4)	Magri *et al*., 2022a
Hedgehog (*Erinaceus europaeus*)	Emilia-Romagna	PCR	80 (4/5)	Magri *et al*., 2022a
Rabbit (*Oryctolagus cuniculus*)	Sicily	PCR	4.2 (3/71)	Abbate *et al*., 2019
Black rat (*Rattus rattus*)	Tuscany	VI & M	2.1 (3/160)	Bettini *et al*., 1980
	Tuscany	VI & M	1.1 (1/94)	Pozio *et al*., 1981
	Calabria	SB; PCR	57.5; 45 (13/22; 9/20)	Di Bella *et al*., 2003
	Montecristo Island (Tuscany)	PCR	15.5 (11/78)	Zanet *et al*., 2014
	Emilia-Romagna	PCR	13.1 (5/39)	Magri *et al*., 2022b
Brown rat (*Rattus norvegicus*)	Sicily	PCR	33.3 (9/22)	Di Bella *et al*., 2003
	Emilia-Romagna	PCR	10.6 (5/47)	Magri *et al*., 2022b
Wood mouse (*Apodemus sylvaticus*)	Tuscany	VI & M	0 (0/139)	Bettini *et al*., 1980
Mouse (*Mus musculus*)	Emilia-Romagna	PCR	10 (4/50)	Magri *et al*., 2022b
Roe deer (*Capreoulus capreolus*)	Emilia-Romagna	PCR	33 (11/33)	Magri *et al*., 2022a

Table 2. Reports of Leishmania *sp. in wild and synanthropic mammals from different Italian regions.* PCR *polymerase chain reaction,* SB *southern blotting,* VI & M *virus inoculation and microscopy.*

Chapter 3

Pandora's box: Diagnosis and molecular targets of *Leishmania infantum*

3.1 Diagnosis of Leishmania infantum*: Clinical and epidemiological aspects*

The diagnostic approach to leishmaniasis greatly differs according to the main purpose. In case of a clinical diagnosis, serology is still considered the gold standard method, especially in the veterinary practice and in epidemiological surveys, while in case of human epidemiological surveys, various techniques can be applied (OIE, 2021). Considering human leishmaniasis, PCR is the most common method applied for the diagnosis; serology is more sensitive in case of VL, while results in case of CL are less reliable (Singh and Sivakumar, 2003). Regarding CanL, quantitative serology is still considered the gold standard, mainly because the antibody-titer can differentiate sick from healthy patients, however even PCR is commonly accepted (Solano-Galego, 2009). The most common techniques used in the diagnosis, detection, identification, and quantification of *Leishmania* spp. are synthetized in table 3.

3.2 Non-DNA-based methods

Non-DNA-based methods are a wide group of techniques that can further be divided in parasitological, serological, and protein-based methods.

3.2.1. *Parasitological methods (for detection purposes)*

Parasitological methods are the most anciently described for the detection and the description of *Leishmania*. The most applied methods are microscopic examination and in vitro parasite culture.

Microscopic examination is usually applied to smears from biopsy of lesions for CL, and needle aspiration smears from spleen, bone marrow and lymph nodes for VL (Fig. 14) (Akhoundi *et al*., 2017). This approach is fast and cheap but of scarce application because it is invasive: biopsies especially in case of VL are not easy to perform. Microscopic examination doesn't allow to discriminate between *Leishmania* species and sensitivity is generally poor because it is strongly related to the number and the spread of the parasites in its host (Reimão *et al*., 2020).

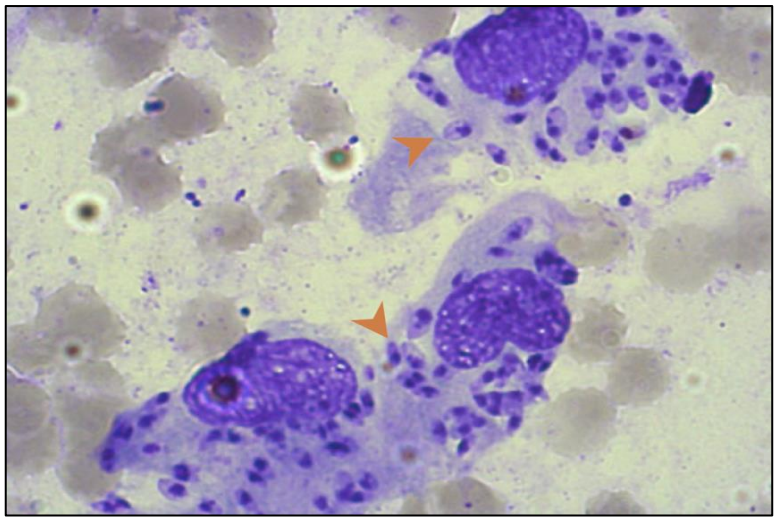

Figure 14. Amastigotes of Leishmania *pointed by orange arrows, isolated in macrophages from a patient with MCL (adapted from Richter* et al.*, 2011).*

To increase sensitivity, biopsy material can be used to inoculate culture medium, but this technique is rarely used in routine medical practice, although the establishment of a culture isolate is of great importance for epidemiological purposes. In fact, many of the techniques that allow strain discrimination require a consistent amount of quality genetic material, thus are culture dependent. Parasite cultivation is moreover important for drugs and vaccine production (Castelli *et al*., 2014). Unfortunately, success to recover *Leishmania* rarely is higher than 70%, is highly time-consuming and requires a good laboratory setup (Akhoundi *et al*., 2017).

The parasitological methods also include xenodiagnosis. In this case the vector is used as a "culture medium" to detect the infection in a mammalian host. Xenodiagnosis can be direct, when vectors are allowed to feed on the host, or indirect when they feed on heparinized blood through a feeder membrane (Singh *et al*., 2020).

Xenodiagnosis is time-consuming and requires a sand fly rearing laboratory and a trained entomologist for dissection and examination of vectors. Nevertheless, it remains a parasitological method of great importance because it can confirm the competence of sand flies as vector or mammals as reservoir (Molina *et al*., 2012).

Moreover, also inoculation of *Leishmania* in experimental animal and dermal diagnostic tests are comprised in the parasitological examination. The first is rarely applied because of the ethic implication, and further requires highly specialized laboratories and is mostly used as complementary tool (Sundar and Rai, 2002). The latter, *Leishmania* skin test (LST) or Montenegro test was considered an extremely important tool for diagnosis in the past century. It is easy to perform with good sensitivity and specificity, but it can't discriminate between past and current infection; in Europe it has been mostly substituted with serological and DNA-based techniques but is still commonly applied in Asia (Sadeghian *et al*., 2013).

Methodology			*Leishmania* Detection In Clinical Samples	*Leishmania* Identification	*Leishmania* Discrimination						*Leishmania* Quantification	Culture Needed	Sensitivity	Specificity
					G	SL	SG	C	S	F				
Non-DNA-based	Parasitological Methods	Microscopic examination	✓	×	✓						✓	×	**	*
		In vitro Parasite Cultures	✓	×	✓						×	✓	*	*
		Isolation in experimental animals	✓	×	✓						×	×	*	**
		Dermal diagnostic test	✓	×	✓						×	×	**	***
		Xenodiagnosis	✓	×	✓						×	×	**	***
	Serological Methods	IFAT	✓	✓	✓						×	×	***	**
		ELISA	✓	×	✓						×	×	**	**
		ICT	✓	✓	✓						×	×	**	**
		Western blot	✓	✓	✓						×	×	***	***
	Protein-based Methods	MLEE	×	✓	✓	✓	✓	✓	✓		×	✓		
		MALDI-TOF	×	✓	✓	✓	✓	✓	✓		×	✓		
DNA-based	PCR-based Methods	PCR*	✓	✓	✓	✓	✓	✓	✓		✓	×	***	**
		MLST	×	×	✓	✓	✓	✓	✓	✓	×	✓	***	***
		MLMT	×	×						✓	×	✓	***	***
	Post-PCR methods	PCR-HMR	✓	✓	✓	✓	✓	✓	✓	✓	×	×	**	***
		PCR-RFLP	✓	✓	✓	✓	✓	✓	✓		×	×	**	***
		Gene Sequencing	✓	✓	✓	✓	✓	✓	✓	✓	×	×	***	***
	Non-PCR-based methods	NASBA	×	✓							×	×	**	***
		LAMP	×	✓							×	×	***	***

Table 3. Comparison of different diagnostic methods of Leishmania species (adapted from Akhoundi et al.*, 2017). G: Genus; SL: Section Level; SG: Subgenus; C: Complex; S: Species; F: Foci. *= low; **=medium;***=high*

**Including different PCR methods i.e. multiplex and nested PCR.*

Abbreviation: MLMT (Multilocus microsatellite typing); PCR-HMR (High resolution melting); PCR-RFLP (Restriction fragment length polymorphism); NASBA (Nucleic acid sequence based amplification); LAMP (Loop-mediated isothermal amplification); ICT (immune-chromatography); ELISA (Enzyme-linked immunosorbent assay); MLEE (Multilocus enzyme electrophoresis); MALDI-TOF (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight).

3.2.2. Serological methods (for diagnostic purposes)

Serological diagnosis is of great importance especially in veterinary routine practice. It is based on the detection of *Leishmania* antigens or anti-*Leishmania* antibodies in serum or urine samples of patients. In canine patients, serological diagnosis is considered the gold standard because it allows the quantification of the antibodies-titer; in human patients it is mostly used for VL because of the prominent humoral response (Reimão *et al*., 2020).

The most used methods are: (*i*) indirect fluorescent antibody test (IFAT); (*ii*) Enzyme-linked immunosorbent assay (ELISA); (*iii*) immunochromatographic strip test (ICT); (*iv*) Western blot.

- IFAT is a serological test largely used in veterinary diagnosis. Test samples (blood sera) are reacted with anti-*Leishmania* antigens presented on acetone-fixed promastigotes on slides followed by a secondary indicator fluorescein-labeled anti-species antibody directed at the specific immune globulin (Fig. 15). The International Office of Epizootics (OIE) refers to IFAT as the gold standard for the diagnosis of CanL, also disciplined by Italian law, and in the regional control plan of VBDs (Venturi *et al*., 2009; OIE, 2021). Dogs with a titer below 1:40 are considered negative, while a titer above 1:160 is suggestive of established infection. In human patients, infection titers range from 1:80 to 1:160 (OIE, 2021). In the case of CanL sensitivity and specificity are

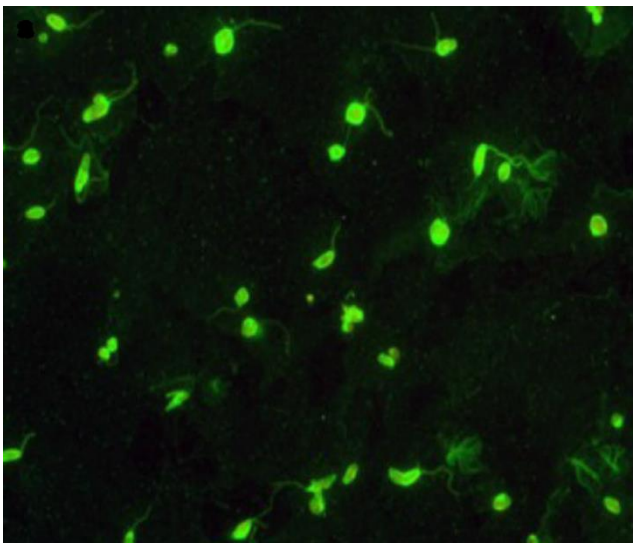

Figure 15. IFAT using as antigen promastigotes of L. infantum *(adapted from Mendoza-Roldan* et al.*, 2021)*

high, while in human patients they decrease in case of coinfection with HIV, or diagnosis of CL or MCL (Barroso-Freitas *et al.*, 2009).

- ELISA has been used as a potential serodiagnostic tool for almost all transmissible diseases, including leishmaniasis (Sundar and Rai, 2002). ELISA can detect and possibly quantify anti-Leishmania antibodies in a sample. Briefly, the Leishmania's antigens are immobilized in a microplate (directly or by a capture antigen) and react with the antibodies of the sample. The reaction produces a color signal indicating the presence of the antigen in the sample; the measurement of the optical density is proportional to the quantity of the antigen in the sample (Shah and Maghsoundlou, 2016). Its sensitivity is high, but the specificity depends upon the antigen used. The most used antigens for *Leishmania* are crude soluble antigens (CSA) present on the membrane of amastigote and promastigote stages, and the kinesin-related recombinant antigen rK39.

On a general note, ELISA can be used for many samples, with different antigens and different types of matrixes at the same time, but it requires specialized professional, sophisticated equipment and is time-consuming, even though rapid kits have been developed. Rapid kits have a sensitivity of 94.7% and specificity ranging from 90.6% to 100%, that are comparable to the standard ELISA technique which has a sensitivity of 80-99.5% and a specificity of 81-100% (Marcondes *et al*., 2011). The lack of discrimination between active disease and clinical healthy patient makes use of rapid kits limited in endemic regions (Elmahallawy *et al.*, 2014).

- A micro-ELISA on strip has also been developed for use in endemic areas, where any sophisticated method can scarcely be employed on large scale (Boelaert *et al.*, 2008). The strip test (Immuno-Chromatographic Test - ICT), is used in the diagnosis of VL, but sensitivity and specificity vary according to the different commercial kit employed and patient's origin (Bangert *et al*., 2018).

- Western blot has been reported as a highly sensitive and specific test that can be applied to different matrixes like serum and urine (Mirzaei *et al*., 2018). Western

blot can give details on antibodies' responses to various antigens; however, it is time consuming, expensive and requires qualified professionals (Patil *et al*., 2012).

3.2.3. Protein based methods (for identification purposes)

Among the protein-based methods for the identification of *Leishmania* spp., the multilocus enzyme electrophoresis (MLEE), is indicated by WHO as the gold standard technique (OIE, 2021). MLEE completely depends on the isolation and cultivation of the parasite, and it differentiates strains based on the profile of a set of proteins in a pH-dependent gel electrophoresis (Reimão *et al*., 2020). Currently the electrophoretic mobility of 15 enzymes has been tested (Pratlong *et al*., 2016). Based on their electrophoretic profile, strains are classified in zymodemes. Only few laboratories in the world can perform MLEE, it is time consuming and has some limitations in the discrimination of strains (Schönian *et al*., 2011).

As earlier mentioned, the Old and the New World use different enzymatic panels so that strains cannot be compared directly. Moreover, MLEE fails to detect nucleotide substitutions, that do not imply aminoacidic changes, and aminoacidic substitutions that do not alter electrophoretic mobility; it does not distinguish similar electrophoretic mobilities that are dependent on distinct genotypes and at last electrophoretic mobility can be modified by post-translational modification (Zemanova *et al*., 2007; Alam *et al.*, 2009). For these reasons, molecular studies do not always agree with the identification of *Leishmania* isolates by MLEE (Schönian *et al*., 2011).

More recently also a mass spectrometry technique has been developed for the identification of *Leishmania*. It is a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) assay. MALDI-TOF consists in ionization of the sample in a specific acid solution, via laser beams from spectrometers the sample is later evaporated towards a sensor; the "time of flight" depends on the molecular weight of the ionized molecules (Fig. 16) (Mouri *et al*., 2014). As main limits, the initial cost of the MALDI-TOF equipment is highly expensive, and it requires a fully equipped

laboratory and trained professionals in the analysis of the results. Furthermore MALDI-TOF needs cultured parasites so it can't be applied directly to clinical samples (Akhoundi *et al*., 2017; Reimão *et al*., 2020).

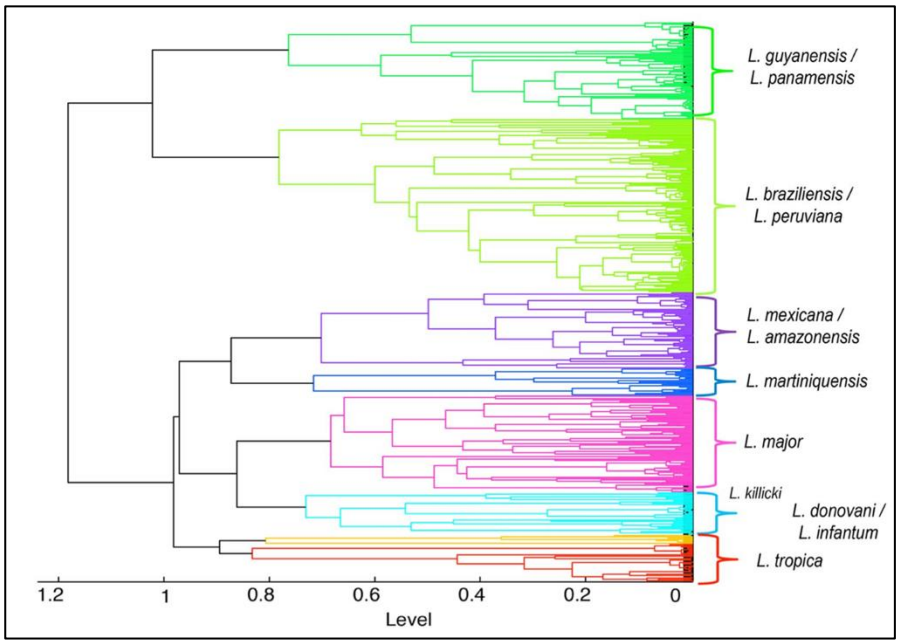

Figure 16. Analysis of MALDI-TOF mass spectrometry of 184 spectra from 46 Leishmania *isolates with distances displayed in relative units (Mouri* et al*., 2014).*

3.3. DNA-based methods (for detection, identification, strain discrimination and quantification purposes)

Since the 1980s, the amplification of DNA via PCR has enabled the development of fast and highly sensitive detection of *Leishmania* in various biological samples (Akhoundi *et al.*, 2017). Molecular methods are routinely applied worldwide for the diagnosis of *leishmaniasis* because they exhibit several advantages, such as feasibility, safety, and reliable results (Thakur *et al.*, 2020).

3.3.1 A kind of magic: PCR assay for detection and identification of Leishmania *spp.*

PCR-based assays are the basis of *Leishmania* detection and typing (Akhoundi *et al.*, 2017; Reimão *et al*., 2020). In most cases, PCR doesn't need cultivation of parasites and it can distinguish even various strains according to the target gene or region. PCR can also be applied to samples with low parasite loads, conversely to microscopy or cultivation (Antinori *et al*., 2007). Conventional (or endpoint) PCR consists in the

partial or total amplification of a DNA region; however, several other PCR assays have been developed.

A variant of conventional PCR is the nested-PCR. It consists of the amplification of a DNA region via two rounds of PCR; two pairs of primers are used, the first amplification targets a long fragment of DNA, the second amplifies a region comprised in the first round (Fig. 17). This technique is often applied when the parasitic load is low but can encompass contamination between the first and the second round (van Eys *et al*., 1992). A variation of nested PCR is the hemi-nested PCR, in which one of the primers used for the first amplification will be used also for the second round (Fig 17).

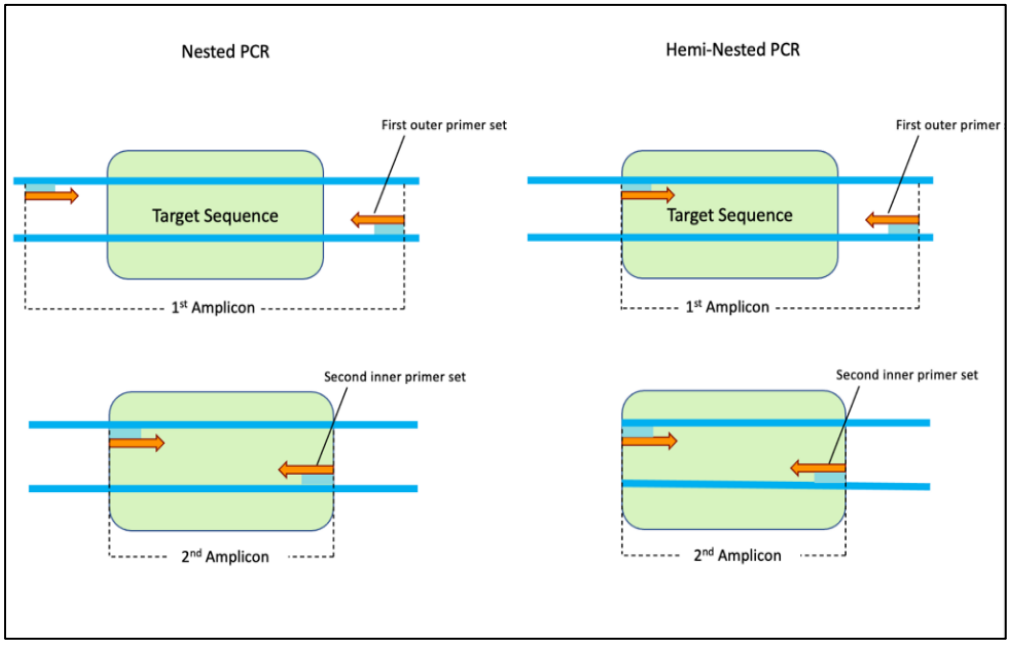

Figure 17. Schematic representation of nested and hemi-nested PCR.

Different DNA targets can also be amplified at same time with different PCR reactions performed simultaneously: this is the case of multiplex PCR. In a multiplex PCR, amplicons of specific DNA targets which vary in size can be produced by using numerous primer sets in one PCR mix only. Multiplex PCR can be used in clinical laboratories, but a relatively low sensitivity makes it a second-choice assay when compared to end-point PCR (Thakur *et al*., 2020).

A more recent PCR assay is the real-time PCR. A real-time PCR is a quantitative PCR that measures the amount of DNA generated by monitoring the amplification of a specific target during each PCR cycle (Fig. 18) (Reimão *et al*., 2020). Real-time PCR is worldwide applied and often it has been described as a valuable tool for the diagnosis of leishmaniasis (Schönian *et al*., 2008). Different targets and protocols have been optimized for detection and quantification of parasite load, and species typing (Moreira *et al*., 2018).

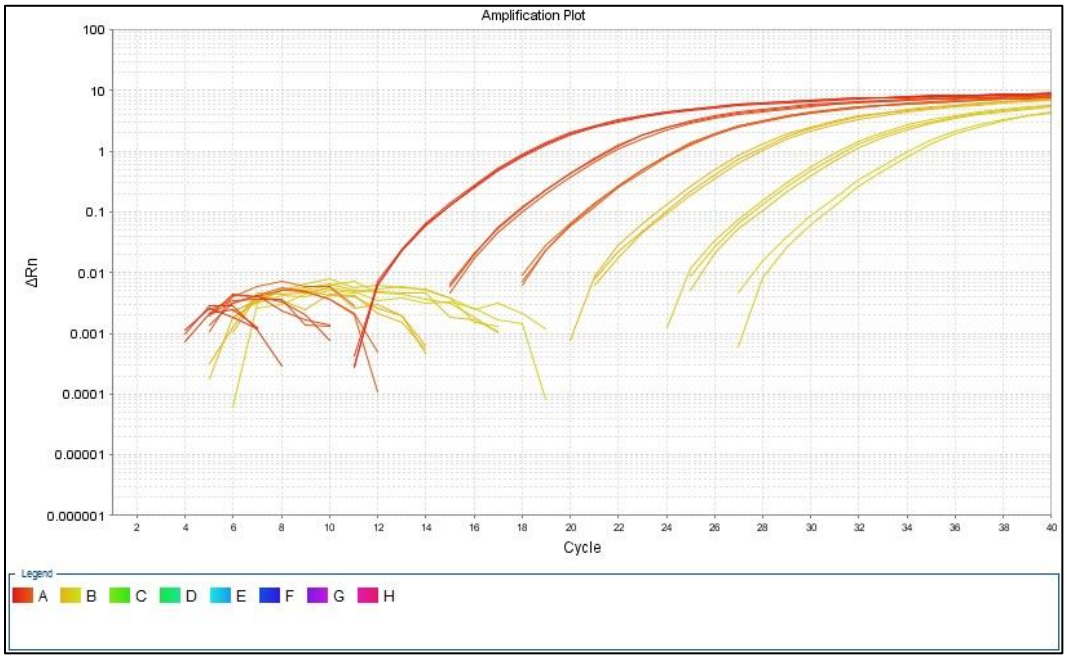

Figure 18. Amplification plot of a real-time PCR.

To discriminate different clinical samples, the Multilocus Sequence Typing (MLST) represents a valuable assay. This method consists in the amplification via PCR followed by DNA sequencing of some housekeeping genes that are evaluated simultaneously. MLST is used mostly (but not only) in endemic areas, it has lower sensitivity than conventional PCR, requires meticulous optimization and often is culture dependent (Thakur *et al*., 2020).

Multilocus Microsatellite Typing (MLMT) is similar to MLST but targets the microsatellites. Microsatellites are repeated motifs of non-coding nucleotides found in all eukaryotic and prokaryotic genomes (Jarne and Lagoda, 1996) and the genome of *Leishmania* is rich in microsatellite sequences (Schönian *et al*., 2011). MLMT can give important insights into the epidemiology of *Leishmania* and allows the characterization

of strains from different geographical areas (Aluru *et al*., 2015). As MLST, MLMT requires meticulous optimization and is culture dependent; however, thanks to its discrimination capacity, MLMT is often used as an alternative to MLEE (Schönian *et al*., 2011).

3.3.2 Post-PCR methods (for identification purposes)

Post-PCR methods are applied to the amplicons of the PCR.

The PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) can discriminate various species depending on the pattern of DNA fragments after digestion with one or more restriction enzymes evaluated by gel electrophoresis. PCR-RFLP is simple and doesn't require too much sophisticated equipment. Several protocols have been described according to different molecular targets; to date the most used to differentiate *Leishmania* spp. targets the ITS region and the heat shock protein 70 (Hsp70) (Akhoundi *et al*., 2017).

A post-PCR method applied to the real-time PCR is the PCR-Hight Resolution Melting (PCR-HRM). PCR-HRM is based on the variations in DNA sequences and uses double-stranded DNA binding dyes for measuring the intensity of fluorescence during dissociation of double stranded to single-stranded DNA amplicons generated from a real-time PCR assay (Reimão *et al*., 2020). This assay can be performed with new generation saturating dyes (i.e., Eva Green or SYTO9) or on an adapted PCR instrument (Moreira *et al*., 2018). Like other real-time PCR protocols, the results may be obtained quickly, with a reduced likelihood of contamination (Galluzzi *et al*., 2018).

A technique mostly used for phylogenetic studies is DNA sequencing. It is based on the incorporation of chain-terminating dideoxynucleotides for the determination of the nucleotide sequence of a specific fragment of DNA (Akhoundi *et al*., 2017). Typing of *Leishmania* can be performed by the analysis of single nucleotide polymorphisms (SNPs) or by comparison of the sample sequence with a reference sequence of *Leishmania* spp. (van der Auwera and Dujardin, 2015). Species discrimination by sequencing is mostly applied to chromosomic regions, but kDNA regions have been

used as well like the cytochrome B gene (van der Auwera and Dujardin, 2015). Minicircles, another component of kDNA, are too variable for species discrimination, however, are useful targets for DNA detection thanks to the high number of copies (Singh *et al*., 1999).

3.3.3 Non-PCR based methods (for diagnostic purposes)

As an alternative to PCR, other techniques targeting DNA can be used for the diagnosis of *leishmaniasis*.

The Loop-mediated isothermal amplification (LAMP) consists in an isothermal amplification that can increase the amount of amplified DNA up to a billion copies in less than an hour. It requires four different primers designed to recognize six distinct regions of a target locus; amplification can be detected by eye as white precipitate or as yellow-green solution after the addition of SYBR green dye (Soroka *et al*., 2021). LAMP is a fast assay that requires only basic laboratory equipment, and sensitivity is high for the diagnosis of both CL and VL (Thakur *et al*., 2020). However, since the amplification occurs at relatively low temperature (60-65°C), the target region shouldn't be GC rich; besides there's a high risk of formation of DNA secondary structures, it has a limited suitable temperature range, and requires accurate optimization (Akhoundi *et al*., 2017).

Developed for the detection of RNA, the nucleic acid sequence-based amplification (NASBA) also exploits the isothermal amplification. NASBA is the only isothermal method that uses RNA as starting material. Amplification is faster when compared to a PCR assay, however NASBA is not suitable for quantification or species discrimination, and ribonuclease contamination can degrade the target RNA (Zanoli and Spoto, 2013).

3.4 Leishmania *identification: Molecular targets*

Identification of *Leishmania* species is highly recommended by WHO (2010) for the correct diagnosis and prognosis of the patient, as well as for management, treatment, and control of the disease.

PCR-based methods combine high sensitivity for direct detection with species specificity: to date no commercial standard tests are available for *Leishmania* species typing. Identification can be achieved with conventional or real-time PCR, as well as with RFLP (Gramiccia and di Muccio, 2018).

As well as other trypanosomatids, *Leishmania* spp. have a unique genomic organization when compared to other eukaryotes. The absence of introns, polycistrons and small chromosomes with high gene densities are some of the traits. The *Leishmania* genome is haploid, organized in 36 chromosomes for the Old World *Leishmania*, and 34 for the New World *Leishmania* (Kazemi, 2011). Moreover, *Leishmania* spp. possess a single mitochondrion including a kinetoplast containing a large network of kinetoplast DNA (kDNA) (Akhoundi *et al*., 2017).

Over the years these distinctive traits have been studied to develop epidemiological or population genetic assays. The most used molecular targets are (Fig 19): (*i*) ribosomal RNA genes; (*ii*) repetitive nuclear sequences; (*iii*) antigen genes like glycoprotein 63 (gp63), heat-shock protein 70 (Hsp70), cysteine protease B (cpB); (*iv*) kDNA comprehensive of maxicircles and minicircles; (*v*) mini-exon genes (ME) (Gramiccia and di Muccio, 2018).

Depending on the purpose of the diagnosis and the epidemiological context, the choice of the molecular target may vary. Targets (and consequently primers) can be genus, subgenus, species, or strain specific. On a general note, the first targets used for the diagnosis of *Leishmania* are high-copy-number targets such as kDNA, SSU rDNA and ME that have been considered the most sensitive (Schönian *et al*., 2003; Gramiccia and di Muccio, 2018).

After the infection has been confirmed by one of the available methods, the second step is the identification at species complex or species level. At this step sensitivity should be less emphasized to allow the discrimination of as many species as possible. ITS1 and Hsp70 are considered the best target for *Leishmania* species identification both in Old and New World (van der Auwera *et al*., 2014).

Discrimination of strains can be achieved by performing assays that target both coding or non-coding multigene targets, like kDNA minicircles, cpB, gp63, ITS, or ME. Unfortunately, they showed some limitations. The main one is the lack of validation, that makes these tests not fully reproducible and comparable between laboratories (Gramiccia and di Muccio, 2018). Moreover, if a test has been developed on a particular *Leishmania* strain, e.g., local strain, the same test could fail if used on global scales remaining still usable locally (Schönian *et al*., 2008).

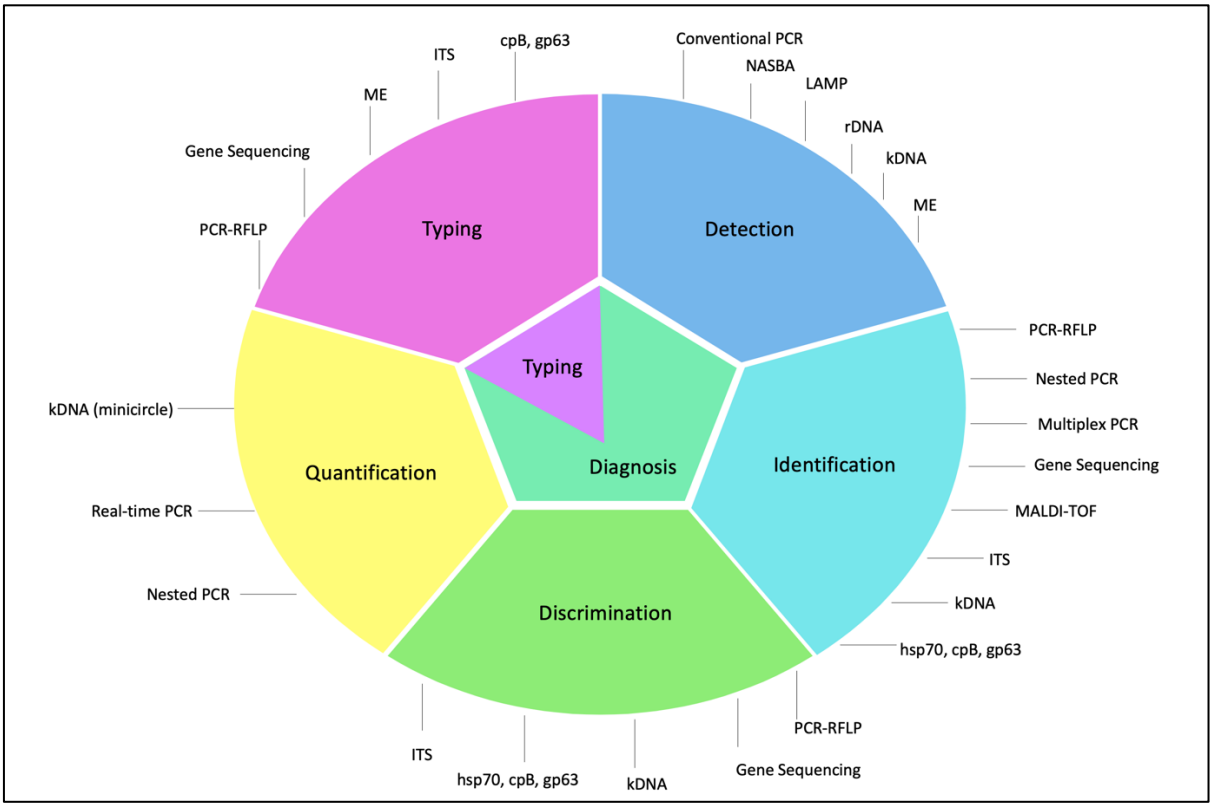

Figure 19. DNA-based methods and molecular targets for Leishmania *diagnosis and typing.*

3.4.1 *Ribosomal DNA (rDNA)*

rDNA belongs to the chromosomal DNA. As in most of the eukaryotes, *Leishmania* ribosomes are composed of two subunits with four rRNA types and more than 70 proteins. The ribosomal RNA transcription units are: the large subunit (LSU) containing the 28S; the 5.8S; the 5S rRNAs and the small subunit (SSU) containing the 18S rRNA (Fig 20) (Torres-Machorro *et al*., 2009). ITS (Internal transcribed spacers) are non-coding DNA spacers. In *Leishmania*, rDNA genes are mostly located on chromosome 27, usually in multiple copies of tandem head-to-tail repeats of 12.5Kb.

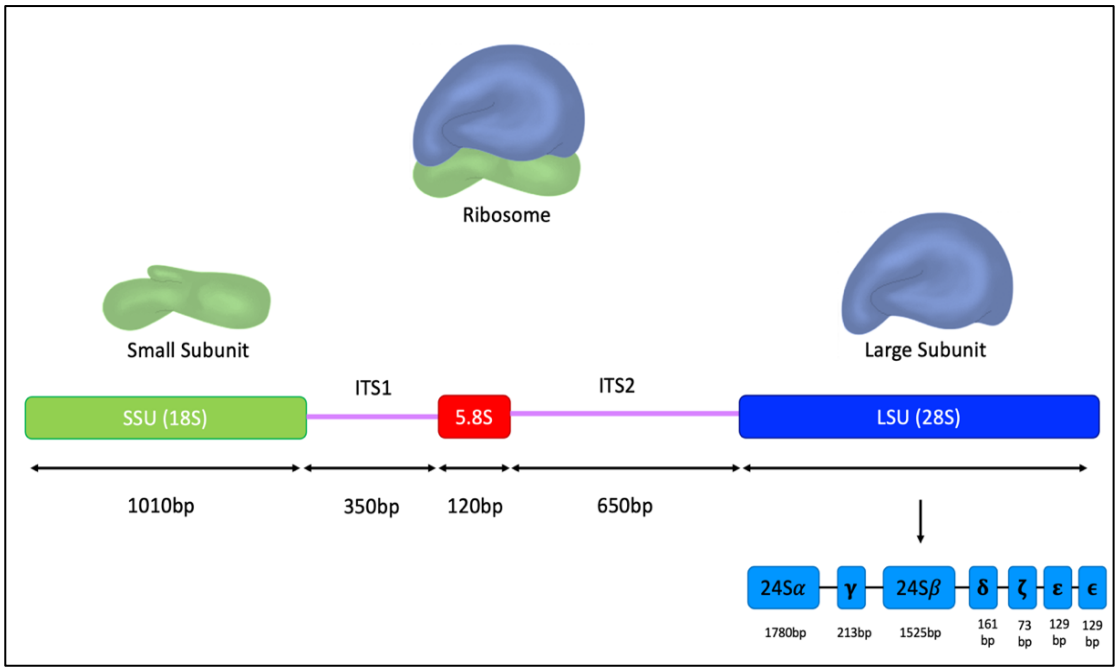

Figure 20. Genetic map of rDNA genes with corresponding fragment length in Leishmania *species.*

As molecular markers, rDNA is mostly used for reconstructing phylogenetic relationships, thanks to its high conservation and its flaking regions (van Eys *et al*., 1992). Among the units, the 18S rRNA is one of the most used markers for phylogenetic purposes because it encodes for the structural subunit of the ribosome. The 28S rRNA catalyzes the peptide bond formation, and in *Leishmania* spp. its structural components differ from the ones described in other eukaryotes. Briefly, it contains two large subunits (24α and 24β) and four small rRNA molecules (γ, ζ, δ, ε) (Soto *et al*., 2004).

As earlier mentioned, rDNA is comprehensive of two non-coding DNA spacers ITS1 and ITS2. The ITS1 length varies according to different *Leishmania* species from

300 to 350bp. It is considered a highly conservative region, but thanks to the development of a PCR-RFLP assay it can be used as molecular marker for species typing (Schönian *et al*., 2003). To date, it is considered one of the best targets for *Leishmania* species determination (Van der Auwera *et al*., 2014).

The rDNA molecular targets are conservative among Trypanosomatidae family, therefore their use in species typing should be carefully evaluated, especially when isolation and cultivation of the parasites are not possible like in the case of diagnosis of *Leishmania* spp. in wild fauna (Díaz-Sáez *et al*., 2014; Merino-Espinosa *et al*., 2016; Calzolari *et al*., 2018).

3.4.2 *Repetitive nuclear sequences*

Repetitive nuclear sequences or repetitive elements are repetitive portions of the genome. They can be classified according to the proliferation state in "living" repeat, which are responsible for changes in the host genome and modulation in the pattern of genes expression, as well as daily maintenance of chromosomes; or in "dead" repeats that constitute a paleontological record (Wickstead *et al*., 2003). Within the *L. donovani* complex, common "dead" repetitive elements have been described. They consist in multiple tandem copies of a 60 bp repeat found in at least six chromosomes (Howard *et al.,* 1991). The redundant nature of these elements makes them a good target to differentiate species based on length polymorphism after end-point PCR or on a PCR-RFLP assay. With reference to the *L. donovani* complex and to the species *L. infantum*, repetitive elements are variable enough to give information at the strain level, especially when applied to a PCR-RLFP assay (Minodier *et al*., 1997).

3.4.3 *Under pressure: Antigen genes*

Antigen genes are of great value in genotyping assays to answer clinical or epidemiological questions (Gramiccia and di Muccio, 2018). Their variable nature makes them a first-choice target to evaluate population structure because subjected to more selective pressure (Guerbouj *et al*., 2001).

One of the most studied target gene is the gp63, which encodes for a major surface glycoprotein widely expressed on the cell membrane. Gp63 has been classified as a virulence factor playing a major role in the bond between the promastigote and the host macrophage and in the interference with the complement fixation (Lieke *et al*., 2008). Molecular tests targeting this region can discriminate *Leishmania* at the species level and, in the case of a PCR assay, RFLP is required (Guerbouj *et al*., 2001; Victoir *et al*., 2003).

Among the possible targets for *Leishmania* species discrimination, heat-shock protein 70 (Hsp70) is considered one of the best, as well as ITS1 (Van der Auwera *et al*., 2014). Hsp70 are chaperonins involved in a considerable wide range of cellular housekeeping activities including folding newly synthesized proteins, translocation of polypeptides into mitochondria and endoplasmic reticulum, disassembly of protein complexes and regulation of protein activities, and stress related activities (Fig. 21) (Rosenzweig *et al*., 2019).

Hps70 of *Leishmania* spp. has been studied in both the Old and the New World. Several primer pairs have been designed and are now applicable to both conventional and nested PCR (Garcia *et al.*, 2004; Montalvo *et al.*, 2010; Montalvo *et al.*, 2012; Van der Auwera *et al.*, 2014).

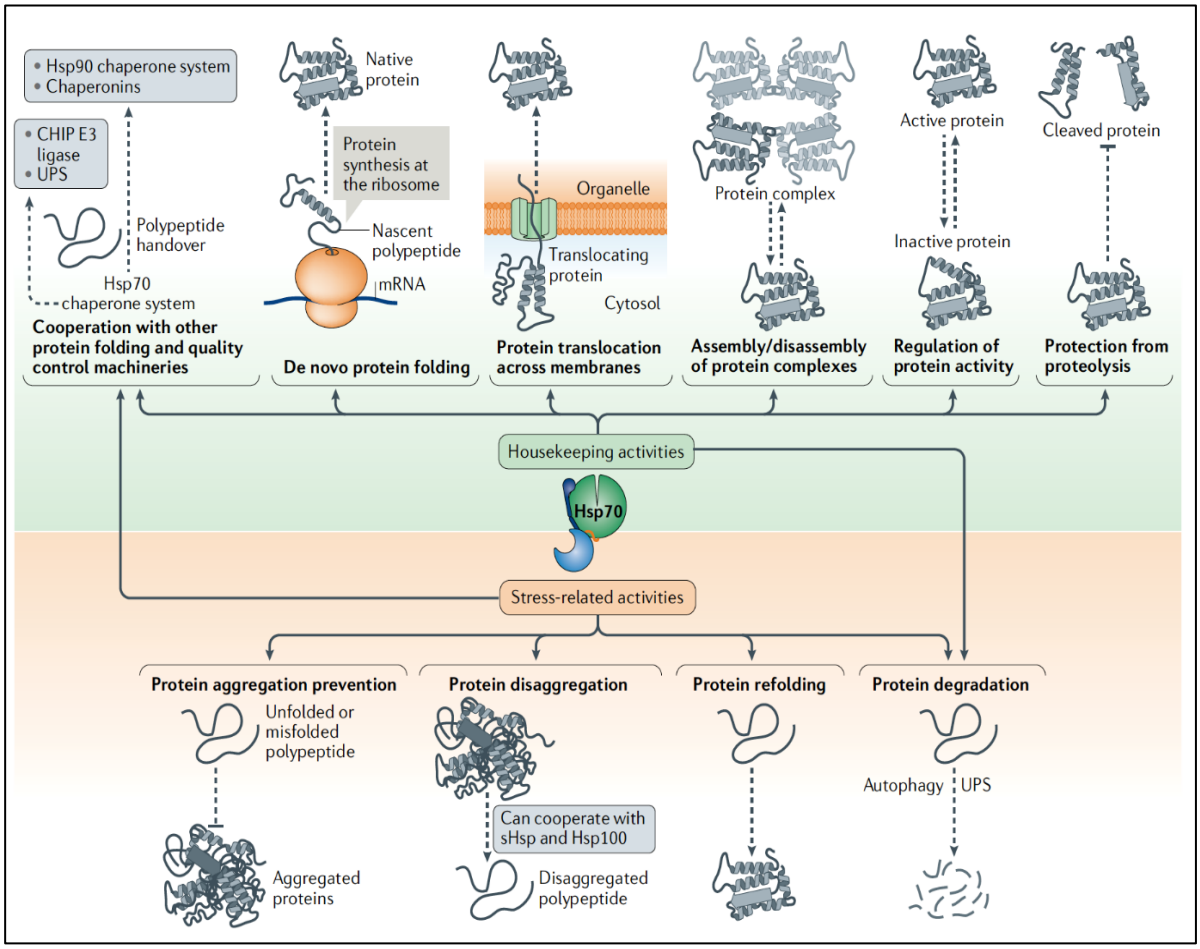

Figure 21. Housekeeping and stress related Hsp70 (Rosenzweig et al.*, 2019).*

Cysteine protease B (cpB) is implicated in several processes including cellular differentiation, nutrition, host cell infection, and evasion of the host immune response (Denise *et al.*, 2003). The cpB genes are multicopy genes located in a single locus with different copy numbers and different nuclear sequences, varying according to the species (Hide *et al.*, 2007). In the *L. donovani* complex, the cpB gene consists of 5 copies tandemly arranged (A, B, C, D, E) (Mundodi *et al.*, 2002). Among them, the last copy has a distinct sequence at its 3' end, where a region of 39-bp indel is described in different species of the *L. donovani* complex. Thanks to the cpB, the *L. donovani* complex can be distinguished in different clusters according to the bp-length. In this locus, the

region of 39 bp can be observed in *L. donovani*, *L. archibaldi* and in some strains of *L. infantum* or deleted in other strains of *L. infantum* (Hide and Bañuls, 2006). Concerning *L. infantum*, the presence of two different strains (with or without the indel) can be exploited in epidemiological surveys; indeed, this differentiation has been proposed for laboratory cultures in end-point PCR assays (Zackay *et al*., 2013; Rugna *et al*., 2017) and for biological samples in a nested PCR assay (Magri *et al*., 2022a - present PhD Thesis).

3.4.4. *The other side: Kinetoplast DNA*

Kinetoplastids have a single mitochondrion, including a special organelle called kinetoplast. This organelle has many unusual properties which are unique to the order Kinetoplastida, like an extensive kinetoplast DNA (kDNA) network and U-insertion/deletion type RNA editing of its mitochondrial transcripts (Lukeš *et al*., 2005).

kDNA is composed by concatenated circular DNAs of two types (Fig. 22): maxicircles present in few dozens of identical copies per network, and minicircles present in several thousand copies per network of similar length but with different sequences (Lukeš *et al*., 2002).

The maxicircles are the most conserved, they encode for proteins and rRNA and are comprehensive of a non-transcribed variable region (VR). Differences in the *Leishmania* species can be found mostly in the VR (Akhoundi *et al*., 2017).

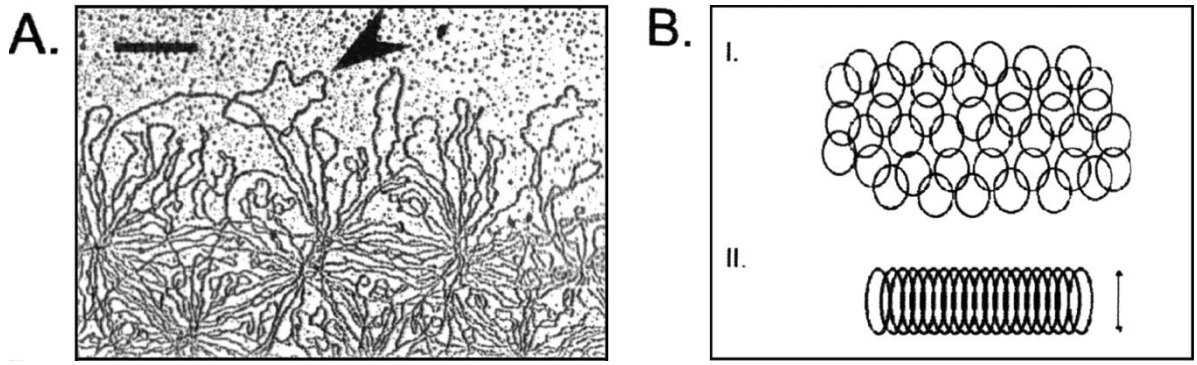

Figure 22. kDNA network structure. (A) Electron micrograph of the periphery of an isolated kDNA network. (B) Diagrams showing the organization of minicircles: (I) Segment of an isolated network showing interlocked minicircle; (II) Section through a condensed network disk in vivo showing stretched-out minicircles. (adapted from Lukeš et al*., 2002).*

Minicircles have species-specific size ranges. Since they are present in thousands of copies per cell, minicircles are an excellent target for the detection of *Leishmania* from biological samples with a low parasitic load (Ceccarelli *et al*., 2014). Despite being a highly variable site, minicircles contain three conserved sequence blocks (CSB-I, CSB-II, CSB-III) which usually are targets of *Leishmania* for sub-generic level assays or phylogenetic investigations (Fig. 23) (Yurčenko *et al*., 2000; Salvatore *et al*., 2016; Akhoundi *et al*., 2017).

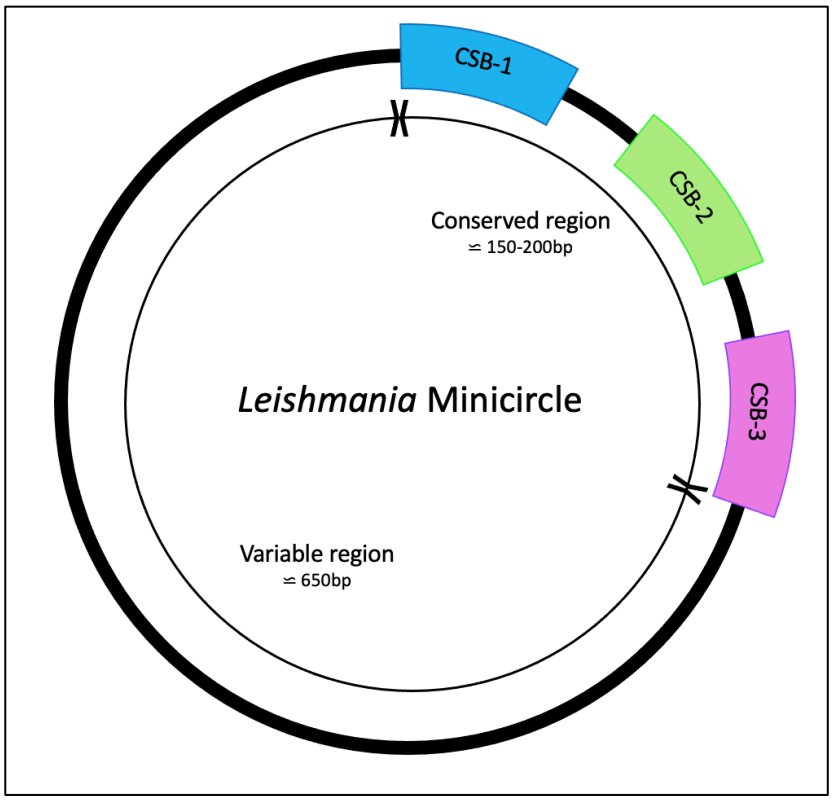

Figure 23. Minicircle of Leishmania *sp. with component fragments.*

3.4.5 *Mini-exon genes*

Mini-exon sequences are present in all kinetoplastids, however their precise function is still not clear, even though they are considered of great importance in cell metabolism. However, the mini-exon derived RNA, enters in the *trans*-splicing reaction with cellular mRNA precursors (Hassan *et al*., 1992). Mini-exon genes are present in 100-200 tandemly repeated copies in the nuclear genome, with each repeat consisting of (*i*) a transcribed very conservative region formed by a 39 nucleotides exon

and a moderately conserved intron of 55-101 nucleotides and (*ii*) a variable non-transcribed spacer (Fernandes *et al*., 1994). Mini-exon genes are present in 100-200 copies per cell so the assays targeting this region can be used for the detection of *Leishmania* spp. (Gramiccia and di Muccio, 2018), moreover the bp-length and the DNA sequence variations of the non-transcribed spacer can be used for species-specific assays (Fernandes *et al*., 1994).

Chapter 4

Aims & Objectives

4.1 *Aims & Objectives*

As the role of dogs as a reservoir of *Leishmania infantum* in the Emilia-Romagna region (Italy) has been questioned since the first notifications of human visceral leishmaniasis (HVL) (Pampiglione, 1975) until more recent HVL cases (Varani *et al*., 2013; Rugna *et al*., 2017), the main aim of this PhD project was to investigate the role of wild and peridomestic mammals as potential animal reservoirs of *L. infantum* in the regional zones where HVL foci are still active, also evaluating the possible role of arthropod vectors other than phlebotomine sandflies as vectors of *Leishmania* spp. in the sylvatic cycle of the protozoa.

In this view the following objectives were undertaken:

- to investigate the presence of *Leishmania* spp. in wild and synanthropic mammals collected nearby reported HVL cases or foci. Such epidemiological surveys were performed considering the studies conducted in other Italian regions and in other European countries, including results of research on the host preferences of sandflies in the Emilia-Romagna region. Therefore, the parasitological survey mainly focused on animal species never tested before in Italy, like *Rattus norvegicus* and *Mus musculus*, or worldwide, such as *Capreolus capreolus*.
- to develop a new molecular assay to distinguish the two strains currently circulating in the Emilia-Romagna region, to be applied on biological samples without the need for parasite isolation or sequencing.
- to collect and review epidemiological data available in Europe on the presence of non-pathogenic trypanosomes, that can often interfere with the molecular diagnosis of other strictly related pathogens, such as *Leishmania* spp..
- to deepen the knowledge on the sylvatic cycle of *L. infantum* by investigating potential *Leishmania* vectors other than phlebotomine sandflies. The presence of the

protozoa was tested in different developmental stages of questing *Ixodes ricinus* ticks collected from rural environment in three parks of Emilia-Romagna region close to reported HVL and CanL cases.

Chapter 5

Materials and Methods

5.1 *Samples from mammals*

Different species of mammals were collected from the provinces of Bologna, Ferrara, Forlì-Cesena, and Ravenna (Emilia-Romagna) between June 2019 and October 2021, focusing primarily on areas where - according to the regional control plan on leishmaniasis - cases of HVL were described (Santi *et al*., 2021).

The carcasses of pest rodents were collected by professional pest control services, the spleen and earlobe of roe deer were provided by ungulates selection hunters, while the carcasses of other wild mammals were collected during park surveillance activities by volunteers and park rangers.

All these samplings were conducted under specific agreements and informative meetings made with professionals and local authorities. Concerning wild mammals, different actions were undertaken for roe deer and other mammals (such as micromammals or wild carnivores). Informative meetings were kept with volunteer park rangers and ungulates selection hunters, with the distribution of informative flyers (Appendix 1 and 2, respectively), to collect animal carcasses found during park surveillance or portions of organs from roe deer hunted and slaughtered.

Overall, samples of organs and tissues from 204 mammals were tested (Fig. 24). When the entire carcass was available, necropsies and samples collection were performed with sterile surgical instruments, and four samples were collected: ear lobe skin, spleen, liver and prescapular lymph nodes (not sampled in 16 pest rodents due to the corruption of the remains).

DNA from these samples was isolated with PureLink® Genomic DNA Mini Kit (Invitrogen/ Thermo Fisher Scientific, Carlsbad, USA) according to the manufacturer's instructions.

Figure 24. Number of specimens tested for each mammal species.

5.2 *Samples from ticks*

Archival DNA extracts from questing *Ixodes ricinus* tick collected from April to October 2012 were examined. Briefly, ticks were collected in 4 sites within 3 parks of the Emilia-Romagna region, in the hilly area of the Apennines, where autochthonous HVL and CanL have been described (Fig. 25) (Mollicone *et al*., 2003; Varani *et al*., 2013).

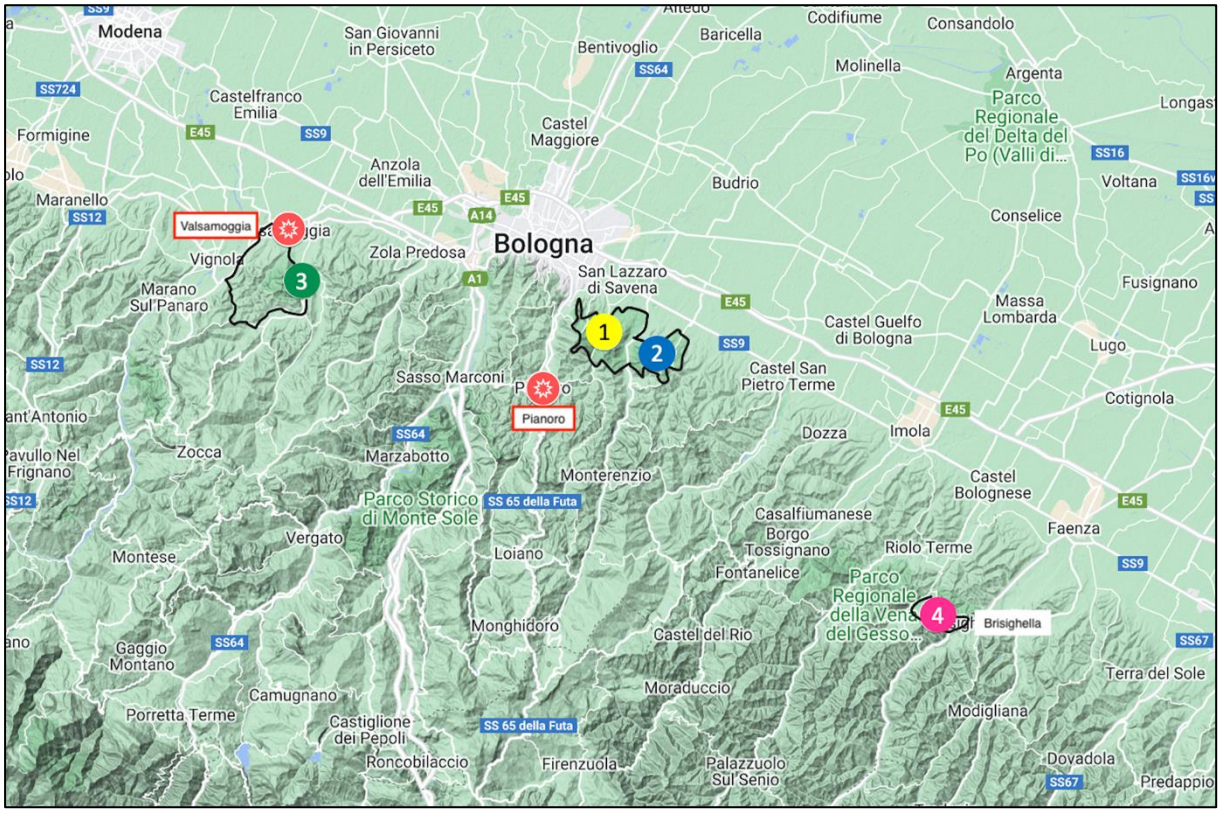

Figure 25. Map of the four sampling sites distributed in three parks of Emilia—Romagna region. Gessi Bolognesi and Calanchi dell'Abbadessa Park: 1 Ca' de Mandorli and 2 Ciagnano. Monteveglio Abbey Park site: 3; Carnè Park site 4. Park borders are marked with black lines.

= Active foci of human visceral leishmaniasis in Valsamoggia and Pianoro (VL single cases have been reported along the whole foothill side).

Ticks were sampled every 15 days by continuously flagging a 1 m^2 white cotton cloth, from transects of 20 m along the uphill side of the pathways, usually reported as having higher tick density than the downhill side, while the picnic areas were flagged completely as described in detail by Aureli *et al*. (2015). The ticks were preserved in 70% ethanol at room temperature and morphological identification was performed following Manilla (1998) and Iori *et al*. (2005). Ticks were processed for DNA extraction as described by Aureli *et al*. (2015) as follows: individual adults and pools consisting of either 5 nymphs or 10 larvae.

Overall, 236 DNA extracts from 7 females, 6 males, 72 nymphs pools (i.e., 380 nymphs) and 151 larvae pools (i.e., 1510 larvae) were analyzed.

5.3 *Real-time PCR*

The presence of *Leishmania* spp. was assessed with a highly sensitive real-time PCR targeting a 71-bp region of minicircle kinetoplast DNA using primer pair Leish71Up (5′-CCAAACTTTTCTGGTCCTYCGGGTAG-3′) and Leish71Do (5′-TGAACGGGATTTCTGCACCCATTTTTC-3′) (Tsakmakidis *et al*., 2017), designed on the CSB of the minicircles (Fig 26).

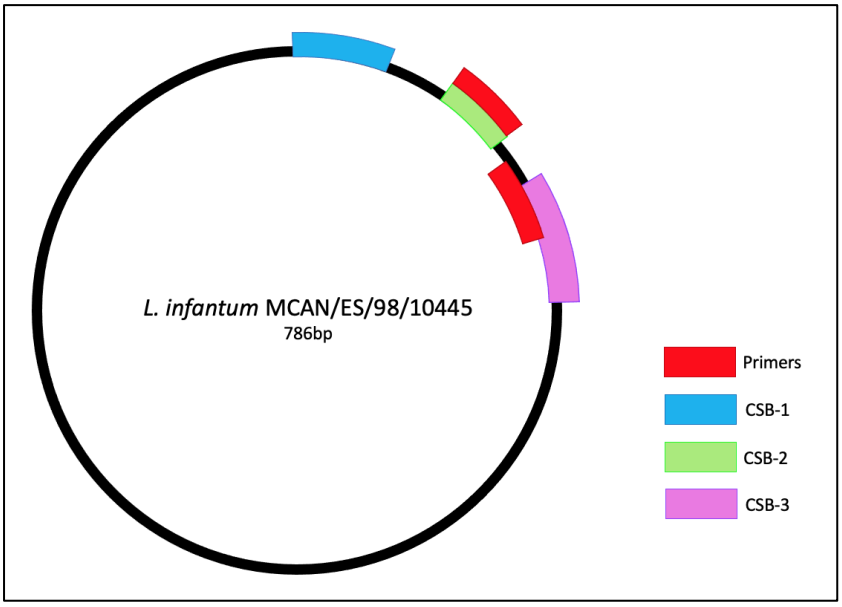

Figure 26. Annealing sites of the primers on the minicircle of L. infantum.

Reactions were carried out in a total volume of 20 µL with 10 µL of PowerUP™ SYBR™ Green master mix (2X), 0.3 µM of each primer and 2 µL of DNA. The thermal cycling profile was adapted to the degraded samples increasing the initial denaturation early described by Tsakmakidis *et al*., 2017. The amplification was performed in StepOnePlus Real-Time PCR System (Applied Biosystems) and the thermal cycling profile was as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s., 60 °C for 30 s. At the end of the amplification, a melting curve analysis was performed from 60 °C to 95 °C, with a slope of 0.3 °C to monitor primer dimers of non-specific product formation. Each sample was amplified in triplicate; the average temperature of melting (Tm) observed was 79.39±0.15 °C.

The standard curve was created with serial dilution of *L. infantum* DNA ranging from 10,000 to 0.1 parasites per reaction. Each reaction was carried out by three replicates per dilution, in three independent experiments. The ct value cut-off was settled at mean ct value of 39.3 which corresponds to 1 parasite per mL of the original parasite suspension.

As a positive control the reference strain *L. infantum* MHOM/TN/80/IPT1, kindly provided by the Unit of Clinical Microbiology, Regional Reference Centre for Microbiological Emergencies (CRREM), St. Orsola-Malpighi University Hospital, Bologna, Italy, was used.

Sample Size Calculator (https://www.calculator.net/sample-size-calculator.html) was used to calculate 95% confidence intervals for the observed prevalence values.

5.4 *Nested PCR*

Samples of the mammals that tested positive to the real-time PCR were subjected to strain identification by a newly developed nested PCR to overstep the degradation of the samples (Magri *et al*., 2022). In a previous study, the discrimination of *L. infantum* strains circulating in the Emilia-Romagna region was based also on the one-step amplification of the gene encoding cysteine protease B (cpB), featuring a 39-nt indel (Rugna *et al*., 2017). However, application of this strategy to tissue samples resulted in the multiple PCR by-products originating from host DNA and preventing diagnosis of *Leishmania* infection. To overcome this problem, a nested PCR protocol was developed by adding a second pair of primers annealing within the amplicon produced at the first amplification stage.

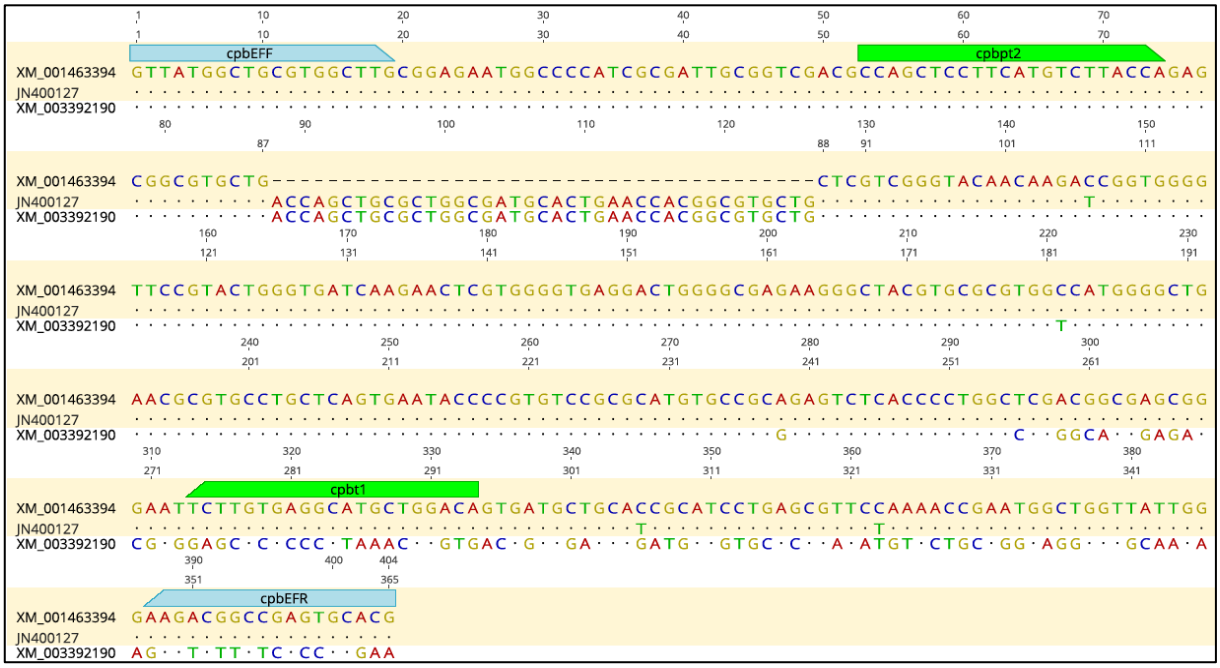

Figure 27. Alignment of partial cpB gene sequences of L. infantum *demonstrating the annealing sites of the primers and the position of the indel. The target gene variant highlighted in the strains JPCM5 and Drep13 is shown along with a non-target variant of the strain JPCM5 (XM_ 001463394, JN400127 and XM_003392190, respectively). Dashes show the characteristic deletion allowing strains discrimination. The PCR products obtained from XM_001463394 and JN400127 are classified as cbpE and cbpF genotypes, respectively when amplified with the external primers (in blue), or as S and L genotypes when amplified with the internal primers (in green).*

As already mentioned, the *L. infantum* genome contains multiple copies of cpB gene, of which only one varies as described above. This copy has a distinct sequence at the 3' end allowing its specific amplification. The cpB sequences were retrieved from GenBank (accession numbers: AJ628943, AY896776, AY896777, AY896778, AY896780, AY896782, AY896791, EU637907, GQ302670, GQ302671, GQ302674, GQ856074, JN400122-JN400131,

XM_001463394). For the first round of PCR, previously reported primers cpbEFF (5'-GTTATGGCTGCGTGGCTTG-3') and cpbEFR (5'-CGTGCACTCGGCCGTCTT-3') were used (Zackay *et al.*, 2013). For the second round, a new primers pair was designed using Geneious Prime (Dotmatics, Boston, USA) software: cpbt1 (5'-TGTCCAGCATGCCTCACAAGA-3') and cpbt2 (5'-CCAGCTCCTTCATGTCTTACCA-3') (Fig. 27).

Reactions were carried out in a total volume of 25 µl with 12.5 µl of PCRBIO Taq Mix Red (PCR Biosystems Ltd, London, UK), 0.3 µM of each primer and 2 µl of DNA in the first round and 1.5 µl of template in the second round. For both rounds, amplification was performed as follows: initial denaturation 94 °C for 4 min., followed by 30 cycles 94 °C for 15 sec., 55 °C for 30 sec., 72 °C for 1 min. and 72 °C for 5 min. final elongation. As a positive control, the reference strain *L. infantum* MHOM/TN/80/IPT1 was used. The amplified fragments were separated on a 1.5% agarose gel stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany). The fragment lengths were 281 bp (long – L variant, no deletion) or 242 bp (short – S variant, deletion) (Fig. 28).

The identity of the PCR products was confirmed by sequencing four samples (two L and two S). The obtained sequences were submitted to GenBank under accession numbers OP186448-OP186451.

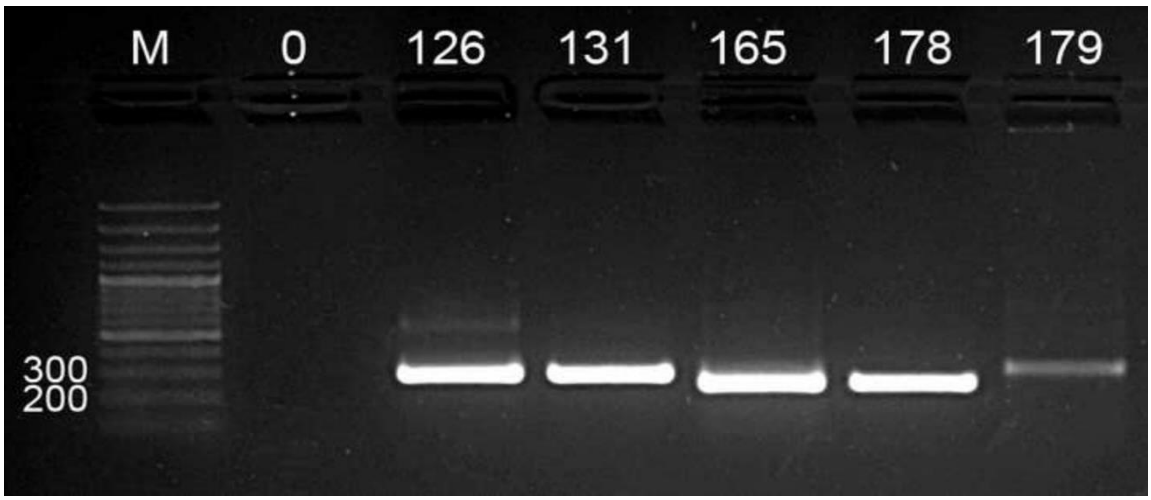

Figure 28. Nested PCR detection of the L and S genotypes of L. infantum. *The 100-bp ladder (Thermo Fisher Scientific, Waltham, USA) is on the left. Lane "0" is a negative amplification control.*

Chapter 6

Autochthonous *Trypanosoma* spp. in European Mammals: A Brief Journey amongst the Neglected Trypanosomes

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Review

Autochthonous *Trypanosoma* spp. in European Mammals: A Brief Journey amongst the Neglected Trypanosomes

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Abstract: The genus *Trypanosoma* includes flagellated protozoa belonging to the family Trypanosomatidae (Euglenozoa, Kinetoplastida) that can infect humans and several animal species. The most studied species are those causing severe human pathology, such as Chagas disease in South and Central America, and the human African trypanosomiasis (HAT), or infections highly affecting animal health, such as nagana in Africa and surra with a wider geographical distribution. The presence of these *Trypanosoma* species in Europe has been thus far linked only to travel/immigration history of the human patients or introduction of infected animals. On the contrary, little is known about the epidemiological status of trypanosomes endemically infecting mammals in Europe, such as *Trypanosoma theileri* in ruminants and *Trypanosoma lewisi* in rodents and other sporadically reported species. This brief review provides an updated collection of scientific data on the presence of autochthonous *Trypanosoma* spp. in mammals on the European territory, in order to support epidemiological and diagnostic studies on Trypanosomatid parasites.

Keywords: *Trypanosoma* spp.; mammals; Europe; epidemiology; *T. theileri*; *T. lewisi*; *T. grosi*

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1. Introduction

The genus *Trypanosoma* includes flagellated protozoans belonging to the Trypanosomatidae family (Euglenozoa, Kinetoplastea) that can infect humans and several animal species [1]. They are mostly dixenous parasites, meaning that the presence of two hosts (commonly one vertebrate and one invertebrate) is required in order to complete their life cycle. Such organisms are capable of parasitizing a wide range of vertebrate hosts, from mammals to birds, fish, amphibians, and reptiles [2].

The most studied species are those causing serious diseases in humans, and are not endemic in the European continent. This group includes species of the *Trypanosoma brucei* complex, mainly responsible for African trypanosomiasis [3], which are usually transmitted cyclically through a salivarian route by the tsetse fly (*Glossina* spp.), or rarely by congenital transmission [4]. In particular, the subspecies *T. brucei gambiense* and *T. brucei rhodesiense* are responsible for human African trypanosomiasis (HAT), also known as sleeping sickness, which can result in death of the patient if untreated [5,6]. A variety of wild and domestic animal species may act as reservoir in endemic countries, especially for *T. brucei rhodesiense* [7,8]. In Europe, the diagnosis of HAT is usually related to travel or migration [9–18], with some differences concerning the species isolated; in general, rhodesiense HAT is more connected with tourism, particularly with travelers returning from short visits to endemic countries, and is the most frequently diagnosed, while gambiense HAT patients had been living in endemic countries for extended period, and therefore is more related to history of migration with economic connections to the endemic countries [18,19].

Moreover, *T. cruzi* is responsible for human American trypanosomiasis, the Chagas disease, typically acquired through stercorarian transmission by triatomine bugs (reduviid insects) vector species, although vertical and iatrogenic transmission are also described [20]. The Chagas disease is endemic in Central and South America, where it

has also been described in more than 100 animal species, whose role as reservoir is well established [21–23]. A large number of cases have also been reported in Europe, both in travelers and, in particular, in migrants from endemic countries; this phenomenon has increased particularly since the 1990s due to massive migrations from Latin America to Italy, Portugal, and Spain [20,24–27], as well as to other European countries such as Belgium, France, Germany, the Netherlands, Switzerland, and the United Kingdom [28].

In domestic animals, animal African trypanosomiasis (AAT, also named nagana) is described as an acute or chronic disease caused by several species of *Trypanosoma* including *T. brucei* subsp. *brucei*, *T. vivax*, *T. congolense*, *T. simiae*, and *T. suis* [7]. These trypanosomes are cyclically transmitted by tsetse fly, although *T. congolense* and *T. vivax* might be mechanically transmitted by Tabanids and Stomoxines [29]. Although evidence for the epidemiological relevance of theirmechanical transmission in Africa are scant, such route has allowed these species to expand their range beyond that of *Glossina* spp. In particular, *T. vivax* has expanded its distribution to South and parts of Central America during European colonization in the last centuries [30]. In Europe, autochthonous cases of nagana have not been described thus far [31,32]. Clinical manifestations may vary according to the species; in particular, *T. congolense* present in Sub-Saharan Africa causes large economical losses in endemic countries [3].

Concerning other animal trypanosomes, such as *T. brucei evansi* and *T. brucei equiperdum*, the possibility of spreading in Europe is different. Classification of these species (previously named as *T. evansi* and *T. equiperdum* [2]) is still subject of debate; since they share important morphological and genetic traits, both parasites should be considered subspecies of *T. brucei* [33–35]. *T. brucei evansi* is the causative agent of the animal disease surra, which can affect a wide range of mammals from different geographical areas—camels, horses, buffalos, and cattle are particularly affected, although other animals, including wildlife, are also susceptible. Being transmitted in a non-cyclic way by tabanids, other flies, vampire bats, or carnivores, surra's spatial distribution is wide, including Africa, Asia, and Latin America [36]. *T. brucei evansi* has been known to be present since 1997 in the Canary Islands [37,38], where the most important population of dromedary camel (*Camelus dromedarius*) in Europe is present [39], and *Stomoxys calcitrans* is commonly involved in its transmission in the archipelago [40]. In 2010, following control programs, in the island of Gran Canaria, about 5% of the camelid population remained positive [40], and it was supposed that small ruminants, rodents, or rabbits could play a role as reservoirs of infection, although no evidence of the parasite in rodents was found [41]. Surra outbreaks have also been reported in dromedary camels and equids (horses and donkeys) from mainland Spain and France following importation of camelids from Canary Island [39,42]; nevertheless, in these cases, sanitary measures were successful in controlling the disease [40]. Furthermore, a single case of Surra was described in Germany in a Jack Russel dog imported from Thailand [43]. Further outbreaks in continental Europe have not been reported. Surveillance measures should be considered by European Countries for current risk of introduction; however, according to the European Food Safety Authority (EFSA), it is currently inconclusive whether *T. brucei evansi* infections (including surra) can be considered eligible to be listed for Union intervention in Animal Health Law [44].

T. brucei equiperdum, the causative agent of dourine in equids, represents an exception amongst trypanosomes, being the only species transmitted directly between hosts through coitus [45]. Dourine was anciently described in North Africa, but the etiological agent was first isolated only at the beginning of the last century by Buffard and Schneider [46]. In Europe, the disease has been described from the XVIII century in Russia, as well as in France, due to introduction of Persian, and Syrian and Spanish stallions, respectively [2]. After the Second World War, the disease spread in Europe, but thanks to several control efforts aimed at eradicating dourine, the disease disappeared from western and central European countries [47]. Although sporadic outbreaks were reported in the 1970s in Italy, dourine remained unreported until 2011, when five outbreaks were confirmed, once again in Italy [48,49]. Such disease is still considered a relevant health issue for equines and

represents a trade barrier in the movement of horses [50]; since it needs no vector for its transmission and can spread with the host, it requires implementation of official control plans [51].

Along with these well-known and studied species, other *Trypanosoma* spp. can infect mammals, and some of them are also diffused in Europe. The aim of this brief review was to gather reports of findings of these neglected species in Europe in order to raise awareness on the presence if these flagellates during epidemiological and diagnostic studies on trypanosomatid parasites on the European territory (Figure 1).

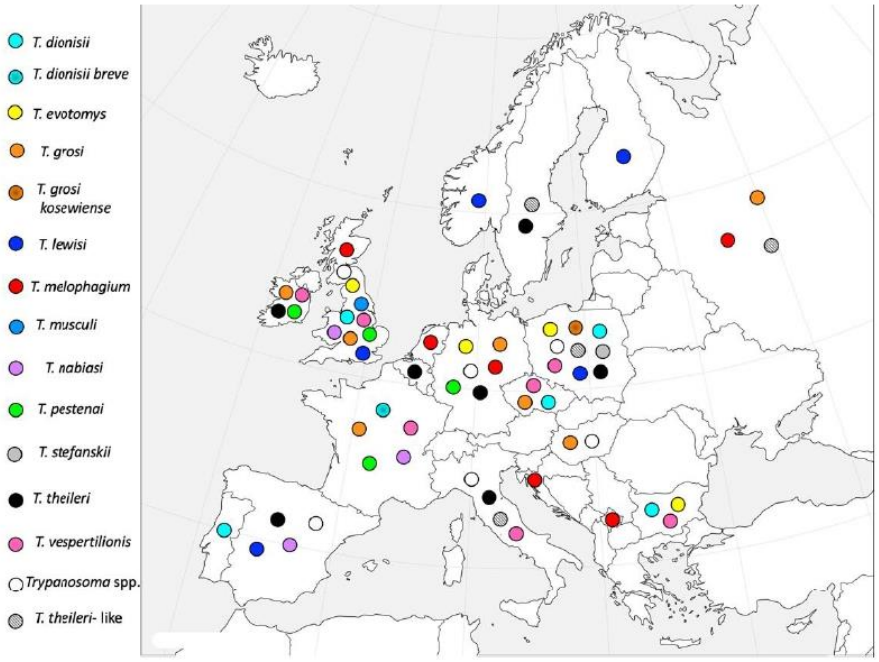

Figure 1. Distribution of the autochthonous *Trypanosoma* species in European mammals.

2. General Taxonomy of the Genus *Trypanosoma*

In order to properly define the distribution of *Trypanosoma* spp. in Europe, we deemed a brief section concerning the classification of this genus to be opportune.

As previously mentioned, trypanosomes are obligate parasites belonging to the Protozoa subkingdom, phylum Euglenozoa, class Kinetoplastea, order Trypanosomatida [1,52]. Kinetoplastea are characterized by the presence of a modified mitochondrion containing a body constituted of a disc-shaped, DNA-containing organelle, known as kinetoplast (from which the class name is derived), located beside the kinetosome at the base of the flagellum [53]. The classification of the Trypanosomatidae family is extremely complicated and still subject of debate amongst parasitologists for several reasons, among which the lack of morphological differences between phylogenetically distinct taxa and of an unequivocal classification approach [1,54].

The genus *Trypanosoma* is usually classified in the Blechomonadinae subfamily, which predominantly hosts dixenous parasites [55] and is conventionally divided in two groups on the basis of the replication site inside the invertebrate host "Salivaria", which develops in the foregut and is transmitted by inoculation, and "Stercoraria", which develops in the hindgut and therefore is transmitted by fecal contamination of skin injuries or mucosae [56]. Although this classification is not strictly taxonomic, it is still widely used because easily recalls life cycle and infection route of trypanosomes and will be also utilized in

this review. In mammalian host, the salivarian trypanosomes reproduce in the trypomastigote stage that has the kinetoplast in terminal or subterminal position and blunt posterior end. The Salivaria group includes four subgenera: *Duttonella*, *Nannomonas*, *Pycnomonas*, and *Trypanozoon*, mainly transmitted by tsetse flies [3,57]. Slight morphological differences between the subgenera have been described: *Duttonella* has rounded posterior end with large and terminal kinetoplast, *Nannomonas* has the kinetoplast in marginal position, while *Pycnomonas* has small and subterminal kinetoplast [58]. Assuming the progressive adaptation of trypanosomes to the tsetse fly as indicative of evolution, researchers have considered the subgenus *Duttonella* (non-cyclic) as the most ancient, and *Trypanozoon* the most recent [56,59,60]. The *Duttonella* subgenus includes *T. vivax*, responsible for nagana diseases in various animal species in Africa, or asymptomatic infections in Central and South America [61]. Concerning the subgenus *Nannomonas*, it includes species of interest in animal health such as *Trypanosoma simiae*, *Trypanosoma godfreyi*, and *Trypanosoma congolense*, also causing nagana in animals [62]. *Pycnomonas* subgenus includes *T. suis*, causing nagana in Suidae in Africa [63]. The *Trypanozoon* subgenus comprises cyclically transmitted trypanosomes extremely relevant for human and animal health, such as *T. brucei* complex, including *T. brucei brucei*, also an agent of nagana disease in animals in Africa, and the HAT causal agents *T. brucei rhodesiense* and *T. brucei gambiense* [64]. Moreover, this subgenus includes *T. brucei evansi*, which causes Surra in a wide range of hosts, and the monoxenous sexually transmitted *T. brucei equiperdum* [36,65].

The Stercoraria group comprises protozoans that, in the mammalian host, reproduce as epimastigote/amastigote forms, and present not reproducing trypomastigote forms in blood. The latter ones have a large kinetoplast, usually not terminal, and pointed end of the body. Stercoraria are mostly considered as non-pathogenic (except for *T. cruzi*) and comprise different subgenera [66]: (i) *Schizotrypanum*, with trypomastigotes typically curved with kinetoplast close to the posterior end of the body, includes *T. cruzi* responsible for Chagas disease or human American trypanosomiasis [67,68]; (ii) *Megatrypanum* are large trypanosomes that in trypomastigote forms have kinetoplast near the nucleolus, far from the posterior end [58] and include, amongst others, the worldwide distributed cyclic species *Trypanosoma melophagium* in sheep and *Trypanosoma theileri* in cattle [2]; (iii) *Herpetomonas*, defined as subgenus by Molyneux [69], includes *Trypanosoma lewisi* as the most studied species, long isolated in rodents worldwide and lately occasionally reported also in humans in Asia and Africa [70,71]. The trypomatigote forms are medium-sized with slender curved body and pronounced free flagellum [2].

According to data based on molecular sequences retrieved from GenBank (non-taxonomic), we found that not all trypanosomes can be classified according to these subgenera; therefore, in this classification, two clades have been introduced: firstly, the clade of "*Trypanosoma* with unspecified subgenus", in which some parasites of wild fauna, such as *Trypanosoma evotomys, Trypanosoma grosi, Trypanosoma nabiasi*, and *Trypanosoma pestanai*, are included. Such parasites are commonly defined as non-pathogenic, as they have rarely been isolated in course of clinical disease. Unexpectedly, according to phylogenetic analysis, *T. theileri* belongs to this clade, although it is taxonomically included in the *Megatrypanum* subgenus. The second clade with no specific subgenus is referred to as "unclassified trypanosomes", further subdivided into "fish trypanosomes" and "other trypanosomes"; the latter includes recently discovered trypanosomes waiting for proper classification. Table 1 reports a schematic classification of *Trypanosoma* spp. on the basis of data retrieved from GenBank taxonomy [72].

Table 1. Classification scheme of the *Trypanosoma* species as retrieved from GenBank taxonomy (25 January 2021), modified by referring them also to Salivaria and Stercoraria groups.

Group	Subgenus/Clade	Species	Subspecies
Salivaria	*Duttonella*	*T. vivax*	
	Nannomonas	*T. congolese*	
		T. godfreyi	
		T. simiae	
	Pycnomonas	*T. suis*	
	Trypanozoon	*T. brucei*	*T. brucei brucei*
			T. brucei gambiense
			T. brucei rhodensiense
			T. brucei equiperdum
			T. brucei evansi
Stercoraria	*Herpetosoma*	*T. lewisi*	
		T. rangeli	
	Megatrypanum	*T. melophagium*	
	Schizotrypanum	*T. cruzi*	
		T. dionisii	*T. dionisii breve*
		T. vespertilionis	
	Trypanosomes with unspecified subgenus	*T. evotomys*	
		T. grayi	
		T. grosi	*T. grosi kosewiense*
		T. pestenai	
		T. theileri	
		T. nabiasi	
	Unclassified trypanosomes	Fish trypanosomes	
		Other unclassified trypanosomes	

3. *Trypanosoma* Species Naturally Occurring in Domestic and Wild Mammals in Europe

As seen in the literature, a wide variety of *Trypanosoma* species have been reported to infect mammals from all European Countries (Table 2). Such species are commonly reported as non-pathogenic trypanosomes. In general, certain characteristics usually distinguish non-pathogenic trypanosome species from the pathogenic ones: (i) the host–parasite relationships are well adapted evolutionarily to both vertebrate and invertebrate host; (ii) infection rates in vectors are high; (iii) infected mammals are usually healthy carriers with inapparent, nonchronic infections; (iv) the host range is extremely restricted; (v) in an invertebrate host, the development of metacyclic trypomastigote occurs in the hindgut and the parasites are shed with the feces; (vi) in the mammalian host, trypanosomes shortly reproduce as epimastigote and/or amastigote forms, after which non-reproductive trypomastigote forms circulate in blood [66]. The only exception to this classification scheme is *T. cruzi*, which is not naturally present in Europe and, although included in the Stercoraria group, is highly pathogenic.

Table 2. *Trypanosoma* spp. naturally occurring in domestic and wild mammals and in vectors in Europe.

Trypanosoma sp.	Country	Host	References
T. dionisii	Bulgaria	Bat and bat flies (*Nycteribia schmidlii*)	[73]
	Czech Republic	Bat and bat flies	[73]
	Poland	Bat and bat flies	[73]
	Portugal	Bat	[74]
	United Kingdom	Bat and bat flies	[75]
T. dionisii breve	France	Bat	[76]
T. evotomys	Bulgaria	*Mus macedonicus*	[77]
	Germany	Voles	[78,79]
	United Kingdom	Voles	[69,80–83]
	Poland	Voles	[84,85]
T. grosi	Czech Republic	*Apodemus agrarius*	[86]
	France	*Apodemus sylvaticus*	[87]
	Germany	Voles	[88]
	Hungary	Voles	[89]
	Ireland	Small rodents	[90]
	United Kingdom	Small rodents	[80,82,90]
	Russia	*Apodemus sylvaticus*	[91,92]
T. grosi kosewiense	Poland	Voles	[93]
T. lewisi	Finland	*Rattus norvegicus, R. rattus*	[94]
	Norway	*Clethrionomys glareolus, Microtus agrestis; Apodemus sylvaticus*	[95]
	Poland	*Rattus norvegicus*	[96]
	United Kingdom	*Rattus norvegicus*	[82]
	Spain	*Rattus norvegicus, R. rattus*	[41]
T. melophagium	Croatia	Sheep ked (*Mallophagus melophagium)*	[97]
	Germany	Sheep ked	[98]
		Sheep	[99,100]
	Holland	Sheep	[101]
	Russia	Sheep	[102]
	United Kingdom	Sheep	[103–106]
		Sheep ked	[107]
	Former Yugoslavia (Croatia and Kosovo)	Sheep	[108]
T. musculi	United Kingdom	Mouse (*Mus musculus*)	[82]
T. nabiasi	France	Wild and domestic rabbits	[109]
	Spain	Rabbits (*Oryctolagus cuniculus*)	[110,111]
	United Kingdom	Rabbits	[112]
T. pestenai	France	European badger (*Meles meles*)	[113,114]
	Germany	Dog	[115]
	Ireland	Badger	[116]
	United Kingdom	Badger	[117–121]
T. stefanskii	Poland	Roe deer (*Capreolus capreolus*)	[122]

Table 2. *Cont.*

Trypanosoma sp.	Country	Host	References
T. theileri	Belgium	Cattle	[123]
	Germany	Cattle	[124,125]
		Fallow deer (*Dama dama*), red deer (*Cervus elaphus*), roe deer	[126,127]
	Italy	Cattle	[128]
		River buffalo (*Bubalus bubalis*)	[129]
	Ireland	Calf	[130]
	Poland	Cattle	[131]
		European bison (*Bison bonasus*)	[132–135]
	Sweden	Cattle	[122]
	Spain	Cattle	[136,137]
T. vespertilionis	Bulgaria	Bat and bat flies	[73]
	Czech Republic	Bat and bat flies	[73]
	France	Bat	[138]
	Italy	Bat	[139–141]
	Ireland	Bat	[142]
	Poland	Bat and bat flies	[73]
	United Kingdom	Bat	[143–145]
		Bat and bat flies	[75]
T. theileri-like	Italy	Sandflies (*Phlebotomus perfiliewi*)	[146]
	Poland	Deer ked (*Lipoptena fortisetosa* and *L. cervi*)	[147]
	Sweden	Roe deer, fallow deer, European elk, red deer, wild boar (*Sus scrofa*)	[148]
	Russia	Horseflies (*Hybomitra tarandina*, *H. muehlfeldi*, *H. bimaculate*, *Chrysops divaricatus*)	[149]
Trypanosoma sp.	Germany	Fallow deer, red deer, roe deer	[150,151]
	Italy	Bat and *Cimex* spp.	[152]
	Hungary	Bat and Cimex spp.	[152]
	Poland	Roe deer, red deer, European elk (*Alces alces*)	[153]
		Roe deer	[154]
	Spain	Bat and *Cimex* spp.	[152]
		Red deer	[155]
		Wild rabbits	[156]
	United Kingdom	Common shrews (*Sorex araneus*)	[157]
		Cattle	[114,158,159]

3.1. *Trypanosoma theileri*

While drafting this review, the presence of *T. theileri* Laveran, 1902 has shown great relevance in Europe. As previously mentioned, this species is usually classified in the *Megatrypanum* subgenus, however, in terms of phylogenetic analysis, it is grouped within the "*Trypanosoma* with unspecified subgenus". *T. theileri* is considered a mildly pathogenic species that typically infects wild and domestic ruminants [131]. Different tabanid species are common vectors of *T. theileri*, transmitting the pathogen by laying infected feces on the skin of the mammalian host or by ingestion of infected insects [127]; however, during a study concerning *Leishmania infantum* in the Emilia-Romagna region (Italy), the presence of *T. theileri*-like trypanosomes has been recently reported in sandflies (*Phlebotomus* spp.) [149],

although their role as vectors has not been established. Exploiting abraded skin or mucosae, *T. theileri* invades the bloodstream of the mammalian host; prepatent period ranges from 4 to 20 days and parasitemia decreases after 2–4 weeks [66]. First isolation was performed in Africa by the veterinary bacteriologist Arnold Theiler, who observed animals with clinical manifestations similar to the "Gall-sickness" (currently Anaplasmosis) during immunization of cattle against rinderpest. He observed *Trypanosoma*-like forms in blood smears and sent them to French and British researchers (Laveran and Bruce, respectively), who both named the parasite as *T. theileri*; however, since Laveran published the description earlier, the species was credited to him [2,160,161]. After the first isolation, reports have been numerous but often incorrect due to the great morphological variability of strains isolated from different animal hosts and geographical areas. In fact, several authors named different new species (e.g., *Trypanosoma frank* from cattle in Germany, *Trypanosoma wrublewskii* from the European bison *Bison bonasu* in Poland, *Trypanosoma americanum* and *Trypanosoma rutherfordi* from cattle in North America [2]), which were lately recognized as *T. theileri* by Herbert [162], making it clear that its distribution was wider than the African continent. Until 1970, reports of *T. theileri* in cattle ranged from Australia [163], to the United Kingdom [158,159], to the USA and Canada [164,165]. Concerning Europe, this species has more recently been reported from cattle also in Belgium, Germany, Italy, Ireland, Poland, and Spain [123,124,128,130,131,137]. Although these infections are generally reported as asymptomatic, clinical manifestations have sometimes been described, as primarily referred by Theiler [161]. Cases of illness were reported mostly in immunocompromised animals consisting of mild leukocytosis, enlargement of the spleen, anemia, weight loss, and considerable drop in milk production, especially if the infection concurs with bovine leukemia virus [128–131]. Water buffalo (*Bubalus bubalis*) is also susceptible to infection, and recent casual findings have been described in Italy in both cattle and water buffalo [128,129], whereas, in Poland, reports in European bison are numerous [132–135].

In Europe, *Megatrypanum* species, often morphologically described as *T. theileri*-like, were also reported in wild ruminants such as roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), and red deer (*Cervus elaphus*) [114,148,150–154,166]. *T. theileri*-like strains were also detected by molecular biology in vectors (i.e., tabanid flies) in Russia [149] and Poland [147]. Studies concerning the characterization of *Megatrypanum* trypanosomes from European Cervidae using isoenzyme analysis and pulsed-field gel electrophoresis suggested that there should be at least two *Megatrypanum* species infecting European deer, one in roe deer, and one in fallow and red deer, and differing from *T. theileri* affecting cattle [127]. More recent studies on phylogenetic analysis of *Megatrypanum* trypanosome from cattle, water buffalo, deer, and antelopes revealed the presence of several host-specific genotypes [167]. Unfortunately, the assessment of pathogenic effects and clinical course of *Trypanosoma* spp. infections in the wild fauna is generally challenging and the diagnosis rely mostly on post-mortem examination, which only in few cases has allowed to detect poor general conditions and small size in infected animals [148,168].

The presence of trypanosomes in roe deer was also reported in Poland by Kingston et al. [122] during one of the few epidemiological studies focused on such parasites in wild fauna in Europe. Blood from hunted roe deer killed between August 1984 and July 1988 revealed a prevalence of 66.6%. The *Trypanosoma* found differed from any others isolated from wild ruminants in central Europe and North America, and a consistent percentage of protozoa lacked a free flagellum, assumed by the authors to be the vector-infective form. Therefore, a new species in the subgenus *Megatrypanum* was described on the basis of morphological traits, namely, *Trypanosoma stefanskii*. No sequence was deposited in GenBank and, to our knowledge, no other reports of this species occur.

On a diagnostic perspective, *T. theileri* and other *Megatrypanum* trypanosomes of ruminants often represent an occasional finding occurring in other investigations [128,149]. For example, Galuppi et al., during cattle blood culture trials for the cultivation of piroplasms [169], observed the presence of *Trypanosoma* sp. (R.G., personal communication).

As it emerges, reports of these trypanosomes are often occasional and the actual prevalence in domestic and wild fauna is not known.

3.2. *Trypanosoma melophagium*

Amongst *Megatrypanum* trypanosomes, *T. melophagium* is species-specific for domestic sheep and is transmitted by the sheep ked *Melophagus ovinus*. Ked become infected through the blood of parasitized animals and, after multiplication in the digestive system of the fly, the metacyclic form develops in the hindgut. Sheep acquire the infection by eating infected ked [2,163]. The first report of *T. melophagium* was from Germany, where in 1905 Pfeiffer observed the presence of "trypanosome-like flagellates" in sheep ked [98]. At first, studies failed in proving the presence of the protozoan in sheep blood and *T. melophagium* was classified as a parasite of the ked gut and named *Crithidia melophagia* [170,171]. Few years later Woodcock succeeded in observing trypanosomes in fresh sheep's blood and identified them as developmental stages of the flagellates described in sheep ked [103]. Interestingly, his work was hardly criticized, and only after almost 10 years of controversy, Nöller [172] and Kleine [173], in separate studies proved not only that *T. melophagium* and *C. melophagia* were actually the same species but also that ked became infected only after feeding on infected sheep. This was finally confirmed by Hoare [104] who, in the same years, found *T. melophagium* in the 80% of the sheep examined in England. Gibson and colleagues observed close genetic similarity between *T. melophagium* and *T. theileri*, suggesting that *T. melophagium* represents a lineage of *T. theileri* that adapted to be transmitted by sheep ked [107]. In more recent years, this protozoan has been eradicated in the United Kingdom as a consequence of the widespread use of pesticides effective against ked [105], with persistency in the Outer Hebrides off the northwest coast of Scotland [106]. Infection with *T. melophagium* is not associated with clinical manifestations, the parasitemia is transitory (3 months), and there is no lasting immunity, and thus sheep can be readily re-infected after several months [104]. Due to the lack of clinical manifestation associated with the infection in sheep, scattered and sporadic reports occurred in Europe, particularly from Germany [100], southeastern Russia [102], former Yugoslavia (currently Croatia and Kosovo) [108], and Turkey [174]. Such sporadic reports could be related to difficulties in the detection of *T. melophagium* in sheep blood samples, possibly due to the low and transitory parasitemia. In fact, a more recent study conducted in Croatia observed a re-emergence of sheep ked in organic farms—the ked were heavily infected by *T. melophagium* (86% of samples), however, none of the 134 sheep from which they had been collected resulted positive at blood smear examination [97].

3.3. *Trypanosoma lewisi*

T. lewisi is a cosmopolitan *Herpetosoma* species, also widely distributed in Europe, responsible for infections in rodents, more specifically in rats. *T. lewisi* is perhaps the best studied non-pathogenic trypanosome amongst the ones here presented, probably for its presence in rats used as laboratory animals [2]. Its first observation dates back to 1850 in France, by Chaussat, who referred its finding as nematode larvae in blood [175]; only almost 30 years later was the parasite recognized as a trypanosome species [176]. Dynamics of infection have been largely studied by Minchin and Thompson, who in 1915 published an extremely detailed work on the development of *T. lewisi* in its vector, the rat flea *Ceratophyllus fasciatus* [177].

In rats, the infection occurs without clinical manifestations, and is primarily characterized by the presence of epimastigote forms in peripheral blood. Rat fleas, while feeding upon the rodent's blood, eliminate feces containing final metacyclic stages, the metatrypanosomes. Through injured skin or mucosae, parasites gain entrance to the host blood stream and multiply as epimastigotes. Several days are needed to detect the parasites in blood and the prepatent period varies according to the parasite load [66]. Recent in vitro studies have also demonstrated the presence of further stages of development and multiplication, such as the "rosette" stage (multiple divisions forms) and the trypomastigote [178].

Although *T. lewisi* is considered cosmopolitan, only few findings have been reported in European wild/synantropic murine population. For instance, it was described in 1970 in Southern Finland, mostly in Helsinki, wherein 36.2% of rats tested positive [94]. Moreover, it was reported in Norway in voles (*Clethrionomys glareolus*, *Microtus agrestis*, and *Apodemus sylvaticus*) [95] and again more recently in voles in Poland [96], but with no prevalence data due to the different aim of the studies (morphological characterization). Rodríguez et al. [41] reported *T. lewisi* in 13% of the rat population examined in the Canary Islands (Spain).

T. lewisi can be transmitted to humans, but only few cases have been described, mostly in children from in Asia and Africa, showing a fatal course if untreated [70,71,179]. Recent studies have demonstrated that *T. lewisi* is resistant to trypanolysis operated by human serum, exhibiting characteristics similar to human pathogenic trypanosomes; therefore, its role as human pathogen might be underestimated [180]. In Europe, no human cases due of *T. lewisi* infection have been described thus far.

3.4. Trypanosoma nabiasi

T. nabiasi was firstly observed in France, where it was reported as an unidentified trypanosome in the blood of wild and domestic rabbits by Jolyet and Nabias [109]. It was lately named as *T. nabiasi* by Raillet in 1895 and considered as a *T. lewisi*-like form in the subgenus *Herpetosoma* [2]. It is now phylogenetically included in the "trypanosomes with unspecified subgenus" clade [72]. The life cycle of *T. nabiasi* comprises the flea *Spilopsyllus cuniculi* as a vector [181]. Rabbits become infected after ingestion of the flea or by contamination of injured skin or mucosae with flea feces. Prepatence may vary from 5 to 12 days, while infection lasts from 4 up to 8 months, during which the parasite can be found in the rabbit blood and is infective for fleas; immunity prevents from reinfection [112,182]. *T. nabiasi* was described in wild rabbits (*Oryctolagus cuniculus*) in Great Britain [112] and in Cottontail rabbits (*Sylvilagus* spp.) in North America [183]. More recently, *T. nabiasi* has been reported from rabbits in Spain in coinfection with *Leishmania infantum*, highlighting possible problems occurring in the diagnosis of leishmaniasis in case of co-presence of these flagellates [110,111].

3.5. Trypanosomes of Small Rodents

Another *T. lewisi*-like protozoan is *T. evotomys*, a parasite of Arvicolinae rodents, referred to the subgenus *Herpetosoma* [2] and now phylogenetically included in the "trypanosomes with unspecified subgenus" clade [72]. Described for the first time by Watson and Hadwen in 1912 in the Canadian vole (*Evotomys saturates*, now *Clethrionomys glareolus*) [183], it was subsequently found in the United Kingdom [69,80–83], Germany [78,79], Poland [84,85], and Bulgaria [77]. The vector is yet to be identified, although fleas are possibly involved. In experimental infection via inoculation, prepatence lasts from 5 to 6 days, during which parasites invade and multiply in lymph nodes and spleen. Patent infection typically lasts 1 month or more in splenectomized hosts [77].

Trypanosoma. grosi is included within the *T. lewisi*-like group. It was firstly described as "very motile vermicules" by Gros [91] in Russian wood mouse (*A. sylvaticus*) and its recognition as *Trypanosoma* sp. occurred several years later in France by Laveran and Pettit [87]. In Russia, it was at first misidentified (*Trypanosoma apodemi* and *Trypanosoma korssaki*) when found in different vole species [84], and then more recently recognized as synonyms of *T grosi* [2]. Since then, this species has been reported in the United Kingdom [80,82,90], Ireland [90], Germany [88], Hungary [89], and the Czech Republic [86]. Moreover, *T. grosi kosewiense* has been described as a new subspecies of *T. grosi* in Poland from voles (*Microtus* spp. and *Apodemus* spp.) and from the yellow-necked mouse (*Apodemus flavicollis*) [93].

T. musculi is a trypanosome of the house mouse (*Mus musculus*), which was first observed in mouse blood in Gambia by Dutton and Todd in 1903 and was defined as a new species of the subgenus *Herpetosoma* by Thiroux in 1905 [184]. It has not been included in any phylogenetic clade yet. Mice acquire the infection from fleas of the genera

Ctenophthalmus, *Leptopsylla*, and *Nosopsylla* [185]. As happened with *T. lewisi*, the role of *T. musculi* as a potential human pathogen has been questioned, mainly due to the biological and morphological characteristics shared between them. *T. musculi* revealed in vivo and in vitro lower sensitivity to human sera than *T. brucei brucei* but higher when compared to *T. lewisi*. Although no case of trypanosomiasis attributed to *T. musculi* has been reported yet, and infection in healthy humans is considered unlikely, some authors suggest caution, especially in immunocompromised patients [186]. In Europe, the only report available in the literature is from a review on protozoans of British small rodents where *T. musculi* is cited as a parasite of *Mus musculus*; no further data are available [82].

Moreover, during a parasitological study conducted on common shrew (*Sorex araneus*) in Northwest England, 9 out of 76 specimens tested positive for *Trypanosoma* spp. It is not possible to identify parasites at the species level on the basis of molecular data available in GenBank. The strain shared great similarity with *T. lewisi*, but formed an outgroup when clustered with *Trypanosoma microti*, *T. evotomys*, *T. musculi*, *T. lewisi*, and *T. grosi* [157].

3.6. Trypanosomes of Bats

Over 30 species of trypanosomes of the *Schizotrypanum* subgenus have been reported in more than 100 Chiroptera species all over the world, including the well-defined *Trypanosoma cruzi*, *T. vespertilionis*, *T. rangeli*, and the globally distributed *T. dionisii* [187]. Trypanosomes in bats were first described in *Miniopterus schreibersii* from Italy in 1899 by Dionisi, who identified it as *T. vespertilionis*; this species was further reported in Italy [140,141], Ireland [142], France [138], and the United Kingdom [143]. In Portugal, Bettencourt and Franca [74] described the species *T. dionisii*, which was later considered as a synonym of *T. vespertilionis*, later reported during specific surveys on trypanosomes in bats in the United Kingdom [144,145]. Only in 1975 did a comparative study based on laboratory culture prove that although these parasites were closely related, they actually differed from a morphological, physiological, and antigenical standpoint [188]. Such species are considered *T. cruzi*-like due to morphological similarities with *T. cruzi* [2]. In the United Kingdom, a later survey conducted on British bats reported the presence of both *T. dionisii* and *T. vespertilionis* [146]. A subspecies of *T. dionisii*, *T. dionisii breve*, was described in France in 1979, and was differentiated on the basis of morphological differences and enzyme electrophoresis [76]; however, to our knowledge, no further reports succeeded.

In bats, trypanosome development follows the *T. cruzi* pattern—infection of the host occurs through injured skin with epimastigote forms invading bloodstream to reach the target organs, namely, striated muscle, cardiac muscle, and stomach muscle (depending on strains/species involved), where parasites multiply as amastigote forms and may form pseudocysts in which trypanosomes multiply by binary fission as epimastigote; rupture of this pseudocysts allows the trypanosome to invade the bloodstream as trypomastigote forms [2]. Vectors of bat trypanosomes are *Cimex* spp. and bat flies (*Nycterida schmidlii*) [75,152]. No report of clinical manifestation has thus far been notified. In a recent study, 381 bat specimens collected in eastern and central Europe between 2015 and 2019 were screened with nested PCR for trypanosomes presence—a part of these tested positive for *T. dionisii* (32.3% in the Czech Republic, 8.3% in Bulgaria, and 16.2% in Poland), while a smaller fraction tested positive for *T. vespertilionis* (3.8% in the Czech Republic). Hematological parameters showed no significant differences between infected and non-infected specimens [73]. In the same year, a survey about the presence of *Trypanosoma* spp. in bats (9 subjects from Hungary, 16 from Italy, and 10 from Spain) and the flies *Nycteribia schmidlii scotti* (71 subjects) parasitizing them, found that in Hungary the prevalence of infection was 33.3% in bats and 35.3% in bat flies, while in Italy it was 43.8% and 11.6%, and in Spain it was 30% and 81.8%, respectively; no species identification was performed [152].

3.7. Trypanosoma pestenai

Amongst the trypanosomes naturally infecting wild carnivores, *T. pestanai* is the only one described in the European territory. *T. pestanai* recognizes the badger (*Meles meles*) as

preferential host and was first reported in Portugal in 1905 [189]. It was then described in France [113], the United Kingdom [117,118,120,121], and Ireland [116]. In particular, in the United Kingdom, the role of the badger flea (*Paraceras melis*) in the transmission of *T. pestanai* has been recognized [119]. As observed for most of non-pathogenic trypanosomes, the infection in badgers seems to be silent, and not associated with alterations of complete blood count [116]. This parasite has been recently reported in Germany in a 12-year-old beagle with a history of occasional travels to Switzerland, and parasitological investigations based on PCR and blood cell cultivation revealed the concurrent presence of *Anaplasma phagocytophilum* [115].

3.8. Trypanosoma spp.

Besides reports concerning the aforementioned *Trypanosoma* species, in some cases identification has been carried out only at the genus level [151,155,156]. For example, Olmeda et al. [155] described the presence of protozoans morphologically referable to the *Megatrypanum* subgenus in blood smear from 17 deer shot in Spain; however, no further data are available. Moreover, in Spain, during a study focused on non-lethal parasites of wild rabbit in a re-stocking program, 125 rabbits of different age classes were examined to test the presence of *Trypanosoma* spp. in blood smear, finding a prevalence of 9.5% in young, 4.4% in juveniles, and 8.2% in adults; no morphological or molecular identification was performed [156]. Such findings suggest that further studies are necessary to increase the knowledge on the *Trypanosoma* species circulating in European mammals.

4. Closing Remarks

The genus *Trypanosoma* includes a wide number of worldwide distributed species that can affect human and animal health. The most important and studied pathogenic species are responsible for African and South American trypanosomiases. As described in this review, the presence of such species in Europe is typically linked to human or animal immigration/travel/introduction from endemic countries. Such reports do not seem to constitute a threat for the European population due to the life cycle and transmission route strictly depending on vectors that are not present in Europe. On the contrary, occasional findings of *T. evansi* might represent a concern, due to the possible spillover to native hosts, favored by the wide range of vectors involved also in a non-cyclic transmission route.

The presence of autochthonous *Trypanosoma* species described in this review, all referable to non-pathogenic stercorarian trypanosomes, has been documented in Europe since the XIX century in both domestic and wild animals. Due to their scant pathogenic effects on the host, these species are more frequently reported as occasional findings during parasitological surveys not specifically focused on trypanosomes and/or during the molecular diagnosis of other strictly related pathogens, such as *Leishmania* spp. [110,111,146].

Although accidental, such findings are far from being trivial, as they provide useful information on the current epidemiological distribution of trypanosomatids in different geographical areas and hosts [148], with relevant implications also for the improvement of diagnostics [110,111]. Studies aimed to improve our knowledge on their epidemiology in Europe should be encouraged, especially considering that environmental changes could increase their spatial distribution.

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References

1. Votýpka, J.; d'Avila-Levy, C.M.; Grellier, P.; Maslov, D.; Lukeš, J.; Yurchenko, V. New Approaches to Systematics of Trypanosomatidae: Criteria for Taxonomic (Re)description. *Trends Parasitol.* **2015**, *31*, 460–469. [CrossRef] [PubMed]
2. Hoare, C.A. *The Trypanosomes of Mammals*, 1st ed.; Blackwell Scientific Publications: Oxford, UK; Edinburgh, UK, 1972; pp. 125–140, 214–219, 219–245, 277–282.
3. Radwanska, M.; Vereecke, N.; Deleeuw, V.; Pinto, J.; Magez, S. Salivarian trypanosomosis: A review of parasites involved, their global distribution and their interaction with the innate and adaptive mammalian host immune system. *Front. Immunol.* **2018**, *9*, 2253. [CrossRef] [PubMed]
4. Linder, A.K.; Priotto, G. The Unknown Risk of Vertical Transmission in Sleeping Sickness—A Literature Review. *PLoS Negl. Trop. Dis.* **2010**, *4*, e783. [CrossRef]
5. Brun, R.; Blum, J.; Chappuis, F.; Burri, C. Human African trypanosomiasis. *Lancet* **2010**, *375*, 148–159. [CrossRef]
6. Blum, J.A.; Neumayr, A.L.; Hatz, C.F. Human African trypanosomiasis in endemic populations and travelers. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 905–913. [CrossRef] [PubMed]
7. Ashcroft, M.T. The importance of African wild animals as reservoirs of trypanosomiasis. *East Afr. Med. J.* **1959**, *36*, 289–297.
8. Auty, H.; Anderson, N.E.; Picozzi, K.; Lembo, T.; Mubanga, J.; Hoare, R.; Fyumagwa, R.D.; Mable, B.; Hamill, L.; Cleaveland, S.; et al. Trypanosome Diversity in Wildlife Species from the Serengeti and Luangwa Valley Ecosystems. *PLoS Negl. Trop. Dis.* **2012**, *6*, e1828. [CrossRef]
9. Junyent, J.M.G.; Rozman, M.; Corachan, M.; Estruch, R.; Urbano-Marquez, A. An unusual course of west African trypanosomiasis in a Caucasian man. *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 931–932. [CrossRef]
10. Braendli, B.; Dankwa, E.; Junghanss, T. East African sleeping sickness (*Trypanosoma rhodesiense* infection) in 2 Swiss travelers to the tropics. *Swiss Med. Wkly.* **1990**, *120*, 1348–1352.
11. Iborra, C.; Danis, M.; Bricaire, F.; Caumes, E.A. Traveler Returning from Central Africa with Fever and a Skin Lesion. *Clin. Infect. Dis.* **1999**, *28*, 679–680. [CrossRef]
12. Jamonneau, V.; Garcia, A.; Ravel, S.; Cuny, G.; Oury, B.; Solano, P.; N'Guessan, P.; N'Dri, L.; Sanon, R.; Frézil, J.L.; et al. Genetic characterization of *Trypanosoma brucei gambiense* and clinical evolution of human African trypanosomiasis in Côte d'Ivoire. *Trop. Med. Int. Health* **2002**, *7*, 610–621. [CrossRef] [PubMed]
13. Ripamonti, D.; Massari, M.; Arici, C.; Gabbi, E.; Farina, C.; Brini, M.; Capatti, C.; Suter, F. African sleeping sickness in tourists returning from Tanzania: The first 2 Italian cases from a small outbreak among European travelers. *Clin. Infect. Dis.* **2002**, *34*, 18–22. [CrossRef]
14. Bisoffi, Z.; Beltrame, A.; Monteiro, G.; Arzese, A.; Marocco, S.; Rorato, G.; Anselmi, M.; Viale, P. African Trypanosomiasis Gambiense, Italy. *Emerg. Infect. Dis.* **2005**, *11*, 1745–1747. [CrossRef] [PubMed]
15. Gautret, P.; Clerinx, J.; Caumes, E.; Simon, F.; Jensenius, M.; Loutan, L.; Shlagenhauf, P.; Castelli, F.; Freedman, D.; Miller, A.; et al. Imported human African trypanosomiasis in Europe, 2005–2009. *Eurosurveillance* **2010**, *14*, 19327. [CrossRef]
16. Gómez-Junyent, J.; Pinazo, M.J.; Castro, P.; Fernández, S.; Mas, J.; Chaguaceda, C.; Pellicé, M.; Muñoz, J. Human African Trypanosomiasis in a Spanish traveler returning from Tanzania. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0005324. [CrossRef]
17. Huits, R.; De Ganck, G.; Clerinx, J.; Büscher, P.; Bottieau, E. A veterinarian with fever, rash and chancre after holidays in Uganda. *J. Travel Med.* **2018**, *25*, 1–2. [CrossRef] [PubMed]
18. Gao, J.-M.; Qian, Z.-Y.; Hide, G.; Lai, D.-H.; Lun, Z.-R.; Wu, Z.-D. Human African trypanosomiasis: The current situation in endemic regions and the risks for nonendemic regions from imported cases. *Parasitology* **2020**, *147*, 922–931. [CrossRef] [PubMed]
19. Simarro, P.P.; Franco, J.R.; Cecchi, G.; Paone, M.; Diarra, A.; Postigo, J.A.R.; Jannin, J.G. Human African Trypanosomiasis in Non-Endemic Countries. *J. Travel Med.* **2012**, *19*, 44–53. [CrossRef]
20. Angheben, A. Chagas Disease in the Mediterranean Area. *Curr. Trop. Med. Rep.* **2017**, *4*, 223–234. [CrossRef]
21. Coura, J.R. The main sceneries of Chagas disease transmission. *The vectors, blood and oral transmissions—A comprehensive review. Mem. Inst. Oswaldo Cruz* **2015**, *110*, 277–282. [CrossRef]
22. Jansen, A.M.; Xavier, S.C.C.; Roque, A.L.R. *Trypanosoma cruzi* transmission in the wild and its most important reservoir hosts in Brazil. *Parasites Vectors* **2018**, *11*, 502. [CrossRef]
23. Jansen, A.M.; Xavier, S.C.C.; Roque, A.L.R. Landmarks of the Knowledge and *Trypanosoma cruzi* Biology in the Wild Environment. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 10. [CrossRef]
24. Angheben, A.; Anselmi, M.; Gobbi, F.; Marocco, S.; Monteiro, G.; Buonfrate, D.; Tais, S.; Talamo, M.; Zavarise, G.; Strohmeyer, M.; et al. Chagas disease in Italy: Breaking an epidemiological silence. *Eurosurveillance* **2011**, *16*, 19969. [CrossRef]
25. Gobbi, F.; Angheben, A.; Anselmi, M.; Postiglione, C.; Repetto, E.; Buonfrate, D.; Marocco, S.; Tais, S.; Chiampan, A.; Mainardi, P.; et al. Profile of *Trypanosoma cruzi* Infection in a Tropical Medicine Reference Center, Northern Italy. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3361. [CrossRef]
26. Repetto, E.C.; Zachariah, R.; Kumar, A.; Angheben, A.; Gobbi, F.; Anselmi, M.; Al Rousan, A.; Toricco, C.; Ruiz, R.; Ledezma, G.; et al. Neglect of a Neglected Disease in Italy: The Challenge of Access-to-Care for Chagas Disease in Bergamo Area. *PLoS Negl. Trop. Dis.* **2015**, *9*, e0004103. [CrossRef]

27. Antinori, S.; Galimberti, L.; Bianco, R.; Grande, R.; Galli, M.; Corbellino, M. Chagas disease in Europe: A review for the internist globalized world. *Eur. J. Intern. Med.* **2017**, *43*, 6–15. [CrossRef]
28. Basile, L.; Jansà, J.M.; Carlier, Y.; Salamanca, D.D.; Angheben, A.; Bartoloni, A.; Seixas, J.; Van Gool, T.; Cañavate, C.; Flores-Chávez, M.; et al. Chagas disease in European countries: The challenge of a surveillance system. *Eurosurveillance* **2011**, *16*, 19968. [CrossRef]
29. Taylor, D.B. Stable Fly (*Stomoxys calcitrans*). In *Reference Module in Biomedical Science*; Elsevier: Amsterdam, The Netherlands, 2020. [CrossRef]
30. Jones, T.W.; Dávila, A.M. *Trypanosoma vivax*—Out of Africa. *Trends Parasitol.* **2001**, *17*, 99–101. [CrossRef]
31. Chamond, N.; Cosson, A.; Blom-Potar, M.C.; Jouvion, G.; D'Archivio, S.; Medina, M.; Droin-Bergère, S.; Huerre, M.; Minoprio, P. *Trypanosoma vivax* Infections: Pushing Ahead with Mouse Models for the Study of Nagana. I. Parasitological, Hematological and Pathological Parameters. *PLoS Negl. Trop. Dis.* **2010**, *4*, e792. [CrossRef]
32. Szöör, B.; Silvester, E.; Matthews, K.R. A Leap Into the Unknown—Early Events in African Trypanosome Transmission. *Trends Parasitol.* **2020**, *36*, 266–278. [CrossRef]
33. Lun, Z.R.; Lai, D.H.; Li, F.J.; Lukes, J.; Ayala, F.J. *Trypanosoma brucei*: Two steps to spread out from Africa. *Trends Parasitol.* **2010**, *26*, 424–427. [CrossRef] [PubMed]
34. Carnes, J.; Anupama, A.; Balmer, O.; Jackson, A.; Lewis, M.; Brown, R.; Cestari, I.; Desquesnes, M.; Gendrin, C.; Hertz-Fowler, C.; et al. Genome and Phylogenetic Analyses of *Trypanosoma evansi* Reveal Extensive Similarity to *T. brucei* and Multiple Independent Origins for Dyskinetoplasty. *PLoS Negl. Trop. Dis.* **2015**, *9*, e3404. [CrossRef]
35. Wen, Y.Z.; Lun, Z.R.; Zhu, X.Q.; Hide, G.; Lai, D.H. Further evidence from SSCP and ITS DNA sequencing support *Trypanosoma evansi* and *Trypanosoma equiperdum* as subspecies or even strains of *Trypanosoma brucei*. *Infect. Genet. Evol.* **2016**, *41*, 56–62. [CrossRef]
36. Desquesnes, M.; Holzmuller, P.; Lai, D.H.; Dargantes, A.; Lun, Z.R.; Jittaplapong, S. *Trypanosoma evansi* and surra: A review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. *Biomed. Res. Int.* **2013**, 194176. [CrossRef]
37. Molina, J.M.; Ruiz, A.; Juste, M.C.; Corbera, J.A.; Amador, R.; Gutiérrez, C. Seroprevalence of *Trypanosoma evansi* in dromedaries (*Camelus dromedarius*) from the Canary Islands (Spain) using an antibody Ab-ELISA. *Prev. Vet. Med.* **1999**, *47*, 53–59. [CrossRef]
38. Gutiérrez, C.; Juste, M.C.; Corbera, J.A.; Magnus, E.; Verloo, D.; Montoya, J.A. Camel trypanosomosis in the Canary Island: Assessment of seroprevalence and infection rates using the card agglutination test (CATT/*T. evansi*) and parasite detection tests. *Vet. Parasitol.* **2000**, *90*, 155–159. [CrossRef]
39. Tamarit, A.; Gutiérrez, C.; Arroyo, R.; Jimenes, V.; Zagalá, G.; Bosch, I.; Sirvent, J.; Alberola, J.; Alonso, I.; Caballero, C. *Trypanosoma evansi* infection in mainland Spain. *Vet. Parasitol.* **2010**, *167*, 74–76. [CrossRef] [PubMed]
40. Gutiérrez, C.; Desquesnes, M.; Touratier, L.; Büscher, P. *Trypanosoma evansi*: Recent outbreaks in Europe. *Vet. Parasitol.* **2010**, *174*, 26–29. [CrossRef] [PubMed]
41. Rodríguez, N.F.; Tejedor-Junco, M.T.; Hernández-Trujillo, Y.; González, M.; Gutiérrez, C. The role of wild rodents in the transmission of *Trypanosoma evansi* infection in an endemic area of the Canary Islands (Spain). *Vet. Parasitol.* **2010**, *174*, 323–327. [CrossRef]
42. Desquesnes, M.; Bossard, G.; Patrel, D.; Herder, S.; Patout, O.; Lepetitcolin, E.; Thevenon, S.; Berthier, D.; Pavlovic, D.; Brugidou, R.; et al. First outbreak of *Trypanosoma evansi* in camels in metropolitan France. *Vet. Record* **2008**, *162*, 750–752. [CrossRef]
43. Defontis, M.; Richartz, J.; Engelmann, N.; Bauer, C.; Schwierk, V.M.; Büscher, P.; Moritz, A. Canine *Trypanosoma evansi* infection introduced into Germany. *Vet. Clin. Pathol.* **2012**, *41*, 369–374. [CrossRef] [PubMed]
44. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare); More, S.; Bøtner, A.; Butterworth, A.; Calistri, P.; Depner, K.; Edwards, S.; Garin-Bastuji, B.; Good, M.; Gortazar Schmidt, C.; et al. Scientific Opinion on the assessment of listing and categorization of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): *Trypanosoma evansi* infections (including Surra). *EFSA J.* **2017**, *15*, 4892. [CrossRef]
45. Scott, D.W.; Miller, W.H. Viral and Protozoal Skin Disease. In *Equine Dermatology*, 2nd ed.; Saunders, Elsevier Science: St. Louis, MO, USA, 2003; pp. 376–394.
46. Buffard, M.; Schneider, G. Le trypanosome de la dourine. *Arc Parasitol.* **1900**, *3*, 124–133.
47. Claes, F.; Büscher, P.; Touratier, L.; Goddeeris, B.M. *Trypanosoma equiperdum*: Master of disguise or historical mistake? *Trends Parasitol.* **2005**, *21*, 316–321. [CrossRef]
48. Pascucci, I.; Di Provvido, A.; Cammà, C.; di Francesco, G.; Calistri, P.; Tittarelli, M.; Ferri, N.; Scacchia, M.; Caporale, V. Diagnosis of dourine in outbreaks in Italy. *Vet. Parasitol.* **2012**, *193*, 30–38. [CrossRef] [PubMed]
49. Calistri, P.; Narcisi, V.; Atzeni, M.; de Massis, F.; Tittarelli, M.; Mercante, M.T.; Ruggieri, E.; Scacchia, M. Dourine Reemergence in Italy. *J. Equine Vet. Sci.* **2012**, *33*, 83–89. [CrossRef]
50. Ahmed, Y.; Hagos, A.; Merga, B.; Van Soom, A.; Duchateau, L.; Goddeeris, B.M.; Govaere, J. *Trypanosoma equiperdum* in the horse—A neglected threat? *Vlaams Diergeneeskundig Tijdschrift* **2018**, *87*, 66–87. [CrossRef]
51. Gizaw, Y.; Megersa, M.; Fayera, T. Dourine: A neglected disease of equids. *Trop. Anim. Health Prod.* **2017**, *49*, 887–897. [CrossRef] [PubMed]
52. Borges, A.R.; Engstler, M.; Wolf, M. 18S rDNA Sequence-Structure Phylogeny of the Trypanosomatida (Kinetoplastea, Euglenozoa) with Special Reference on Trypanosoma. *Res. Sq.* **2020**, *15*. [CrossRef]

53. Roberts, L.; Janovy, J., Jr. *Foundations of Parasitology*, 6th ed.; McGraw-Hill: New York, NY, USA, 2000; pp. 55–83.
54. Kaufer, A.; Ellis, J.; Stark, D.; Barratt, J. The evolution of trypanosomatid taxonomy. *Parasites Vectors* **2017**, *10*, 287. [CrossRef]
55. Votýpka, J.; Suková, E.; Kraeva, N.; Ishemgulova, A.; Duží, I.; Lukeš, J.; Yurchenko, V. Diversity of Trypanosomatids (Kinetoplastea: Trypanosomatidae) Parasitizing Fleas (Insecta: Siphonaptera) and Description of a New Genus *Blechomonas* gen.n. *Protist* **2013**, *164*, 763–781. [CrossRef]
56. Deplazes, P.; Eckert, J.; Mathis, A.; von Samson-Himmelstierna, G.; Zahner, H. Phylum Euglenozoa. In *Parasitology in Veterinary Medicine*, 1st ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2016; pp. 59–79. [CrossRef]
57. Gibson, W. Liaisons dangereuses: Sexual recombination among pathogenic trypanosomes. *Res. Microbiol.* **2015**, *166*, 459–466. [CrossRef]
58. Marquardt, W.C.; Demaree, R.S.; Grieve, R.B. Trypanosomes and Trypanosomiasis. In *Parasitology & Vector Biology*, 2nd ed.; Harcourt/Academic Press: San Diego, CA, USA, 2000; pp. 37–55.
59. Stevens, J.R.; Noyes, H.A.; Schofield, C.J.; Gibson, W. The molecular evolution of Trypanosomatidae. *Adv. Parasitol.* **2001**, *48*, 1–56. [CrossRef]
60. Gibson, W. Species concepts for trypanosomes: From morphological to molecular definitions? *Kinetoplast. Biol. Dis.* **2003**, *2*, 10. [CrossRef]
61. Osório, A.L.A.R.; Madruga, C.R.; Desquesnes, M.; Soares, C.O.; Ribeiro, L.R.R.; Costa, S.C.G.D. *Trypanosoma* (*Duttonella*) *vivax*: Its biology, epidemiology, pathogenesis, and introduction in the New World—A review. *Mem. Inst. Oswaldo Cruz* **2008**, *103*, 1–13. [CrossRef]
62. Garcia, H.A.; Rodrigues, C.M.; Rodrigues, A.C.; Pereira, D.L.; Pereira, C.L.; Camargo, E.P.; Hamilton, P.B.; Teixeira, M.M. Remarkable richness of trypanosomes in tsetse flies (*Glossina morsitans morsitans* and *Glossina pallidipes*) from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). *Infect. Genet. Evol.* **2018**, *63*, 370–379. [CrossRef]
63. Hutchinson, R.; Gibson, W. Rediscovery of *Trypanosoma* (*Pycnomonas*) *suis*, a tsetse-transmitted trypanosome closely related to *T. brucei*. *Infect. Genet. Evol.* **2015**, *36*, 381–388. [CrossRef] [PubMed]
64. Auty, H.; Torr, S.J.; Michoel, T.; Jayaraman, S.; Morrison, L.J. Cattle trypanosomosis: The diversity of trypanosomes and implications for disease epidemiology and control. *Rev. Sci. Tech.* **2015**, *34*, 587–598. [CrossRef] [PubMed]
65. Echeverria, J.T.; Soares, R.L.; Crepaldi, B.A.; de Oliveira, G.G.; da Silva, P.M.P.; Pupin, R.C.; Martins, T.B.; Kellermann Cleveland, H.P.; Ramos, C.A.N.; Borges, F.A. Clinical and therapeutic aspects of an outbreak of canine trypanosomiasis. *Rev. Bras. Parasitol. Vet.* **2019**, *8*. [CrossRef]
66. Mansfield, J.M. Nonpathogenic Trypanosomes of mammals. In *Parasitic Protozoa, Taxonomy, Kinetoplastids and Flagellates of Fish*; Kreier, J.P., Ed.; Academic Press, Inc.: New York, NY, USA, 1977; Volume 1, pp. 297–327.
67. Sutherland, C.S.; Yukich, J.; Goeree, R.; Tediosi, F.A. Literature Review of Economic Evaluations for a Neglected Tropical Disease: Human African Trypanosomiasis ("Sleeping Sickness"). *PLoS Negl. Trop. Dis.* **2015**, *9*, e0003397. [CrossRef]
68. De Fuentes-Vincente, J.A.; Gutiérrez-Cabrera, A.E.; Flores-Villegas, A.L.; Lowenberger, C.; Benelli, G.; Salazar-Schettino, P.M.; Córdoba-Aguilar, A. What makes an effective Chagas disease vector? Factors underlying *Trypanosoma cruzi*-triatomine interactions. *Acta Trop.* **2018**, *183*, 23–31. [CrossRef]
69. Molyneux, D.H. The morphology and biology of *Trypanosoma (Herpetosoma) evotomys* of the bank-vole, *Clethrionomys glareolus*. *Parasitology* **1969**, *59*, 843. [CrossRef]
70. Lun, Z.R.; Reid, S.A.; Lai, D.H.; Li, F.J. Atypical human trypanosomiasis: A neglected disease or just an unlucky accident? *Trends Parasitol.* **2009**, *25*, 107–108. [CrossRef]
71. Desquesnes, M.; Yangtara, S.; Kunphukhieo, P.; Chalermwong, P.; Jittapalapong, S.; Herder, S. Zoonotic trypanosomes in South East Asia: Attempts to control *Trypanosoma lewisi* using veterinary drugs. *Exp. Parasitol.* **2016**, *165*, 35–42. [CrossRef] [PubMed]
72. Schoch, C.L.; Ciufo, S.; Domrachev, M.; Hotton, C.L.; Kannan, S.; Khovanskaya, R.; Leipe, D.; Mcveigh, R.; O'Neill, K.; Robbertse, B.; et al. NCBI Taxonomy: A comprehensive update on curation, resources and tools. *Database* **2020**, baaa062. [CrossRef]
73. Linhart, P.; Band'ouchová, H.; Zukal, J.; Votypka, J.; Kokurewicz, T.; Dundarova, H.; Apoznanski, G.; Heger, T.; Kubickova, A.; Nemcova, M.; et al. Trypanosomes in eastern and central European bats. *Acta Vet. Brno* **2020**, *89*, 69–78. [CrossRef]
74. Bettencourt, A.; França, C. Sur un trypanosome de la chauve-souris. *CR Soc. Biol.* **1905**, *57*, 306.
75. Gardner, R.A.; Molyneux, D.H. Schizotrypanum in British bats. *Parasitology* **1988**, *97*, 43–50. [CrossRef] [PubMed]
76. Baker, J.R.; Miles, M.A. *Trypanosoma (Schizotrypanum) dionisii breve* n. subsp. from Chiroptera. *Syst. Parasitol.* **1979**, *1*, 61–65. [CrossRef]
77. Mitkovska, V.; Chassovnikarova, T.; Dimitrov, H. First record of *Trypanosoma* infection in Mediterranean mouse (*Mus macedonicus*, Petrov & Ružić, 1983) in Bulgaria. *ZooNotes* **2014**, *64*, 1–6.
78. Krampitz, H.E. Kritisches zur Taxonomie und Systematik parasitischer Säugetier-Trypanosomen mit besonderer Beachtung einiger der in Wülmäusen verbreiteten spezifischen Formen. *Zschr. Tropenmed.* **1961**, *12*, 117.
79. Walter, G.; Liebisch, A. Studies of the ecology of some blood protozoa of wild small mammals in North Germany. *Acta Trop.* **1980**, *37*, 31–40.
80. Elton, C. The health and parasites of a wild mouse population. *Proc. Zool. Lond.* **1931**, *101*, 657–721. [CrossRef]
81. Turner, C.M.R.; Cox, F.E.G. Interspecific interactions between blood parasites in a wild rodent community. *Ann. Trop. Med. PH* **1985**, *79*, 463–465. [CrossRef] [PubMed]

82. Cox, F.E.G. Protozoan parasites of British small rodents. *Mammal. Rev.* **1987**, *17*, 59–66. [CrossRef]
83. Noyes, H.A.; Ambrose, P.; Barker, F.; Begon, M.; Bennet, M.; Bown, K.J.; Kemp, S.J. Host specificity of *Trypanosoma* (Herpetosoma) species: Evidence that bank voles (*Clethrionomys glareolus*) carry only one *T.(H.) evotomys* 18S rRNA genotype but wood mice (*Apodemus sylvaticus*) carry at least two polyphyletic parasites. *Parasitology* **2002**, *124*, 185–190. [CrossRef] [PubMed]
84. Karbowiak, G.; Sinski, E. The occurrence and morphological characteristics of a *Trypanosma evotomys* strain from North Poland. *Acta Parasitol.* **1996**, *41*, 105–107.
85. Bajer, A.; Welc-Falęciakc, R.; Bednarska, M.; Alsarraf, M.; Behnke-Borowczyk, J.; Siński, E.; Behnke, J.M. Long-Term Spatiotemporal Stability and Dynamic Changes in the Haemoparasite Community of Bank Voles (*Myodes glareolus*) in NE Poland. *Environ. Microbiol.* **2014**, *68*, 196–211. [CrossRef]
86. Karbowiak, G.; Stanko, M.; Fričová, J.; Wita, I.; Hapunik, J.; Peťko, B. Blood parasites of the striped field mouse *Apodemus agrarius* and their morphological characteristics. *Biologia* **2009**, *64*, 1219. [CrossRef]
87. Laveran, A.; Pettit, A. Sur le trypanosome du mulot, *Mus sylvaticus*. *CR Soc. Biol.* **1909**, *67*, 564.
88. Krampitz, H.E. Ueber das Europäische Waldmaustrypanosom, *Trypanosoma grosi* Laveran et Pettit 1909 (Promonadina, Trypanosomidae). *Zschr. Parasitenk.* **1959**, *19*, 232. [PubMed]
89. Šebek, Z. Blood Parasites of Small Mammals in Western Hungary. *Parasitol. Hung.* **1978**, *11*, 17–22.
90. Alharbi, B. Arthropod-Borne Infections in the United Kingdom and Saudi Arabia. Ph.D. Thesis, University of Salford, Salford, UK, 2018.
91. Gros, G. Observations et inductions microscopiques sur quelques parasites. *Bull. Soc. Imp. Nat. Mosc.* **1845**, *18*, 380.
92. Yakimoff, W.L.; Korssak, D.W. Hämatoparasitologische Notizen. II. Ein Trypanosoma der Feldmaus im ostasiatischen Ufergebiete. *Cbl. Bakt.* **1910**, *55*, 370.
93. Karbowiak, G.; Wita, I. *Trypanosoma* (Herpetosoma) *grosi kosewiense* subsp.n., the Parasite of the Yellow-Necked Mouse *Apodemus flavicollis* (Melchior, 1834). *Acta Protozool.* **2004**, *43*, 173–178. [CrossRef]
94. Rislakki, V. Studies on the Prevalence and Effect of *Trypasnosoma lewisi* Infection in Finnish Rats. *Acta Vet. Scand.* **1971**, *12*, 448–450. [CrossRef] [PubMed]
95. Wiger, R. Seasonal and annual variations in the prevalence of blood parasites in cyclic species of small rodents in Norway with special reference to *Clethrionomys glareolus*. *Ecography* **1979**, *2*. [CrossRef]
96. Karbowiak, G.; Wita, I.; Czaplińska, U. The occurrence and ultrastructure of *Trypanosoma* (*Herpetosoma*) *lewisi* (Kent, 1880) Laveran and Mesnil, 1901, the parasite of rats (*Rattus norvegicus*) in Poland. *Wiad. Parazytol.* **2009**, *55*, 249–258.
97. Martinković, F.; Matanović, K.; Rodrigues, A.C.; Garcia, H.A.; Teixeira, M.M.G. *Trypanosoma* (*Megatrypanum*) *melophagium* in the sheep ked *Melophagus ovinus* from organic farms in Croatia: Phylogenetic interferences support restriction to sheep and sheep keds and close relationship with trypanosomes from other ruminant species. *J. Eukaryot. Microbiol.* **2012**, *59*, 134–144. [CrossRef]
98. Pfeiffer, E. Ueber trypanosomenahnliche Plagellaten im Darm von *Melophagus ovinus*. *Zschr. Hyg. Infektkr.* **1905**, *30*, 324–330. [CrossRef]
99. Behn, P. Weitere Trypasomenbefunde beim Schafe. *Berl. Tierärztl. Wschr.* **1912**, *50*, 934.
100. Büscher, G.; Friedhoff, K.T. The Morphology of Ovine *Trypanosoma melophagium* (Zoomastigophorea: Kinetoplastida) 1. *J. Protozool.* **1984**, *31*, 98–101. [CrossRef]
101. Douwes, J.A. Trypanosomen bij het Schaap (*Trypanosoma Melophagium*, Flu). *Tijdschr. Diegeneesk* **1920**, *47*, 408.
102. Bozhenko, V.P.; Zeiss, A.L. Trypanosomiasis of sheep. *Rev. Microbiol.* **1928**, *7*, 417–420.
103. Woodcock, H.M. A reply to Miss Porter's note entitled "Some remarks on the genera *Crithidia*, *Herpetomonas* and *Trypanosoma*". *Parasitology* **1911**, *4*, 150. [CrossRef]
104. Hoare, C.A. An experimental study of the sheep-trypanosome (*T. melophagium* Flu, 1908) and its transmission by the sheep-ked (*Melophagus ovinus* L.). *Parasitology* **1923**, *15*, 365. [CrossRef]
105. Small, R.W. A review of *Melophagus ovinus* (L.), the sheep ked. *Vet. Parasitol.* **2005**, *130*, 141–155. [CrossRef]
106. Craig, B.H.; Tempest, L.J.; Pilkington, J.G.; Pemberton, J.M. Metazoan-protozoan parasite co-infections and host body weight in St Kilda Soay sheep. *Parasitology* **2008**, *135*, 433–441. [CrossRef]
107. Gibson, W.; Pilkington, J.G.; Pemberton, J.M. *Trypanosoma melophagium* from the sheep ked *Melophagus ovinus* on the island of St. Kilda. *Parasitology* **2010**, *137*, 1799–1804. [CrossRef] [PubMed]
108. Katic, R.V. Researches on *Trypanosoma melophagium* and its Incidence in Yugoslavia. *Jug. Vet. Glasnik* **1940**, *20*, 373–384.
109. Jolyet, F.; Nabias, B. Sur un hématozoarie du lapin domestique. *J. Méd. Bordx.* **1891**, *20*, 325.
110. Díaz-Sáez, V.; Merino-Espinosa, G.; Morales-Yuste, M.; Corpas-López, V.; Pratlong, F.; Morillas-Márquez, F.; Martín-Sánchez, J. High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: Epidemiological consequences. *Vet. Parasitol.* **2014**, *202*, 119–127. [CrossRef]
111. Merino-Espinosa, G.; Corpas-López, V.; Morillas-Márquez, F.; Díaz-Sáez, V.; Martín-Sánchez, J. Genetic variability and infective ability of the rabbit trypanosome, *Trypanosoma nabiasi* Railliet 1895, in southern Spain. *Infect. Genet. Evol.* **2016**, *45*, 98–104. [CrossRef]
112. Grewal, M.S. The life-cycle of the British rabbit trypanosome, *Trypanosoma nabiasi* Railliet, 1985. *Parasitology* **1957**, *47*, 100. [CrossRef] [PubMed]

113. Rioux, J.-A.; Albaret, J.-L.; Bres, A.; Dumas, A. Présence de *Trypanosoma pestanai* Bettencourt et França, 1905, chez les Blaireaux du sud de la France. *Ann. Parasitol. Hum. Comp.* **1966**, *41*, 281–288. [CrossRef]
114. Dirie, M.F.; Bornstein, S.; Wallbanks, K.R.; Stiles, J.K.; Molyneux, D.H. Zymogram and life-history studies on trypanosomes of the subgenus *Megatrypanum*. *Parasitol. Res.* **1990**, *76*, 669–674. [CrossRef] [PubMed]
115. Dyachenko, V.; Steinmann, M.; Bangoura, B.; Selzer, M.; Munderloh, U.; Daugschies, A. Co-infection of *Trypanosoma pestanai* and *Anaplasma phagocytophilum* in a dog from Germany. *Vet. Parasitol. Reg.* **2017**, *9*, 110–114. [CrossRef] [PubMed]
116. McCarthy, G.; Shiel, R.; O'Rourke, L.; Murphy, D.; Corner, L.; Costello, E.; Gormley, E. Bronchoalveolar lavage cytology from captive badgers. *Vet. Clin. Pathol.* **2009**, *38*, 381–387. [CrossRef]
117. Peirce, M.A.; Neal, C. *Trypanosoma* (*Megatrypanum*) *pestanai* in British badgers (*Meles meles*). *Int. J. Parasitol.* **1974**, *4*, 439–440. [CrossRef]
118. Macdonald, D.W.; Anwar, M.; Newman, C.; Woodroffe, R.; Johnson, P.J. Inter-annual differences in the age-related prevalences of Babesia and *Trypanosoma* parasites of European badgers (*Meles meles*). *J. Zool.* **1999**, *247*, 65–70. [CrossRef]
119. Lizundia, R.; Newman, C.; Buesching, C.D.; Ngugi, D.; Blake, D.; Wa Sin, Y.; Macdonald, D.W.; Wilson, A.; McKeever, D. Evidence for a role of the Host-Specific Flea (*Paraceras melis*) in the Transmission of *Trypanosoma* (*Megatrypanum*) *pestanai* to the European Badger. *PLoS ONE* **2011**, *6*, e16977. [CrossRef]
120. Sin, Y.W.; Annavi, G.; Dugdale, H.L.; Newman, C.; Burke, T.; Macdonald, D.W. Pathogen burden, co-infection and major histocompatibility complex variability in the European badger (*Meles meles*). *Mol. Ecol.* **2014**, *23*, 5072–5088. [CrossRef] [PubMed]
121. Ideozu, E.J.; Whiteoak, A.M.; Tomlinson, A.J.; Robertson, A.; Delahay, R.J.; Hide, G. High prevalence of trypanosomes in European badgers detected using ITS-PCR. *Parasites Vectors* **2015**, *8*, 480. [CrossRef] [PubMed]
122. Kingston, N.; Bobek, B.; Perzanowski, K.; Wita, I.; Maki, L. Description of *Trypanosoma* (*Megatrypanum*) *stefanskii* sp. n. from Roe Deer (*Capreolus capreolus*) in Poland. *Proc. Helminthol. Soc. Wash.* **1992**, *59*, 89–95.
123. Verloo, D.; Brandt, J.; Van Meirvenne, N.; Büscher, P. Comparative in vitro isolation of *Trypanosoma theileri* from cattle in Belgium. *Vet. Parasitol.* **2000**, *89*, 129–132. [CrossRef]
124. Bittner, L.; Wöckell, A.; Snedec, T.; Delling, C.; Böttcher, D.; Klose, K.; Köller, G.; Starke, A. Case report: Trace Mineral Deficiency with concurrent Detection of *Trypanosoma theileri* in a Suckler Cow Herd in Germany. *Anim Biol.* **2019**, *21*, 84.
125. Frank, G. Ueber den Befund von Trypanosomen bei einem in Stein-Wingert (Westerwald, Regierungsbezirk Wiesbaden) verendeten Rinde. *Zschr. Infektionskrankh Haustiere* **1909**, *5*, 313.
126. Böse, R.; Friedhoff, K.T.; Olbrich, S.; Büscher, G.; Domeyer, I. Transmission of *Trypanosoma theileri* to cattle by Tabanidae. *Parasitol. Res.* **1987**, *73*, 421–424. [CrossRef]
127. Böse, R.; Petersen, K.; Pospichal, H.; Buchanan, N.; Tait, A. Characterization of *Megatrypanum* trypanosomes from European Cervidae. *Parasitology* **1993**, *107*, 55–61. [CrossRef] [PubMed]
128. Amato, B.; Mira, F.; Lo Presti, V.D.M.; Guercio, A.; Russotto, L.; Gucciardi, F.; Vitale, M.; Lena, A.; Loria, G.R.; Puleio, R.; et al. A case of bovine trypanosomiasis caused by *Trypanosoma theileri* in Sicily, Italy. *Parasitol. Res.* **2019**, *118*, 2723–2727. [CrossRef]
129. Greco, A.; Loria, G.R.; Dara, S.; Luckins, T.; Sparagano, O. First Isolation of *Trypanosoma theileri* in Sicilian cattle. *Vet. Res. Commun.* **2000**, *24*, 471–475. [CrossRef]
130. Doherty, M.L.; Windle, H.; Vooheis, H.P.; Larkin, H.; Casey, M.; Clery, D.; Murray, M. Clinical disease associated with *Trypanosoma theileri* infection in a calf in Ireland. *Vet. Record* **1993**, *132*, 653–656. [CrossRef]
131. Werszko, J.; Szewczyk, T.; Steiner-Bogdaszewska, Ż.; Wróblewski, P.; Karbowiak, G.; Laskowski, Z. Molecular detection of *Megatrypanum* trypanosomes in tabanid flies. *Med. Vet. Entomol.* **2019**, 5p. [CrossRef]
132. Karbowiak, G.; Wita, I.; Czaplińska, U. *Trypanosoma* (*Megatrypanum*) *Wrublewskii*, Wladimiroff et Yakimoff 1909, the parasite of European bison (*Bison bonasus* L). In Proceedings of the Trudy 5 Respublikanskoj Nauchno-Prakticheskoj Konferentsii, Vitebsk, Belarus, 21–22 September 2006; pp. 268–270. (In Russian).
133. Karbowiak, G.; Wita, I.; Czaplińska, U. Pleomorfizm of trypanosomes occurring in *Bison bonasus* L. *Wiad Parazytol.* **2007**, *53*, 54.
134. Karbowiak, G.; Demiaszkiewicz, A.W.; Pyziel, A.M.; Wita, I.; Moskowa, B.; Werszko, J.; Bień, J.; Goździk, K.; Lachowicz, J.; Cabaj, W. The parasitic fauna of the European bison (*Bison bonasus*) (Linnaeus, 1758) and their impact on the conservation. Part 1: The summarising list of parasites noted. *Acta Parasitol.* **2014**, *53*, 363–371. [CrossRef]
135. Wladimiroff, A.; Yakimoff, W. Bemerkung zur vorstehenden Mitteilung Wrublewskis. *Zentralbl Bakteriol. Orig A* **1909**, *48*, 164.
136. Espinosa, A.V.; Gutiérrez, C.; Gracia, E.; Moreno, B.; Chacón, G.; Sanz, P.V.; Büscher, P.; Touratier, L. Presencia de *Trypanosoma theileri* en bovinos en España. *Albéitar Publ. Vet. Indep.* **2006**, *93*, 36–37.
137. Villa, A.; Gutierrez, C.; Garcia, E.; Moreno, B.; Chaćon, G.; Sanz, P.V.; Büscher, P.; Touratier, L. Presence of *Trypanosoma theileri* in Spanish Cattle. *Ann. N. Y. Acad. Sci.* **2008**, *1149*, 352–354. [CrossRef] [PubMed]
138. Chatton, E.; Courrier, R. Un *Schizotrypanum* chez les chauve-souris (*Vesperugo pipistrellus*) en Basse-Alsace. Schizotrypanose et goître endémique. *CR Soc. Biol.* **1921**, *84*, 943.
139. Dionisi, A. La malaria in alcune specie di pipistrelli. *Atti Soc. Studi Malaria* **1899**, *1*, 133.
140. Battaglia, M. Alcune ricerche su due tripanosomi (*Trypanosoma vespertilionis-Trypanosoma lewisi*). *Ann. Med. Navale* **1904**, *10*, 517.
141. Gonder, R. *Trypanosoma vespertilionis* (Battaglia). *Centralbl. f Bakteriol.* **1910**, *53*, 293–302.
142. Mettam, A.E. On the presence of a trypanosome in an Irish bat. *Dublin J. Med. Sci.* **1907**, *124*, 417–419. [CrossRef]
143. Petrie, G.F. Observations relating to the structure and geographical distribution of certain trypanosomes. *J. Hyg.* **1905**, *5*, 191–200. [CrossRef]

144. Coles, A.C. Blood parasites found in mammals, birds and fishes in England. *Parasitology* **1914**, *7*, 17–61. [CrossRef]
145. Molyneux, D.H.; Bafort, J.M. Observations on the trypanosome of *Pipistrellus pipistrellus* in Britain, *Trypanosoma* (*Schizotrypanum*) *vespertilionis*. *Ann. Soc. Belg. Med. Trop.* **1971**, *51*, 335–348.
146. Calzolari, M.; Rugna, G.; Clementi, E.; Carra, E.; Pinna, M.; Bergamini, F.; Fabbi, M.; Dottori, M.; Sacchi, L.; Votýpka, J. Isolation of a Trypanosome Related to *Trypanosoma theileri* (Kinetoplastea: Trypanosomadidae) from *Phlebotomus perfiliewi* (Diptera: Pychodidae). *BioMed Res. Int.* **2018**, 8p. [CrossRef]
147. Werszko, J.; Steiner-Bogdaszewska, Ż.; Jeżewski, W.; Szewczyk, T.; Kuryło, G.; Wołkowycki, M.; Wróblewski, P.; Karbowiak, G. Molecular detection of *Trypanosoma* spp. in *Lipoptena cervi* and *Lipoptena fortisetosa* (Diptera: Hippoboscidae) and their potential role in the transmission of pathogens. *Parasitology* **2020**, *147*, 1629–1635. [CrossRef]
148. Neumüller, M.; Nilsson, K.; Påhlson, C. *Trypanosoma* spp. In Swedish game animals. *Parasitol. Res.* **2012**, *110*, 135–139. [CrossRef] [PubMed]
149. Ganyukova, A.I.; Zolotarev, A.V.; Malysheva, M.N.; Frolov, A.O. First record of *Trypanosoma theileri*-like flagellates in horseflies from Northwest Russia. *Protistology* **2018**, *12*, 223–230. [CrossRef]
150. Friedhoff, K.T.; Petrich, J.; Hoffmann, M.; Büscher, G. Trypanosomes in Cervidae in Germany. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **1984**, *256*, 286–287. [CrossRef]
151. Hoffmann, M.; Büscher, G.; Friedhoff, K.T. Stercorarian Trypanosomes from Deer (Cervidae) in Germany. *J. Protozool.* **1984**, *31*, 581–584. [CrossRef]
152. Szentiványi, T.; Markotter, W.; Dietrich, M.; Clément, L.; Ançay, L.; Brun, L.; Genzoni, E.; Kearney, T.; Seamark, E.; Estók, P.; et al. Host conservation through their parasites: Molecular surveillance of vector-borne microorganisms in bats using ectoparasitic bat flies. *Parasite* **2020**, *27*, 72. [CrossRef] [PubMed]
153. Kingston, N.; Drozdz, J.; Rutkowska, M. *Trypanosoma* sp. in red deer (*Cervus elaphus*) and elk (*Alces alces*) in Poland. *Proc. Helrninthol. Soc. Wash.* **1985**, *52*, 144–145.
154. Kingston, N.; Bobek, B. A trypanosome in roe deer, *Capreolus capreolus*, in southern Poland. *Proc. Helminthol. Soc. Wash.* **1985**, *52*, 143.
155. Olmeda, A.S.; San Miguel, J.M.; Luzón, M. Primera denuncia de Trypanosoma (*Megatrypanum*) en ciervos (*Cervus elaphus*) de la Península Ibérica. *Galemys* **2001**, *13*, 149–153.
156. Reglero, M.; Vicente, J.; Rouco, C.; Villafuerte, R.; Gortazar, C. *Trypanosoma* spp. infection in wild rabbits (*Oryctolagus cuniculus*) during a restocking program in southern Spain. *Vet. Parasitol.* **2007**, *149*, 178–184. [CrossRef]
157. Bray, D.P.; Bown, K.J.; Stockley, P.; Hurst, J.L.; Bennett, M.; Birtles, R.J. Haemoparasites of common shrews (*Sorex araneus*) in northwest England. *Parasitology* **2007**, *134*, 819–826. [CrossRef] [PubMed]
158. Hoyte, H.M. The morphology of *Trypanosoma theileri* in the blood of cattle, and the rediscovery of *Theileria mutans* in England. *Z. Parasitenkd.* **1972**, *38*, 183–199. [CrossRef]
159. Wells, E.A. Isolation of *Trypanosoma theileri* Laveran 1902, from Cattle in Scotland. *Nature* **1965**, *4986*, 847. [CrossRef] [PubMed]
160. Laveran, A. Sur un nouveau trypanosome des bovidés (*Trypanosoma theileri*). *CR Acad. Sci.* **1902**, *134*, 512–514.
161. Theiler, A. A new Trypanosoma, and the disease caused by it. *J. Comp. Pathol. Ther.* **1903**, *16*, 193–209. [CrossRef]
162. Herbert, I.V. *Trypanosoma theileri* Laveran, 1902. A cosmopolitan parasite of cattle. *Vet. Bull.* **1964**, *34*, 563.
163. Turner, A.W.; Murname, D. Trypanosomes in the Blood of Victorian Animals. I. A Preliminary Note on the Occurrence of *Trypanosoma theileri* in the Blood of Cattle. *J. Sci. Ind. Res.* **1930**, *3*, 120–121.
164. Woo, P.; Soltys, M.A.; Gillick, A.C. Trypanosomes in Cattle in Southern Ontario. *Can. J. Vet. Res.* **1970**, *34*, 142–147.
165. Matthews, D.M.; Kingston, N.; Maki, L.; Nelms, G. *Trypanosoma theileri* Laveran, 1902, in Wyoming cattle. *Am. J. Vet. Res.* **1979**, *40*, 623–629. [PubMed]
166. Kingston, N.; Nikander, S. Poron, *Rangifer tarandus*, *Trypanosoma* sp. Suomessa (*Trypanosoma* sp. in reindeer (*Rangifer tarandus*) in Finland). *Suomen Elainlddkarilehti* **1985**, *91*, 1.
167. Garcia, H.A.; Rodrigues, A.C.; Martinkovic, F.; Minervino, A.H.; Campaner, M.; Nunes, V.L.B.; Paiva, F.; Hamilton, P.B.; Teixeira, M.M.G. Multilocus phylogeographical analysis of *Trypanosoma* (*Megatrypanum*) genotypes from sympatric cattle and water buffalo populations supports evolutionary host constraint and close phylogenetic relationships with genotypes found in other ruminants. *Int. J. Parasitol.* **2011**. [CrossRef] [PubMed]
168. Kocan, A.A. Blood-Inhabiting Protozoan Parasites. In *Parasitic Diseases of Wild Mammals*, 2nd ed.; Samuel, W.M., Margo, J.P., Kocan, A.A., Eds.; Iowa State University Press: Iowa City, IA, USA, 2001; pp. 520–522.
169. Galuppi, R.; Bonoli, C.; Aureli, S.; Cassini, R.; Marcer, F.; Foley, J.E.; Tampieri, M.P. Comparison of diagnostic methods to detect piroplasms in asymptomatic cattle. *Vet. Parasitol.* **2012**, *183*, 364–368. [CrossRef] [PubMed]
170. Turner, A.W.; Murnane, D. On the presence of the non-pathogenetic *Trypanosoma melophagium* in the blood of Victorian sheep, and its transmission by *Melophagus ovinus*. *Aust. J. Exp. Biol. Med. Sci.* **1930**, *7*, 5–8. [CrossRef]
171. Flu, P.C. Ueber die Flagellaten im Darm von *Melophagium ovinus*. *Protist* **1908**, *12*, 147.
172. Nöller, W. Beitrag zur Kenntnis des Schaftrypanosomas. *Arch. Schiffs Tropenhyg.* **1919**, *23*, 99.
173. Kleine, F.K. Beitrang zum Kenntnis des *Trypanosoma melophagium*, Flu. *Deutsch Tierärztl.* **1919**, *27*, 408.
174. Nalbantoglu, S.; Karaer, Z. *Trypanosoma melophagium* in blood cell culture. *Ankara Univ. Vet. Fak. Derg.* **2008**, *55*, 173–176.
175. Chaussat, J.B. Recerches Microscopiques Appliquées à la Pathologiée des Hématozoàires. Ph.D. Thesis, Faculté de Médicine de Paris, Paris, France, 1850.

176. Lewis, T.R. Flagellated organisms in the blood of healthy rats. *Q. J. Microsc. Sci.* **1879**, *19*, 109.
177. Minchin, E.A.; Thompson, J.D. The Rat-*Trypanosoma lewisi*, in its relation to the Rat-Flea, *Ceratophyllus fasciatus*. *J. Cell Sci.* **1915**, *2*, 463–681.
178. Zhang, X.; Li, S.J.; Li, Z.; He, C.Y.; Hide, G.; Lai, D.H.; Lun, Z.R. Cell cycle and cleavage events during in vitro cultivation of bloodstream forms of *Trypanosoma lewisi*, a zoonotic pathogen. *Cell Cycle* **2019**, *18*, 552–567. [CrossRef] [PubMed]
179. Truc, P.; Büscher, P.; Cuny, G.; Gonzatti, M.I.; Jannin, J.; Joshi, P.; Juyal, P.; Lun, Z.R.; Mattioli, R.; Pays, E.; et al. Atypical human infections by animal trypanosomes. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2256. [CrossRef] [PubMed]
180. Lun, Z.R.; Wen, Y.Z.; Uzureau, P.; Lecordier, L.; Lai, D.H.; Lan, Y.G.; Desquesnes, M.; Geng, G.Q.; Yang, T.B.; Zhou, W.L.; et al. Resistance to normal human serum reveals *Trypanosoma lewisi* as an underestimated human pathogen. *Mol. Biochem. Parasitol.* **2015**, *199*, 58–61. [CrossRef]
181. Brumpt, E. Evolution de *Trypanosoma Lewisi*, *Duttoni*, *Nabiasi*, *Blanchardi*, chez les puces et les punaises. Transmission par les dejections. Comparaison avec *T. cruzi*. *Bull. Soc. Pathol. Exot.* **1913**, *6*, 167–171.
182. Channon, H.A.; Wright, H.D. Observations on Trypanosomiasis of Rabbits and its natural Mode of Transmission. *J. Pathol. Bacteriol.* **1927**, *30*, 253–260. [CrossRef]
183. Watson, E.A.; Hadwen, S. Trypanosomoses found in Canadian mammals. *Parasitology* **1912**, *5*, 21. [CrossRef]
184. Thiroux, A. Rescherches morphologiques et expérimentales sur *Trypanosoma duttoni* (Thiroux) *Ann. Inst. Pasteur.* **1905**, *19*, 564.
185. Monroy, F.P.; Dusanic, D.G. The Kidney Form of *Trypanosoma musculi*: A Distinct Stage in the Life Cycle? *Parasitol. Today* **2000**, *6*, 107–110. [CrossRef]
186. Zhang, X.; Hong, X.K.; Li, S.J.; Lai, D.H.; Hide, G.; Lun, Z.R.; Wen, Y.Z. The effect of normal human serum on the mouse trypanosome *Trypanosoma musculi* in vitro and in vivo. *Exp. Parasitol.* **2018**, *184*, 115–120. [CrossRef]
187. Austen, J.; Van Kampen, E.; Egan, S.; O'Dea, M.; Jackson, B.; Ryan, U.; Irwin, P.J.; Prada, D. First report of *Trypanosoma dionisii* (Trypanosomatidae) identified in Australia. *Parasitology* **2020**, *147*, 1801–1809. [CrossRef] [PubMed]
188. Soria, C.A.; Dusanic, D.G. Comparative studies of *Trypanosoma vespertilionis* Battaglia and *Trypanosoma dionisii* Bettencourt & França. *J. Protozool.* **1975**, *22*, 509–513. [CrossRef] [PubMed]
189. Bettencourt, A.; França, C. Sur un trypanosome du blairau (*Meles taxus*, Schreib). *CR Soc. Biol.* **1905**, *57*, 306.

Chapter 7

Detection of *Leishmania* sp. kDNA in questing *Ixodes ricinus* (Acari, Ixodidae) from the Emilia-Romagna Region in northeastern Italy

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BRIEF REPORT

Detection of *Leishmania* sp. kDNA in questing *Ixodes ricinus* (Acari, Ixodidae) from the Emilia-Romagna Region in northeastern Italy

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Abstract

To date, sand flies (Phlebotominae) are the only recognized biological vectors of *Leishmania infantum*, the causative agent of human visceral leishmaniasis, which is endemic in the Mediterranean basin and also widespread in Central and South America, the Middle East, and Central Asia. Dogs are the main domestic reservoir of zoonotic visceral leishmaniasis, and the role of secondary vectors such as ticks and fleas and particularly Rhipicephalus sanguineus (the brown dog tick) in transmitting *L. infantum* has been investigated. In the present paper, the presence of *Leishmania* DNA was investigated in questing *Ixodes ricinus* ticks collected from 4 rural areas included in three parks of the Emilia-Romagna Region (north-eastern Italy), where active foci of human visceral leishmaniasis have been identified. The analyses were performed on 236 DNA extracts from 7 females, 6 males, 72 nymph pools, and 151 larvae pools. Four samples (1.7%) (i.e., one larva pool, 2 nymph pools, and one adult male) tested positive for *Leishmania* kDNA. To the best of our knowledge, this is the first report of the presence of *Leishmania* kDNA in questing *I. ricinus* ticks collected from a rural environment. This finding in unfed larvae, nymphs, and adult male ticks supports the hypothesis that *L. infantum* can have both transstadial and transovarial passage in *I. ricinus* ticks. The potential role of *I. ricinus* ticks in the sylvatic cycle of leishmaniasis should be further investigated.

Keywords Leishmaniasis · *Leishmania* sp. · *Ixodes ricinus* · Ticks

Introduction

Leishmania infantum (Kinetoplastea, Trypanosomatida) is the causative agent of human visceral leishmaniasis, an important zoonosis endemic in the Mediterranean basin and also widespread in Central and South America, the Middle East, and Central Asia (Alvar et al. 2012). The parasite is naturally transmitted to humans by phlebotomine sand flies and, in the peridomestic cycle, the dog is traditionally recognized as a reservoir (Podaliri Vulpiani et al. 2011), for its high susceptibility to the infection and heavy skin parasitism (Dantas-Torres 2007).

Sand flies are the only recognized biological vectors for *L. infantum*, and their rapid geographical spread is followed by the spread of leishmaniasis into previously free areas (Dujardin et al. 2008), although secondary routes of transmission (i.e., transfusions, vertical in utero transmission, and venereal transmission) of little epidemiological relevance have been reported in dogs (de Freitas et al. 2006; Silva et al. 2009; Boggiatto et al. 2011). However, a possible role of secondary vectors such as ticks and fleas has been suggested (Coutinho et al. 2005; 2007). In particular, the brown dog tick *Rhipicephalus sanguineus* has received a lot of attention mainly due to its parasitic life cycle and its close relationship with dogs in both rural and urban areas, being highly adapted to live within human dwellings (Dantas-Torres 2010). Although a considerable amount of research has been carried out to investigate the presence of *L. infantum* in *R. sanguineus* collected from dogs (Colombo et al. 2010; Dantas-Torres et al. 2010a; Solano-Gallego et al. 2012; Campos and Costa 2014; Medeiros-Silva et al. 2015) and possible transmission routes (e.g., ticks bites,

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ingestion of infected ticks) (McKenzie 1984; Coutinho et al. 2005), their role in the transmission of *L. infantum* has been debated (Otranto and Dantas-Torres 2010) and is still questioned. *L. infantum* DNA was also detected in the castor bean tick *Ixodes ricinus*: particularly, Trotta et al. (2012) found it in ticks collected from dogs in Central Italy, and subsequently Salvatore et al. (2014) detected it in *I. ricinus* from both dogs and cats in Northern Italy, areas where human visceral leishmaniasis is endemic. To the best of our knowledge, no previous research has been performed to establish the presence of *Leishmania* spp. in questing ticks from rural environments. The present study is focused on the search of *Leishmania* spp. in *I. ricinus* questing ticks collected from three parks of Emilia-Romagna region (northeastern Italy), in hilly areas where human visceral leishmaniasis has long been described.

Material and methods

Sampling

The analyses were performed on DNA extracts from questing *Ixodes ricinus* ticks collected from April to October 2010 in 4 sites within 3 parks of the Emilia-Romagna region (Fig. 1). The parks are located along the hilly area of the Apennines, where the presence of autochthonous cases of leishmaniasis has been described in both humans and dogs (Mollicone et al. 2003; Varani et al. 2013). The area is characterized by a series of gypsum outcrops, closed valleys, cliffs, forested mountains, and gray calanques alternated with farmland. The sampling sites are natural pathways and picnic areas with habitual human attendance. Questing ticks were collected every 15 days by continuously flagging with a 1 m^2 white cotton cloth, from transects of 20 m along the uphill side of the pathways, usually reported as having higher tick density than the downhill side, while the picnic areas were flagged completely as described in detail by Aureli et al. (2015). Collected ticks were preserved in 70% ethanol at room temperature. Following morphological identification performed according to Manilla (1998) and Iori et al. (2005), ticks (individual adults, and pools consisting of either 5 nymphs or 10 larvae) were processed for DNA extraction as described by Aureli et al. (2015). Overall, 236 DNA extracts from 7 females, 6 males, 72 nymphs pools (i.e., 380 nymphs) and 151 larvae pools (i.e., 1510 larvae) were analyzed.

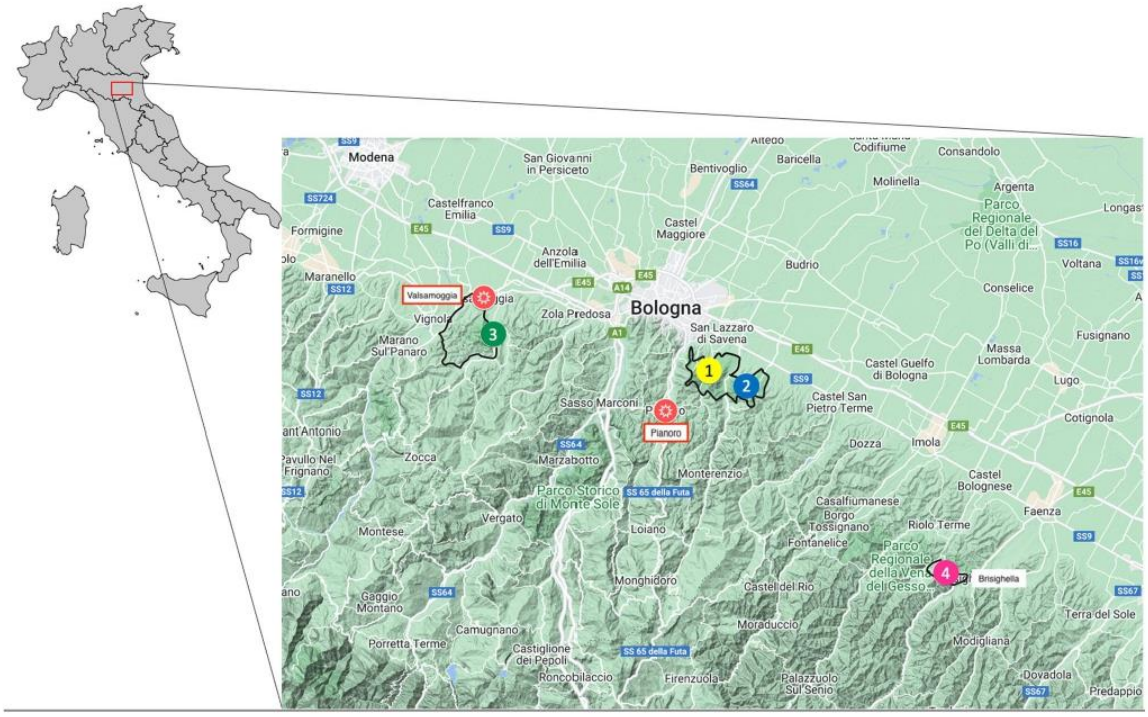

Fig. 1 Map of the four sampling sites distributed in three parks of Emilia-Romagna region. Gessi Bolognesi and Calanchi dell'Abbadessa Park: "number 1 in yellow circle" Ca' de Mandorli and "number 2 in blue circle" Ciagnano. Monteveglio Abbey, Park site: "number 3 in green circle"; Carnè Park site "number 4 in pink circle". Park borders are marked with black lines. "Asterisk symbol in red circle"Active foci of human visceral leishmaniasis in Valsamoggia and Pianoro (VL single cases have been reported along the whole foothill side)

DNA analysis

To detect the presence of *Leishmania* kDNA, a real-time PCR protocol was performed targeting a 71-bp region of the minicircle kinetoplast DNA using the primer pair Leish71Up (5′-CCAAACTTTTCTGGTCCTYCGGGTAG-3′) and Leish71Do (5′-TGAACGGGATTTCTGCACCCATTTTTC-3′) (Tsakmakidis et al. 2017) and following the conditions reported by Magri et al. (2022a, b).

Results and discussion

Out of the 236 *I. ricinus* DNA extracts, 4 (1.7%) tested positive for *Leishmania* sp. in 2 of the four sites examined: 2 nymph pools (5.4%) and 1 adult male (33.3%) from Monteveglio Abbey Park and 1 larva pool (2.3%) from Carné Park (Table 1).

Previous research mainly investigated the brown dog tick as a possible vector of *L. infantum*, and several studies showed the presence of *L. infantum* DNA in *R. sanguineus* collected from dogs affected by canine leishmaniasis (Coutinho et al. 2005; Paz et al. 2010; Campos and Costa 2014; Medeiros-Silva et al. 2015; Viol et al 2016) and from seronegative dogs living in endemic areas (Solano-Gallego et al. 2012). Nevertheless, the finding of *Leishmania* DNA in *R. sanguineus* ticks could be expected, given their blood feeding habits. Further work speculated that brown ticks can transmit canine leishmaniasis: Dantas-Torres et al. (2010a) reported the presence of *L. infantum* kDNA in the salivary glands of *R. sanguineus* ticks, corroborating the hypothesis that ticks could inject *Leishmania* parasites while blood feeding. Colombo et al. (2010) found viable *Leishmania* by RNA analysis in ticks 7 to 10 days after their removal from the dogs, showing that the parasite could survive for a long period in ticks, even after ecdysis had occurred in laboratory conditions. Dantas-Torres et al (2010b) demonstrated the transovarial passage of *L. infantum* kDNA in artificially infected *R. sanguineus*, and a subsequent study (Dantas-Torres et al. 2011) reported the detection and quantification of *L. infantum* DNA in field-collected engorged females and in their eggs and larvae. The transstadial and transovarian transmission of *L. infantum* in *R. sanguineus* was further confirmed by Dabaghmanesh et al. (2016). Medeiros-Silva et al. (2015), isolated *Leishmania* spp. in cultures from salivary glands and intestines of ticks collected from dogs; interestingly, it was possible to culture the parasite also from pools of unfed male ticks suggesting that *Leishmania* could persist in the brown tick after blood digestion (Medeiros-Silva et al. 2015). *L. infantum* DNA was also reported from questing *Rhipicephalus* spp. from Israel (Mumcuoglu et al. 2022). Finally, further studies demonstrated the capability of *R. sanguineus* nourished on infected dogs to transmit the parasites to hamsters (Almeida et al. 2016). Based on these findings, although sand flies are the recognized vectors of *Leishmania,* a minor role of the dog brown tick could not be excluded.

Concerning tick species other than *R. sanguineus*, also *I. ricinus* collected from dogs in central Italy was found positive for *L. infantum* (Trotta et al. 2012). Moreover, Salvatore et al (2014) found *Leishmania* kDNA in *I. ricinus* ticks removed from 4 dogs and 1 cat living in areas of northeastern Italy where canine leishmaniasis is endemic, although no anamnestic data related to infection in these animals were reported.

Table 1 Number of specimens examined according to sampling sites and developmental stages. T = total; P = positive

	Gessi Bolognesi and Calanchi dell'Abadessa Park									
	1 Cà de Mandorli		2 Ciagnano		3 Monteveglio Abbey Park		3 Carnè Park		Total	
	T	*P*	*T*	*P*	*T*	*P*	*T*	*P*	*T*	*P*
Adult females	3	0	1	0	3	0	0	-	7	0
Adult males	2	0	1	0	3	1 (33.3%) (95% *CI*: 0.0–53.3)	0	-	6	1 (16.6%) (95% *CI*: 0.0–46.37)
Nymphs (pools)	24	0	11	0	37	2 (5.4%) (95% *CI*: 0.0–12.7)	0	-	72	2 (2.8%) (95% *CI*: 0.0–6.61)
Larvae (pools)	13	0	74	0	21		43	1 (2.3%) (95% *CI*: 0.0–35.5)	151	1 (0.66%)
Total	42	0	87	0	64	3 (4.7%) (95% *CI*: 0.0–9.9)	43	1	236	4 (1.7%) (95% *CI*: 0.05–3.3)

T total; *P* positive

In the Argentine Patagonia, Millan et al. (2016) observed the presence of *Leishmania* DNA in the gray fox *Pseudalopex griseus* and in pools of *Amblyomma tigrinum* ticks collected from both positive and negative foxes, in a remote non-endemic area of South America, where dogs are scarce and sand flies are not known to be present, supporting the hypothesis that *L. infantum* could maintain a sylvatic cycle in the studied area, not involving dogs or sand flies.

Interestingly, in the same areas where the present study was carried out, a high positivity rate for *L. infantum* (33.3%) was observed in the roe deer *Capreolus capreolus* (Magri et al. 2022a, b), and blood meal preference for this species was found in sand flies (Calzolari et al. 2022), suggesting the possible involvement of *C. capreolus* (frequently hosts of the adult stages of *I. ricinus*) in the epidemiology of leishmaniasis in the area under study.

Conclusions

To the best of our knowledge, this is the first description of *Leishmania* DNA in questing *I. ricinus* ticks collected from a rural environment. This finding in unfed larvae, nymphs and males support the hypothesis that, even in this tick species, *L. infantum* could have both transstadial and transovarial transmission. The percentage (1.7%) of ticks positive to *Leishmania* DNA obtained in our study appears lower than the one reported in sand flies in other research (2.9–57.1%), stressing the fact that phlebotomine flies are the sole *Leishmania* efficient proven vector (Aransay et al. 2000; Gómez-Saladín et al. 2005; Ergunay et al. 2014; González et al. 2017; Latrofa et al. 2018). Nevertheless, a role of *I. ricinus* in a sylvatic cycle, albeit minor, could not be excluded in the endemic areas under study.

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Data availability Data supporting the conclusions of this article are included within the article and its supplementary tables.

Declarations

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References

Almeida VA, da Hora TN, Júnior NFL, Carvalho FS, da Silva AL, Wenceslau AA, Albuquerque GR, Silva FL (2016) Detection of *Leishmania infantum* DNA in hamsters infested with ticks collected from naturally infected dogs. Rev Bras Med Vet 38(4):329–333

Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, WHO Leishmaniasis Control Team (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7(5):e35671. https://doi.org/10.1371/journal.pone.0035671

Aransay AM, Scoulica E, Tselentis Y (2000) Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Appl Environ Microbiol 66(5):1933–1938. https://doi.org/10.1128/AEM.66.5.1933-1938.2000

Aureli S, Galuppi R, Ostanello F, Foley JE, Bonoli C, Rejmanek D, Rocchi G, Orlandi E, Tampieri MP (2015) Abundance of questing ticks and molecular evidence for pathogens in ticks in three parks of Emilia-Romagna region of Northern Italy. Ann Agric Environ Med 22(3):459–466. https://doi.org/10.5604/12321966.1167714

Boggiatto PM, Gibson-Corley KN, Metz K, Gallup JM, Hostetter JM, Mullin K, Petersen CA (2011) Transplacental transmission of Leishmania infantum as a means for continued disease incidence in North America. PLoS Negl Trop Dis 5(4):e1019. https://doi.org/10.1371/journal.pntd.0001019

Calzolari M, Romeo G, Bergamini F, Dottori M, Rugna G, Carra E (2022) Host preference and *Leishmania infantum* natural infection of the sand fly *Phlebotomus perfiliewi* in northern Italy. Acta Trop 26:106246. https://doi.org/10.1016/j.actatropica.2021.106246

Campos JH, Costa FAL (2014) Participation of ticks in the infectious cycle of canine visceral Leishmaniasis in Teresina, Piauí, Brazil. Rev Inst Med Trop Sao Paulo 56(4):297–300. https://doi.org/10.1590/S0036-46652014000400005

Colombo FA, Ororizzi RMFN, Laurenti MD, Galati EAB, Canavez F, Pereira-Chioccola VL (2010) Detection of *Leishmania (Leishmania) infantum* RNA in fleas and ticks collected from naturally infected dogs. Parasitol Res 109:267–274. https://doi.org/10.1007/s00436-010-2247-6

Coutinho MT, Linardi PM (2007) Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? Vet Parasitol 147(3–4):320–325. https://doi.org/10.1016/j.vetpar.2007.04.008

Coutinho MTZ, Bueno LL, Sterzik A, Fujiwara RT, Botelho JR, De Maria M, Genaro O, Linardi PM (2005) Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. Vet Parasitol 128:149–155. https://doi.org/10.1016/j.vetpar.2004.11.011

Dabaghmanesh T, Asgari Q, Moemenbellah-Fard MD, Soltani A, Azizi K (2016) Natural transovarial and transstadial transmission of *Leishmania infantum* by naïve *Rhipicephalus sanguineus* ticks blood feeding on an endemically infected dog in Shiraz, south of Iran. Tans R Soc Trop Med Hyg 110:408–413. https://doi.org/10.1093/trstmh/trw041

Dantas-Torres F (2007) The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania (Leishmania) infantum* and *Leishmania (Viannia) braziliensis*. Vet Parasitol 149(3–4):139–146. https://doi.org/10.1016/j.vetpar.2007.07.007

Dantas-Torres F (2010) Biology and ecology of the brown dog tick Rhipicephalus Sanguineus. Parasit Vectors 3:26. https://doi.org/10.1186/1756-3305-3-26

Dantas-Torres F, Lorusso V, Testini G, De Paiva-Cavalcanti M, Figueredo AL, Stanneck D, Mencke N, Brandão-Filho SP, Alved LC, Otranto D (2010) Detection of *Leishmaniainfantum* in *Rhipicephalus sanguineus* ticks from Brazil and Italy. Parasitol Res 106:857–860. https://doi.org/10.1007/s00436-010-1722-4

Dantas-Torres F, Martins TF, de Paiva-Cavalcanti M, Figueredo LA, Lima BS, Brandão-Filho SP (2010) Transovarial passage of *Leishmania infantum* kDNA in artificially infected *Rhipicephalus sanguineus*. Exp Parasitol 125:184–185. https://doi.org/10.1016/j.exppara.2010.02.003

Dantas-Torres F, Latrofa MS, Otranto D (2011) Quantification of *Leishmania infantum* DNA in females, eggs and larvae of *Rhipicephalus sanguineus*. Parasit Vectors 4:56. https://doi.org/10.1186/1756-3305-4-56

de Freitas E, Melo MN, da Costa-Val AP, Michalick MS (2006) Transmission of *Leishmania infantum* via blood transfusion in dogs: potential for infection and importance of clinical factors. Vet Parasitol 137(1–2):159–167. https://doi.org/10.1016/j.vetpar.2005.12.011

Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M (2008) Spread of vector-borne diseases and neglect of Leishmaniasis. Europe Emerg Infect Dis 14(7):1013–1018. https://doi.org/10.3201/eid1407.071589

Ergunay K, Kasap OE, Orsten S, Oter K, Gunay F, Yoldar AZ, Dincer E, Alten B, Ozkul A (2014) Phlebovirus and *Leishmania* detection in sandflies from eastern Thrace and northern Cyprus. Parasites Vectors 7:575. https://doi.org/10.1186/s13071-014-0575-6

Gómez-Saladín E, Doud CW, Maroli M (2005) Short report: surveillance of *Leishmania* sp. among sand flies in Sicily (Italy) using a fluorogenic real-time polymerase chain reaction. Am J Trop Med Hyg 72:138–141

González E, Álvarez A, Ruiz S, Molina R, Jiménez M (2017) Detection of high *Leishmania infantum* loads in *Phlebotomus perniciosus* captured in the leishmaniasis focus of southwestern Madrid region (Spain) by real time PCR. Acta Trop 171:68–73. https://doi.org/10.1016/j.actatropica.2017.03.023

Iori A, Di Giulio A, De Felici S (2005) Zecche d'Italia. In: Cringoli G, Iori A, Rinaldi L, Veneziano V, Genchi C (eds.). Mappe parassitologiche: Zecche. Rolando Editore, Napoli 52–163

Latrofa MS, Iatta R, Dantas-Torres F, Annoscia G, Gabrielli S, Pombi M, Gradoni L, Otranto D (2018) Detection of *Leishmania infantum* DNA in phlebotomine sand flies from ad area where canine leishmaniosis is endemic in southern Italy. Vet Parasitol 253:39–42. https://doi.org/10.1016/j.vetpar.2018.02.006

Magri A, Galuppi R, Fioravanti M, Caffara M (2022a) Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy) Vet Res Commun. https://doi.org/10.1007/s11259-022-09925-4.

Magri A, Bianchi C, Kostygov A, Caffara M, Galuppi R, Fioravanti M, Jurčenko V (2022b) Survey on the presence of *Leishmania infantum* in wild animals from the province of Bologna (North-eastern Italy). XXXII Congresso SoIPa – Naples (Italy), 27–30 June 2022b: 306

Manilla G (1998) Fauna d'Italia. Acari Ixodida. Edizioni Calderini, Bologna, Italy

McKenzie KK (1984) A study of the transmission of canine leishmaniasis by the tick, *Rhipicephalus sanguineus*, and an ultrastructural comparison of the promastigote. PhD Dissertation, Oklahoma State University

Medeiros-Silva V, Gurgel-Gonçalves R, Nitz N, D'Anduraim Morales LE, Cruz LM, Sobrai IG, Boité MC, Ferreira GEM, Cupolillo E, Romero GAS (2015) Successful isolation of *Leishmania infantum* from *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) collected from naturally infected dogs. BMC Vet Res 11:258. https://doi.org/10.1186/s12917-015-0576-5

Millán J, Travaini A, Zanet S, López-Bao JV, Trisciuoglio A, Ferroglio E, Rodríguez A (2016) Detection of *Leishmania* DNA in wild foxes and associated ticks in Patagonia, Argentina, 2000 km south of its known distribution area. Parasites Vectors 9:241. https://doi.org/10.1186/s13071-016-1515-4

Mollicone E, Battelli G, Gramiccia M, Maroli M, Baldelli R (2003) A stable focus of canine leishmaniosis in the Bologna Province, Italy. Parassitologia 45:85–88

Mumcuoglu KY, Arslan-Akveran GA, Aydogdu S, Karasartova D, Kosar A, Savci U, Keskin A, Taylan-Ozkan, (2022) Pathogens in ticks collected in Israel: II Bacteria and Protozoa Found *in Rhipicephalus Sanguineus* Sensu Lato and *Rhipicephalus Turanicus*. Ticks Tick Borne Dis 13:101986. https://doi.org/10.1016/j.ttbdis.2022.101986

Otranto D, Dantas-Torres F (2010) Fleas and ticks as vectors of *Leishmania* spp. to dogs: caution is needed. Vet Parasitol 168:173–174. https://doi.org/10.1016/j.vetpar.2009.11.016

Paz GF, Ribeiro MFB, de Magalhães DF, Sathler KPB, Morais MH, Fiúza VOP, Brandão ST, Werneck GL, Fortes-Dias CL, Dias ES (2010) Association between the prevalence of infestation by *Rhipicephalus sanguineus* and *Ctenocephalides felis felis* and the presence of anti-*Leishmania* antibodies: a case–control study in dogs from a Brazilian endemic area. Prev Vet Med 97:131–133. https://doi.org/10.1016/j.prevetmed.2010.08.006

Podaliri Vulpiani M, Iannetti L, Paganico D, Iannino F, Ferri N (2011) Methods of control of the *Leishmania infantum* dog reservoir: state of the Art. Vet Med Int 2011:215964. https://doi.org/10.4061/2011/215964

Salvatore D, Aureli S, Baldelli R, di Francesco A, Tampieri MP, Galuppi R (2014) Molecular evidence of *Leishmania infantum* in *Ixodes ricinus* ticks from dogs and cats, in Italy. Vet Ital 50(4):307–312. https://doi.org/10.12834/VetIt.83.1222.2.

Silva FL, Oliveira RG, Silva TM, Xavier MN, Nascimento EF, Santos RL (2009) Venereal transmission of canine visceral leishmaniasis. Vet Parasitol 160(1–2):55–59. https://doi.org/10.1016/j.vetpar.2008.10.079

Solano-Gallego L, Rossi L, Scroccaro AM, Montarsi F, Caldin M, Furlanello T, Trotta M (2012) Detection of *Leishmania infantum* DNA mainly in *Rhipicephalus sanguineus* male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasites Vectors 5:98. https://doi.org/10.1186/1756-3305-5-98

Trotta M, Nicetto M, Fogliazza A, Montarsi F, Caldin M, Furlanello T, Solano-Galego L (2012) Detection of *Leishmania infantum*, *Babesia canis*, and rickettsiae in ticks removed from dogs living in Italy. Ticks Tick Borne Dis 3:293–296. https://doi.org/10.1016/j.ttbdis.2012.10.031

Tsakmakidis I, Angelopoulou K, Dovas CI, Dokianakis E, Tamvakis A, Symeonidou I, Antoniou M, Diakou A (2017) *Leishmania* infection in rodents in Greece. Trop Med Int Health 22(12):1523–1532. https://doi.org/10.1111/tmi.12982

Varani S, Cagarelli R, Melchionda F, Attard L, Salvadori C, Finarelli A, Gentilomi G, Tigani R, Rangoni R, Todeschini R, Scalone A, Di Muccio T, Gramiccia M, Gradoni L, Viale P, Landini M (2013)

Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. Euro Surveill 18(29):3–6

Viol MA, Guerrero FD, de Oliveira BCM, de Aquino MCC, Loiola SH, de Melo GD, de Souza Gomes AH, Kanamura CT, Garcia MV, Andreotti R, de Lima VMF, Bresciani KDS (2016) Identification of *Leishmania* spp. promastigotes in the intestines, ovaries, and salivary glands of *Rhipicephalus sanguineus* actively infesting dogs. Parasitol Res 115:3479–3484. https://doi.org/10.1007/s00436-016-5111-5

Chapter 8

Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy)

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Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy)

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Abstract

Leishmaniasis is a neglected vector-borne parasitic disease caused in Italy only by the species *Leishmania infantum* of the *Leishmania donovani* complex, which is the causative agent of the zoonotic visceral leishmaniasis (VL), and the sporadic cutaneous leishmaniasis (CL) in humans, and of the canine leishmaniasis (CanL). The disease is considered endemic in southern, central, and insular Italian regions and recognizes phlebotomine sand flies as vector and dogs as main reservoir. However, a specific north-eastern region, namely Emilia-Romagna, always showed a peculiar epidemiological situation when compared to the other northern Italian regions and recent studies are indeed questioning the role of dog as main reservoir of *L. infantum*. Due to their synanthropic relationship with humans, rodents have been tested for *Leishmania* spp. in several European countries. The aim of this study was to assess the presence of *Leishmania* spp. in peridomestic rodents in the Emilia-Romagna

Region. The study was carried out on 136 peridomestic rodents collected by professional rodent control services: 47 brown rats (*Rattus norvegicus*), 39 black rats (*Rattus rattus*) and 50 mice (*Mus musculus*). Specimens of earlobe skin, spleen, liver and prescapular lymph nodes were tested with a real-time PCR. Fifteen (11 %) rodents, tested positive for *L. infantum*. Positivity was obtained from different target organs; notably 33% of the rodents tested positive in earlobe skin samples. These findings revealed the presence of *Leishmania* spp. in peridomestic rodents of the Emilia-Romagna Region, also in two species never tested before in Italy, namely *R. norvegicus* and *M. musculus*.

Keywords: Leishmaniasis, Italy, *Mus musculus, Rattus norvegicus*, *Rattus rattus*

Background

Leishmaniasis is a neglected vector-borne parasitic disease endemic in southwestern Europe. With reference to Italy, *Leishmania infantum* of the *Leishmania donovani* complex is the only species responsible for visceral leishmaniasis (VL), for sporadic cutaneous leishmaniasis (CL) in humans and for canine leishmaniasis (CanL) (Gramiccia and Gradoni 2005; Rugna et al. 2018). The parasite is transmitted by phlebotomine sand flies, and in Italy dogs have long been recognized as the major reservoir in southern, central and insular regions, where the disease is considered endemic. Among the northern Italian regions, Emilia-Romagna has always had a different epidemiological scenario: CL has been widely reported since 1934, and between 1950-1958 up to 2,670 cases were diagnosed in the province of Forlì (Pampiglione 1975). In contrast, until the early 1970's, in this region VL appeared sporadically, with only 4 autochthonous cases observed, one in the province of Bologna and 3 in the province of Forlì. In the same period and within the same area, no ascertained autochthonous cases of CanL were reported (Pampiglione 1975). In 1971-1972, in two municipalities of Bologna province located in a foothill area a dramatic outbreak of VL was reported, involving 60 patients with a lethality of 21.7% (Pampiglione 1975). Since then, the geographic distribution of human and canine leishmaniasis has notably increased and the disease spread even in other regions of northern Italy, where many autochthonous cases of VL, CL and CanL have been reported (Gaspari et al. 2017; Mendoza-Roldan et al. 2020). This epidemiological change may be due to environmental issues, occurrence of competent insect vectors and movement of infected dogs from endemic areas (Santi et al. 2014). However, such changes might not be sufficient to explain the recurrent VL and CL

56 foci recorded in Emilia-Romagna Region (Gaspari et al. 2017), especially considering that
57 molecular studies carried out on strains isolated from autochthonous cases of VL are
58 questioning the role of dogs as reservoirs of *L. infantum* in this region, as earlier suggested
59 (Pampiglione 1975; Rugna et al. 2018).
60 The role of wildlife has long been recognized as crucial in the transmission and maintenance
61 of zoonotic agents and several sylvatic species are known to be susceptible to leishmaniasis.
62 Considering their synanthropic relationship with humans and their abundance the role of
63 rodents as possible leishmaniasis reservoirs has been questioned in different European
64 countries (Alcover at al. 2021). Several studies established the presence of *L. infantum* in
65 these hosts in Greece (Papadogiannakis et al. 2009; Tsakmakidis et al. 2017), Portugal
66 (Helhazar et al. 2013) and Spain (Navea-Pérez et al. 2015; Galán-Puchades et al. 2019;
67 Martín-Sánchez et al. 2020).
68 In Italy, a study performed in Sicily detected *L. infantum* by PCR in 45% of black rats, even
69 if in this region the role of the dog as reservoir has been well established (Di Bella et al.
70 2003). However, a study performed in Montecristo Island (Tuscany), revealed the presence
71 of *L. infantum* in the 15.5% of rodents examined, even in the absence of domestic carnivores
72 (Zanet et al. 2014).
73 The aim of this survey was to assess the presence of *Leishmania* spp. in peridomestic rodents
74 collected in the Emilia-Romagna Region, Italy.
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76
77

Materials and Methods

From June 2019 to June 2021, 136 peridomestic rodent carcasses were sampled during pest control programs from the provinces of Ferrara, Forlì-Cesena and Ravenna (Emilia Romagna) (figure 1): 47 brown rats, *Rattus norvegicus* (20 females and 27 males), 39 black rats, *R. rattus* (21 females and 18 males), 50 mice, *Mus musculus* (22 females and 28 males) were collected from the territory by professional rodent control services and stored at -20 °C before processing.

The entire carcass was examined; species, sex and age classes were identified by morphological and metrical evaluation (CDC). Necropsies and samples collection were performed with sterile surgical instruments and when possible, according to the state of the carcasses, 25 mg of tissue were collected from earlobe skin, prescapular lymph node and liver, and 10 mg from the spleen (Helhazar et al. 2013). Due to the corruption of the remains, lymph nodes were not collected from 16 subjects. Samples were placed in sterile 1.5 ml tubes and stored at -20 °C.

DNA extraction was performed with PureLink® Genomic DNA Mini Kit (Invitrogen, ThermoFisher Scientific) following the manufacturer's instructions. For DNA amplification a real-time PCR protocol was performed targeting a 71-bp region of the minicircle kinetoplast DNA using primer pair Leish71Up (5'-CCAAACTTTTCTGGTCCTYCGGGTAG-3') and Leish71Do (5'-TGAACGGGATTTCTGCACCCATTTTTC -3') (Tsakmakidis et al. 2017). Reactions were carried out in a total volume of 20 µL with 10 µL of PowerUP™ SYBR™ Green master mix (2X), 0.3 µM of each primer and 2 µL of DNA. The amplification was performed in

StepOnePlus Real-Time PCR System (Applied Biosystems) and the thermal cycling profile was as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 sec., 60 °C for 30 sec. At the end of the amplification, a melting curve analysis was performed from 60 °C to 95 °C, with a slope of 0.3 °C to monitor primer dimers of non-specific product formation. Each sample was amplified in triplicate, the average temperature of melting (Tm) observed was 79.39 ± 0.15 °C and the average standard deviation observed in cts was 0.65. The standard curve was created with serial dilution of *L. infantum* DNA ranging from 10,000 to 0.1 parasites per reaction. Each reaction was carried out by three replicates per dilution, in three independent experiments. The ct value cut-off was settled at mean ct value of 39.3 which corresponds to 1 parasite per mL of the original parasite suspension.

As a positive control the reference strain *L. infantum* MHOM/TN/80/IPT1, kindly provided by the Unit of Clinical Microbiology, Regional Reference Centre for Microbiological Emergencies (CRREM), St. Orsola-Malpighi University Hospital, Bologna, Italy, was used. Confidence intervals were calculated by R Studio (RStudio Team 2020).

Results and Discussion

Out of 136 subjects examined, 15 (11 %; 95% CI=5.7-16.3) were positive for *Leishmania* spp. In particular, 10.6% (95% CI=1.8-19.4) of brown rats, 12.8% (95% CI=2.5-23.7) of black rats and 10% (95% CI=1.7-18.3) of mice (Table 1). Of the five positive mice, three tested positive in two target organs - spleen and earlobe skin or spleen and liver or spleen and lymph nodes - the remaining two subjects tested positive only in lymph nodes or liver, respectively. The geographical distribution of the positive subjects appears homogeneous between the sampled sites (figure 1).

122 The present survey assessed the presence of *Leishmania* spp. in synanthropic rodents of the
123 Emilia-Romagna Region. The conditions settled by the WHO (2010) for a species to be
124 recognized as reservoir is the prevalence of infection > 20% and the availability of the
125 parasite in blood and skin in sufficient amount to be ingested by a sand fly. In the
126 Mediterranean area such conditions were globally assessed only for *M. musculus*, while *R.*
127 *norvegicus* and *R. rattus* showed lower prevalence of infections (16.4% and 9.9%,
128 respectively) (Alcover et al. 2021).
129 The prevalence values observed in the current study are below the average found in
130 Portugal or Spain (Barcelona) where the 33.3% of examined rodents (*M. musculus* and *R.*
131 *norvegicus*, Helhazar et al. 2013; *R. norvegicus,* Galán-Puchades et al. 2019) resulted positive,
132 or the one reported in Granada (Spain) in mice (88.9%) (Martín-Sánchez et al. 2020) or in
133 different rodent species (*R. rattus*, *M. musculus* and *Apodemus sylvaticus*) (27%) by Navea-
134 Pérez et al. (2015), whilst it is higher than the prevalence observed in brown rats (5.9%) in
135 Greece (Papadogiannakis et al. 2009). Further studies performed in Greece by Tsakmakidis
136 et al. (2017) on spleen of *R. norvegicus*, *R. rattus* and *M. musculus* revealed a prevalence of
137 19.58% comparable to the one herein reported. The majority of the studies evaluated the
138 presence of the parasite in more than one target organ including skin, liver, spleen and blood
139 (Helhazar et al. 2013; Martín-Sánchez et al. 2020; Navea-Pérez et al. 2015; Tsakmakidis et al.
140 2017) while few studies examined only the spleen as target organ (Galán-Puchades et al.
141 2019; Papadogiannakis et al. 2009). Testing more than one target tissue allow to increase the
142 possibility to detect *Leishmania* spp. as observed also in our study. Three *M. musculus* here
143 examined showed the presence of the parasite DNA in two different target organs (spleen

144 + lymph nodes and spleen + liver). Although the spleen is traditionally recognized as
145 *Leishmania* spp. target organ for PCR in different animal species (Papadogiannakis et al.
146 2009), our results showed the presence of *Leishmania* spp. in the earlobe skin samples from
147 33 % of the positive rodents pointing out that this tissue should be also considered. In fact,
148 wild animals are frequently collected in decomposition state and the putrefaction of the
149 target tissues, like visceral organs, may affect the integrity of the kinetoplast DNA (Mũnoz-
150 Madrid et al. 2013).
151 In Italy, the role of black rats in the transmission of *L. infantum* has long been investigated,
152 starting from surveys performed in Tuscany in the 1980's (Pozio et al. 1985). Further studies
153 showed that *Phlebotomus perniciosus* and *P. perfiliewi* are attracted to *R. rattus* and that these
154 sand fly species become infected when they feed on black rats experimentally infected with
155 *L. infantum* (Pozio et al. 1985). More recent study carried out in Calabria (Italy) by Di Bella
156 et al. (2003) showed 45% positivity in the spleen of 22 *R. rattus* although in this region the
157 role of dogs as reservoirs has long been established. Zanet et al. (2014), reported 15.5%
158 prevalence in black rats examined in the Montecristo Island (Tuscany, Italy) where *L.*
159 *infantum* was recorded even in absence of domestic carnivore hosts. This value is similar to
160 the one (12.8%) obtained in the same host in our study, that moreover provided also data
161 on *R. norvegicus* and *M. musculus* (10.6% and 10% respectively) species not previously tested
162 for *L. infantum* in Italy.
163 Leishmaniasis in Emilia-Romagna has a peculiar epidemiological scenario compared to the
164 other Northern Italian regions. Recently Rugna et al. (2018), by Multilocus Microsatellite
165 Typing (MLMT) detected differences between *Leishmania* strains from men and sand flies to

166 the ones from dogs. The MLMT profiles showed all canine samples belonged to one group
167 genetically related to Mediterranean MON-1 strain and similar to the VL samples from other
168 Italian regions, while all but one VL Emilia-Romagna case, and the isolates from sand fly
169 fell into a different group. Therefore, in this region the co-circulation of two distinct groups
170 of *L. infantum* seems to occur, and the VL in humans could have different cycles involving
171 *P. perfiliewi* as a vector (Rugna et al. 2018; Calzolari et al. 2019) and might include other
172 vertebrates, besides dogs, as reservoirs.
173 In two of the three provinces studied, Ravenna and Forlì-Cesena, foci of VL, usually located
174 in hilly areas, were historically described. The rodent samples analyzed were collected in
175 an area not higher than 50 m above sea level, where the density of phlebotomines is scant
176 and, according to leishmaniasis regional control plan, in 2020 only CL cases have been
177 reported (Santi et al. 2021). Further research should focus on studying which strains
178 circulate in this area.
179 Also notable is the presence of a positive brown rat in the province of Ferrara, where
180 autochthonous cases of leishmaniasis in both dogs and humans have never been recorded:
181 the specimen was collected in a locality on the border between the provinces of Ferrara and
182 Ravenna where the phlebotomine population is recorded as being moderate (Santi et al.
183 2021). This finding, considering the consistent increase in geographical distribution of the
184 disease and its vector, will require further investigation.
185 *L. infantum* is a vector-borne parasite and in its epidemiology many mammal species are
186 involved, hence identifying which one may act as a reservoir in the Emilia-Romagna Region
187 is an ambitious task due to the presence of different environments i.e. hilly or flatlands and

different distribution of sylvatic and peridomestic animals, which may possibly be involved in the parasite cycle. Even if the presence of the parasite in mammalian hosts is crucial, in order to fully understand his meaning as main reservoir or epiphenomena it should be associated with studies on the blood preferences of the phlebotomine vector.

The total prevalence observed in the present study (11%), despite being lower to the one required from WHO (2010) to establish a role of reservoir is comparable to the Mediterranean's one. As reported in previous studies, this value is far from being trivial: considering their close relationship with humans, their ability to colonize new environments and their impact on human health, rodents should not be neglected for their potential role in the transmission of Leishmaniasis, especially in urban areas (Alcover et al. 2021).

Although these preliminary findings are not sufficient to prove the role of peridomestic rodents as reservoirs of *L. infantum*, they nevertheless indicate the opportunity to further investigate their possible role in the epidemiology of different strains of *L. infantum* circulating in the Emilia-Romagna Region.

List of abbreviations

VL = Visceral Leishmaniasis

CanL = Canine Leishmaniasis

CL = Cutaneous Leishmaniasis

Declarations:

-Ethics approval and consent to participate

No ethical approval is officially required since the rodents examined had been subjected to pest control are considered pest species.

- *Consent for publication*

Not applicable

- *Availability of data and materials*

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

- *Competing interests*

The authors declare that they have no competing interests

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- *Authors' contributions*

MF and RG conceived the study. AM performed field work. AM and MC performed laboratory work and analyzed data. AM and MC wrote the first draft of the manuscript. MF and RG reviewed the manuscript. All authors read and approved the final manuscript

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References

Alcover MM, Riera MC and Fisa R (2021). Leishmaniosis in rodents caused by *Leishmania infantum*: a review of studies in the Mediterranean area. Frontiers in Veterinary Science 8:702687.

Calzolari M, Carra E, Rugna G, Bonilauri P, Bergamini F, Bellini R, et al. (2019) Isolation and molecular typing of Leishmania infantum from Phlebotomus perfiliewi in a re-emerging focus of Leishmaniasis, Northeastern Italy. Microorganisms 7:644. https://doi.org/10.3390/microorganisms7120644.

CDC. Domestic rodent field identification. CDC Pictorial Keys. Atlanta, USA. Available from: https://www.cdc.gov/nceh/ehs/docs/pictorial_keys/rodents.pdf.

Di Bella C, Vitale E, Russo G, Greco A, Millazzo C, Aloise G, et al. (2003) Are rodents a potential reservoir for *Leishmania infantum* in Italy? J Mt Ecol 7(Suppl.):125-129.

Galán-Puchades MT, Gómez-Samblás M, Suárez-Morán JM, Osuna A, Sanxis-Furló J, Pascual J, et al. (2019) Leishmaniasis in norway rats in Sewers, Barcelona, Spain. Emerg Infect Dis 25(6):1222-1224. https://doi.org/10.3201/eid2506.181027.

Gaspari V, Ortalli M, Foschini MP, Baldovini C, Lanzoni A, Cagarelli R, et al. (2017) New Evidence of Cutaneous Leishmaniasis in North-Eastern Italy. JEADV 31(9): 1534–40. https://doi.org/10.1111/jdv.14309.

Gramiccia M, Gradoni L (2005) The current status of zoonotic Leishmaniases and approaches to disease control. Int J Parasitol 35(11-12):1169-80. https://doi.org/10.1016/j.ijpara.2005.07.001.

253 Helhazar M, Leitão J, Duarte A, Tavares L, Pereira da Fonseca I (2013) Natural infection of
254 synanthropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania*
255 *infantum* in Sesimbra and Sintra – Portugal. Parasites Vectors 6:88.
256 https://doi.org/10.1186/1756-3305-6-88.
257 Martín-Sánchez J, Torres-Medina N, Corpas-López V, Morillas-Márquez F, Díaz-Sáez V.
258 2020 Vertical transmission may play a greater role in the spread of *Leishmania infantum*
259 in synanthropic *Mus musculus* rodents than previously believed. Transbound Emerg
260 Dis. 67:1113–1118. https://doi.org/10.1111/tbed.13436.
261 Mendoza-Roldan J, Benelli G, Panarese R, Iatta R, Furlanello T, et al. (2020) *Leishmania*
262 *infantum* and *Dirofilaria immitis* infections in Italy, 2009–2019: changing distribution
263 patterns. Parasites Vectors. 13:193. https://doi.org/10.1186/s13071-020-04063-9.
264 Mũnoz-Madrid R, Belinchón-Lorenzo S, Iniesta V, Fernández-Cotrina J, Parejo JC, Monroy
265 I, et al. (2013) First detection of *Leishmania infantum* kinetoplast DNA in hair of wild
266 mammals: Application of qPCR method to determine potential parasite reservoirs.
267 Acta Trop 128:706-709. http://dx.doi.org/10.1016/j.actatropica.2013.08.009.
268 Navea-Pérez HM, Díaz-Sáez V, Corpas-Lóez V, Merino-Espinosa G, Morillas-Márquez F,
269 Martín-Sánchez J (2015). *Leishmania infantum* in wild rodents: reservoirs or just
270 irrelevant incidental hosts? Parasitol Res: 114:2363-2370
271 https://doi.org/10.1007/s00436-015-4434-y
272 Pampiglione S (1975) La Leishmaniosi viscerale in Italia. Ann San pubbl 35(6)1021-1028.
273 Papadogiannakis E, Spannakos G, Kontos V, Menounos PG, Tegos N, Vakalis N (2009)
274 Molecular detection of *Leishmania infantum* in wild rodents (*Rattus norvegicus*) in

Greece. Zoonoses and Public Health 57:23-25. https://doi.org/10.1111/j.1863-2378.2009.01264.x.

Pozio E, Maroli M, Gradoni L, Gramiccia M (1985) Laboratory transmission of *Leishmania infantum* to *Rattus rattus* by the bite of experimentally infected *Phlebotomus perniciosus*. Trans R Soc Trop Med Hyg 79(4):524–526. https://doi.org/10.1016/0035-9203(85)90085-9.

RStudio Team (2020) RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Santi A, Renzi M, Baldelli R, Calzolari M, Caminiti A, Dell'Anna S, et al. (2014) A surveillance program on canine Leishmaniasis in the public kennels of Emilia-Romagna Region, Northern Italy. Vector Borne Zoonotic Dis 14(3):206-11. https://doi.org/10.1089/vbz.2013.1362.

Santi A, Rossi A, Galletti G, Casadei G, Tamba M (2021) Piano Regionale di controllo della leishmaniosi risultati anno 2020. Ordine dei veterinari di Reggio Emilia http://www.ordineveterinarireggioemilia.it/userfiles/files/Relazione_Piano_Leishmania_2020.pdf

Tsakmakidis I, Angelopoulou K, Dovas CI, Dokianakis E, Tamvakis A, Symeonidou I, Antoniou M, Diakou A (2017) Leishmania infection in rodents in Greece. Trop Med Int Health 22(12):1523-1532. https://doi.org/10.1111/tmi.12982

Rugna G, Carra E, Bergamini F, Calzolari M, Salvatore D, Corpus F, et al. (2018) Multilocus microsatellite typing (MLMT) reveals host-related population structure in *Leishmania*

296 *infantum* from northeastern Italy. PLoS Negl Trop Dis 12(7):e0006595.
297 https://doi.org/10.1371/journal.pntd.0006595.
298 Zanet S, Sposimo P, Trisciuoglio A, Giannini F, Strumia F, Ferroglio E (2014) Epidemiology
299 of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in
300 absence of domestic reservoir and definitive host. Vet Parasitol 199:247- 249.
301 https://doi.org/10.1016/j.vetpar.2013.10.023.

Table 1: Real time PCR positive samples

ID	Specimen	Locality	Real-Time PCR			
			Earlobe Skin	Spleen	Liver	Lymph Node
57	*Mus musculus*	Bizzuno (RA)	ct= 32.7 (87)	ct= 29.68 (676)	Negative	NA
59	*Mus musculus*	Bizzuno (RA)	Negative	ct= 30.77 (316)	ct= 31.97 (143)	NA
67	*Mus musculus*	Bizzuno (RA)	Negative	Negative	Negative	ct= 33.61 (47)
98	*Mus musculus*	S. Alberto (RA)	Negative	ct= 36.71 (5.8)	Negative	ct= 37.07 (4.5)
111	*Mus musculus*	Bizzuno (RA))	Negative	Negative	ct= 35.9 (10)	Negative
4	*Rattus norvegicus*	Ravenna (RA)	ct= 34.25 (30.9)	Negative	Negative	Negative
86	*Rattus norvegicus*	Godo (RA)	Negative	Negative	ct= 36.47 (6.8)	Negative
141	*Rattus norvegicus*	Ravenna (RA)	ct= 37.75 (2.9)	Negative	Negative	Negative
175	*Rattus norvegicus*	Forlì (FC)	Negative	Negative	ct= 36.27 (7.8)	Negative
178	*Rattus norvegicus*	Argenta (FE)	Negative	Negative	ct= 36.67 (5.8)	Negative
37	*Rattus rattus*	Forlì (FC)	Negative	ct= 36.47 (6.8)	Negative	Negative
60	*Rattus rattus*	San Pietro in Campiano (RA)	ct= 36.86 (6.2)	Negative	Negative	Negative
95	*Rattus rattus*	Montaletto di Cervia (RA)	Negative	ct= 37.44 (6.2)	Negative	Negative
179	*Rattus rattus*	Montaletto di Cervia (RA)	Negative	Negative	Negative	ct= 36.63
206	*Rattus rattus*	Longastrino (RA)	ct= 37.89 (2.6)	Negative	Negative	Negative

Legend: Ct values are reported as mean ct of observed in different target organs with the estimated quantity of parasites/ml (mean standard deviation observed ±0.65). Localities are as well reported with reference to the province: Ferrara (FE), Forlì-Cesena (FC) and Ravenna (RA).

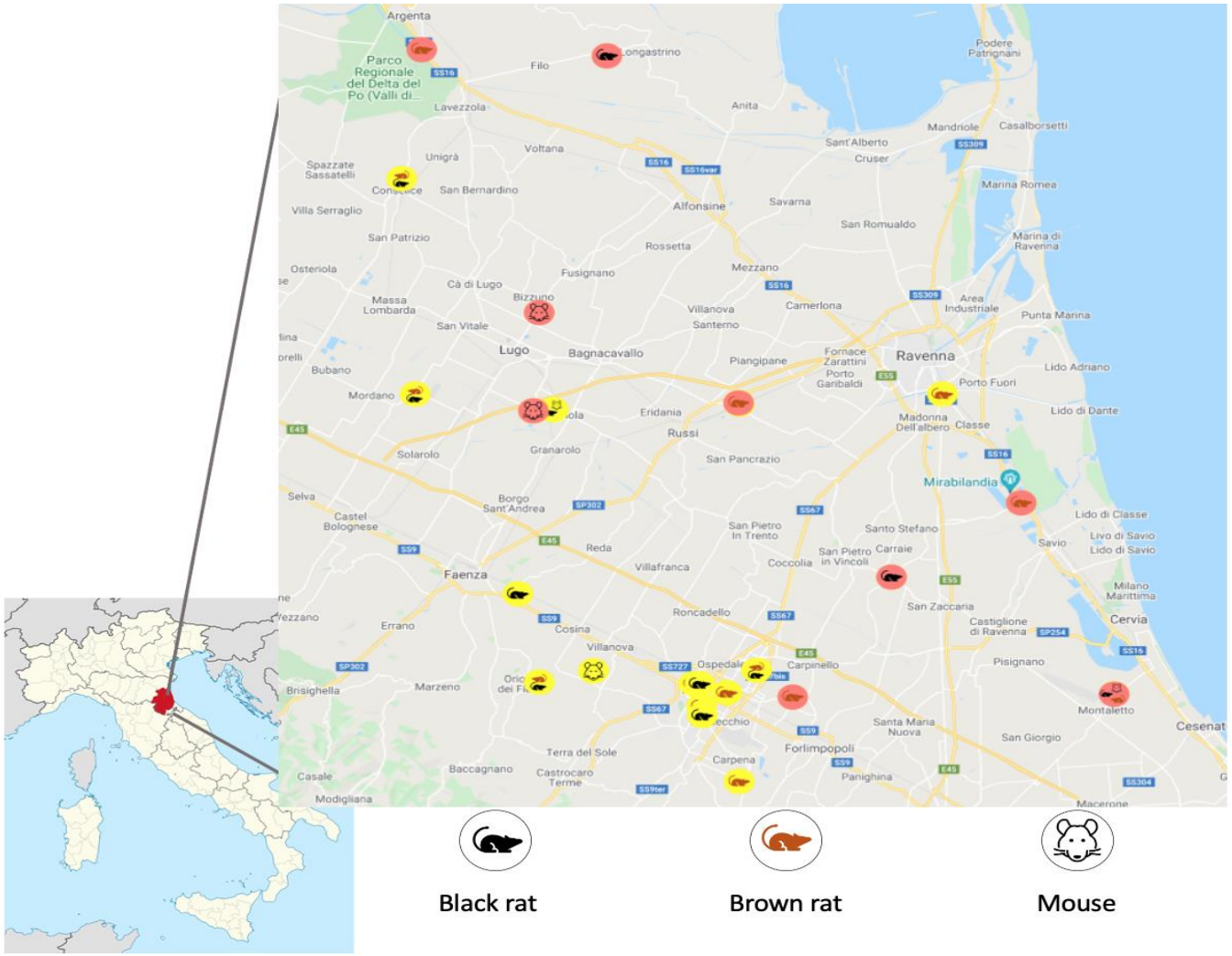

Figure 1. Map of the sampling area in the Emilia-Romagna Region. Dots are representative for sampling sites; red dots: at least one specimen positive, yellow dots: all the specimen negative.

Chapter 9

Roe deer (*Capreolus capreolus*) is a novel potential reservoir for human visceral leishmaniasis in Emilia-Romagna region of Northeastern Italy

Magri, A., Bianchi, C., Chmelová, L., Caffara, M., Galuppi, R., Fioravanti, M., Yurchenko, V., Kostygov, A.Y. (2022a). Roe deer (*Capreolus capreolus*) are a novel potential reservoir for human visceral leishmaniasis in the Emilia-Romagna region of northeastern Italy. International Journal for Parasitology, https://doi.org/10.1016/j.ijpara.2022.09.002.

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Roe deer (*Capreolus capreolus*) are a novel potential reservoir for human visceral leishmaniasis in the Emilia-Romagna region of northeastern Italy ☆

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ABSTRACT

Leishmaniasis is a complex human disease caused by intracellular parasites of the genus *Leishmania*, predominantly transmitted by the bite of sand flies. In Italy, leishmaniasis is caused exclusively by *Leishmania infantum*, responsible for the human and canine visceral leishmaniases (HVL and CVL, respectively). Within the Emilia-Romagna region, two different foci are active in the municipalities of Pianoro and Valsamoggia (both in the province of Bologna). Recent molecular studies indicated that *L. infantum* strains circulating in dogs and humans are different, suggesting that there is an animal reservoir other than dogs for human visceral leishmaniasis in the Emilia-Romagna region. In this work, we analyzed specimens from wild animals collected during hunts or surveillance of regional parks near active foci of human visceral leishmaniasis for *L. infantum* infection in the province of Bologna. Out of 70 individuals analyzed, 17 (24%) were positive for *L. infantum*. The infection prevalence in hedgehogs (*Erinaceus europaeus*), roe deer (*Capreolus capreolus*), badgers (*Meles meles*), and bank voles (*Myodes glareolus*) was 80, 33, 25, and 11%, respectively. To distinguish the two strains of *L. infantum* we have developed a nested PCR protocol optimized for animal tissues. Our results demonstrated that most (over 90%) of *L. infantum* infections in roe deer were due to the strain circulating in humans in the Emilia-Romagna region.

1. Introduction

Leishmaniasis is a complex of tropical and subtropical vector-borne diseases with up to 1 million new cases recorded annually (WHO, 2022. Leishmaniasis, https://www.who.int/en/news-room/-fact-sheets/detail/leishmaniasis. (accessed August 16, 2022)). It is caused by sandfly-transmitted parasitic flagellates of the genus *Leishmania* (Euglenozoa: Kinetoplastea: Trypanosomatidae), whose development in vertebrates occurs intracellularly (Kostygov et al., 2021). The disease has three main clinical forms: cutaneous (skin sores), mucocutaneous (destruction of skin and mucosa), and visceral (systemic inflammation focusing on liver, spleen, and bone marrow) (Bruschi and Gradoni, 2018). The latter form is the most dangerous as mortality in untreated patients can exceed 95% (WHO, 2022. Leishmaniasis, https://www.who.int/en/news-room/-fact-sheets/detail/leishmaniasis. (accessed August 16, 2022)). Leishmaniasis in Italy is caused only by *Leishmania infantum* (Lukeš et al., 2007), which is responsible for human and canine visceral leishmaniases (HVL and CVL, respectively). In the Emilia-Romagna region (ER) of northeastern Italy, HVL outbreaks have been described since the 1970s, mostly in the foothill areas (Pampiglione, 1975). From the 1990s, the outbreaks became recurrent and *L. infantum* was documented even in the non-endemic territories of northern Italy (Maroli et al., 2008; Abdalmaula et al., 2013; Cesinaro et al., 2017; Ferroglio et al., 2018). Currently in the Bologna province of ER, two different foci are active in the municipalities of Pianoro and Valsamoggia (Varani et al., 2013; Ortalli et al., 2020).

The HVL caused by *L. infantum* is considered zoonotic, i.e. mammals other than humans are always involved in circulation of the parasite (Bruschi and Gradoni, 2018). For example, in Italy, dogs were proposed for that role, however, a wide range of wildlife species have also been implicated as potential reservoirs in Europe: carnivores (canids, felids, mustelids, badgers, etc.), lagomorphs

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank under accession numbers **OP186448-OP186451**.

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0020-7519/

(hares and rabbits), insectivores (hedgehogs and shrews), rodents (various mice and rats, squirrels, etc.), and bats (Bruschi and Gradoni, 2018; Cardoso et al., 2021). Wild ruminants might also serve as reservoirs, but they have been sampled rather scarcely. To the best of our knowledge, there were no reports on *L. infantum* infections in these animals either in Europe, or on other continents thus far.

Recent studies demonstrated that the *L. infantum* population in ER is heterogeneous and can be split into two different strains which are exclusively present (besides sandflies) in either human patients or dogs (Rugna et al., 2017, 2018). Notably, in other regions of Italy, the parasites of humans are genetically similar to those that infect dogs in the ER region. This implies that parasites causing HVL in ER have distinct animal reservoirs, as initially suggested in 1974 (Pampiglione et al., 1974). In line with this, analysis of the vectors' blood meal preferences in this area showed a strong bias toward wild mammals (Calzolari et al., 2022).

In the present study, 70 individuals of nine species of wild mammals collected in municipalities of Bologna located near the active foci of HVL were screened for the presence of *Leishmania* spp. This was done using real-time PCR for kinetoplast DNA (kpDNA) as well as a newly developed nested PCR protocol allowing precise strain differentiation. In addition to the known reservoirs such as mice, rats and hedgehogs, we detected *L. infantum* in roe deer, thereby presenting the first known report of such an infection in ruminants.

2. Materials and methods

2.1. Collected material

From June 2019 to December 2020, organs and entire carcasses of 70 wild mammals belonging to nine species were sampled near active HVL foci in the Pianoro and Valsamoggia municipalities of Bologna (Table 1; Fig. 1). In particular, ear lobes and spleen from roe deer, as well as carcasses of hares, were provided by professional hunters, while carcasses of other mammals revealed during park surveillance were collected by volunteers and park rangers. When entire carcasses were available, four samples were taken from each of them during necropsies: the ear lobe skin, spleen, liver, and prescapular lymph nodes. The necropsy details have been described elsewhere (Magri et al., 2022). DNA from these samples was isolated with PureLink® Genomic DNA Mini Kit (Invitrogen/ Thermo Fisher Scientific, Carlsbad, USA) according to the manufacturer's instructions.

2.2. Real-time PCR

The presence of *Leishmania* spp. was assessed with a highly sensitive real-time PCR targeting a 71-bp region of minicircle kpDNA using primer pair Leish71Up (5′-ccaaacttttctggtcctycgggtag-3′) and Leish71Do (5′-tgaacgggatttctgcacccatttttc-3′) (Tsakmakidis et al., 2017). The details of amplification and parasite quantification were described previously Magri et al. (2022).

Table 1
Summary of the samples collected in Italy.

Species	Locality	Individuals positive/total
Roe deer (*Capreolus capreolus*)	Pianoro	11/33
Hare (*Lepus europaeus*)		0/13
Hedgehog (*Erinaceus europaeus*)		3/3
	Valsamoggia	1/2
Badger (*Meles meles*)		1/4
Bank vole (*Myodes glareolus*)		1/9
Beech marten (*Martes foina*)		0/1
European polecat (*Mustela putorius*)		0/1
Common shrew (*Sorex araneus*)		0/3
Fox (*Vulpes vulpes*)		0/1

2.3. Nested PCR

Discrimination of *L. infantum* strains based on the one-step amplification of the gene encoding cysteine peptidase B (*cpb*), featuring a 39-nucleotide (nt) indel, was previously proposed for laboratory cultures (Zackay et al., 2013; Rugna et al., 2017). However, application of this strategy to tissue samples resulted in multiple PCR by-products originating from host DNA and prevented diagnosis of *Leishmania* infection. To overcome this problem, we developed a nested PCR protocol by adding a second pair of primers annealing within the amplicon produced at the first amplification stage. It is important to note that the *L. infantum* genome contains multiple copies of the *cpb* gene, of which only one varies as described above. This copy has a distinct sequence at the 3′ end allowing its specific amplification. The *cpb* sequences were retrieved from GenBank (accession numbers: AJ628943, AY896776, AY896777, AY896778, AY896780, AY896782, AY896791, EU637907, GQ302670, GQ302671, GQ302674, GQ856074, JN400122-JN400131, XM_001463394). For the first round of PCR, previously reported primers cpbEFF (5′-gttatggctgc gtggcttg-3′) and cpbEFR (5′-cgtgcactcggccgtctt-3′) were used (Zackay et al., 2013). For the second round, a new primer pair was designed using Geneious Prime (Dotmatics, Boston, USA) software: cpbt1: 5′-tgtccagcatgcctcacaaga-3′ and cpbt2: 5′-ccagctcctt catgtcttacca-3′ (Fig. 2).

Leishmania infantum strain identity was determined for 17 samples which tested positive here by real-time PCR and for 15 previously reported positive samples from black rats (*Rattus rattus*), brown rats (*Rattus norvegicus*), and mice (*Mus musculus*) collected in the ER region (Magri et al., 2022). Reactions were carried out in a total volume of 25 µl with 12.5 µl of PCRBIO Taq Mix Red (PCR Biosystems ltd, London, UK), 0.3 µM of each primer and 2 µl of DNA in the first round and 1.5 µl of template in the second round. For both rounds, amplification was performed as follows: initial denaturation 94 °C for 4 min, followed by 30 cycles 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 5 min final elongation. As a positive control, the reference strain *L. infantum* MHOM/TN/80/IPT1 was used. The amplified fragments were separated on a 1.5% agarose gel stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany). The fragment lengths were 281 bp (long – L variant, no deletion) or 242 bp (short – S variant, deletion) (Fig. 3). The identity of the PCR products was confirmed by sequencing four samples (two long and two short). The Sample Size Calculator (https://www.calculator.net/sample-size-calculator.html) was used to calculate 95% confidence intervals (CIs) for the observed prevalence values.

3. Results

3.1. Real-time PCR

In the real-time PCR screening, 17 out of 70 analyzed individuals (24% overall prevalence) tested positive (Table 2). The presence of leishmaniae was detected in earlobe skin of 11 roe deer out of 33 analyzed (prevalence 33%; 95% CI: 17.3, 49.4). In one of these samples, the parasites were also detected in the spleen. Four out of five hedgehogs (prevalence 80%; 95% CI: 44.9, 100), showed signal either in the spleen or in the ear lobe skin (three and one individual, respectively). Leishmaniae were also detected in the liver and spleen of one bank vole (prevalence 11%; 95% CI: 0, 31.6 as well as

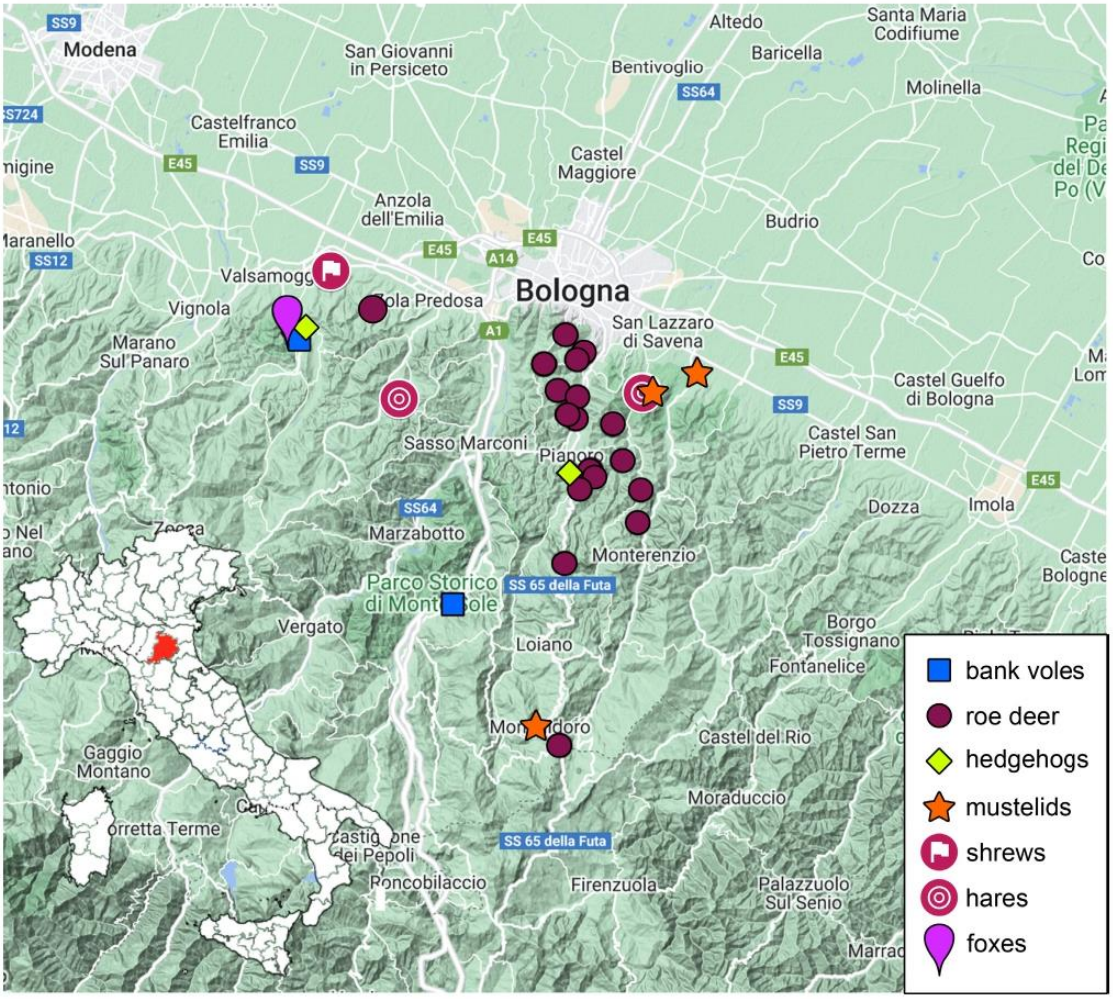

Fig. 1. Map of the region in Italy where the samples were collected.

in the liver of a single badger (prevalence 25%; 95% CI: 0, 67.4) (Table 2).

3.2. Nested PCR

The 17 samples which tested positive in the present study, and 15 isolates from mice and two species of rats from which *Leishmania* has been detected in a previous study (Magri et al., 2022), were subjected to strain identification by the newly developed nested PCR. For seven out of the combined 32 samples, the *Leishmania* strain identity could not be determined due to the lack of PCR products. This apparently can be explained by the low content of parasite DNA and/or its degradation due to tissue decomposition. The real-time PCR used here is more resistant to the latter issue, since it targets a much shorter fragment (71 bp versus 365–404 bp amplified at the first step of the nested PCR). Moreover, the kpDNA fragment originates from a multicopy template (mini-circles) compared with the single-copy nuclear gene target of the nested PCR. For the remaining 25 samples, the *L. infantum* strain identity was determined as L (long) or S (short). The L and S genotypes in our work correspond to the *cbp*F (documented in humans and sandflies in the ER region) and *cbp*E (documented in dogs in the ER region and humans elsewhere) genotypes, respectively (Rugna et al., 2017, 2018). In total, we documented 16 L and nine S genotypes. The host species distribution for the analyzed genotypes was as follows: roe deer – 10 L (91%), one S (9%); hedgehogs – three L (75%) and one S (25%); mice – two L (40%), one S (20%), and two samples were unidentified due to PCR failure; black rats – one L (20%), three S (60%), and one unidentified sample; brown rats – no L, three S (60%) and two unidentified samples.

4. Discussion

In Europe, several studies aimed to assess the presence of *L. infantum* in wild and peri-domestic animals. Even though the role of these animals as reservoirs has been demonstrated in certain outbreaks (Molina et al., 2012; Helhazar et al., 2013; Tsakmakidis et al., 2017), it is still uncertain if they can also act as accidental hosts or amplifiers (Tomassone et al., 2018).

In the current study, samples from 70 individuals of nine wildlife species, collected in the proximity of HVL foci of the ER region,

Fig. 2. Alignment of partial cysteine peptidase B gene sequences of *Leishmania infantum* demonstrating the annealing sites of the used primers and the position of the indel. The target gene variant (highlighted) in the strains JPCM5 and Drep 13 is shown together with a non-target variant of the strain JPCM5 (GenBank accession numbers XM_001463394, JN400127, and XM_003392190, respectively). Nucleotides identical to those in the first sequence are replaced with dots. Dashes show the characteristic deletion allowing strain discrimination. The PCR products obtained from XM_001463394 and JN400127 are classified as *cbp*E and *cbp*F genotypes, respectively, when amplified with the external primers (in blue), or as S and L genotypes when amplified with the internal primers (in green).

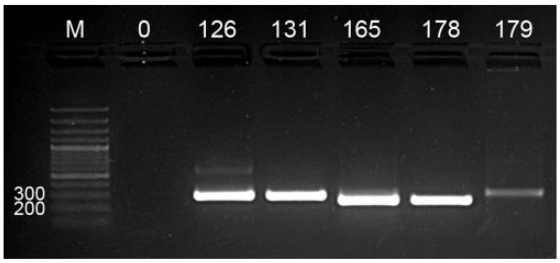

Fig. 3. Nested PCR detection of the L (specimens 126, 131, and 179) and S (specimens 165 and 178) genotypes of *Leishmania infantum*. The 100-bp ladder (Thermo Fisher Scientific, Waltham, USA) is on the left. Lane "0" is a negative amplification control.

were screened for *Leishmania* spp. and 17 of them tested positive (prevalence 24%). Of particular interest is the fact that approximately one-third of the examined roe deer were positive. Moreover, the majority of them (10/11) harbored the strain associated with HVL in ER. This suggests that roe deer may represent a natural reservoir of *L. infantum* in general and HVL in the ER region in particular.

The parasite load, especially in the skin, can reflect infectiousness in the natural life cycle (Courtenay et al., 2014). It was previously estimated that in order to serve as a reservoir, the host species should demonstrate an infection prevalence over 20% with the parasite detectable in blood or skin in sufficient amounts to be ingested by a sand fly (Roque and Jansen, 2014; Alemayehu and Alemayehu, 2017). A reservoir species should be sufficiently abundant and long-lived, thereby providing sufficient frequency of contacts with the vector (WHO, 2022). In Europe (as in the rest of the Old World), leishmaniae are mainly transmitted by *Phlebotomus* spp. (Torres-Guerrero et al., 2017). Even though these sandflies have been considered generally opportunistic (De Colmenares et al., 1995), the analysis of blood meal of *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* in the ER region revealed a high presence of roe deer blood (Calzolari et al., 2022). Taken together, these findings suggest the role of roe deer in the epidemiology of *L. infantum* in northern Italy. Whether other wild animals such as badgers, hedgehogs or bank voles may play a similar role remains to be investigated further, because the number of specimens analyzed thus far is not sufficient to make any solid conclusions.

In order to differentiate *L. infantum* strains circulating in human and animals, we took advantage of the *cpb* locus possessing a 39-bp deletion in some isolates (Hide and Bañuls, 2006; Chaouch et al., 2013). It has been previously reported that in the ER region the autochthonous human isolates were endowed with a longer sequence, while those circulating in dogs possessed the above-mentioned deletion, implying that causative agents of HVL and CVL in this region are different (Rugna et al., 2017, 2018). Here, we report that most of the wild animals collected in the proximity of active HVL foci in the ER region (10 roe deer, three hedgehogs) tested positive for the strain associated with humans. This strain has been previously documented in one mouse and one black rat from the Romagna area where, according to the leishmaniasis regional control authority, cases of the autochthonous HVL were reported last year (Santi et al., 2022). The strain circulating in dogs in the ER region was documented in one hedgehog and one roe deer in the same area, and in rodents from the Ravenna province, an area with cases of CVL in kennels.

In conclusion, we revealed two strains of *L. infantum* circulating in the wild and synanthropic fauna of the ER region of Italy. The strain causing HVL in ER was documented in roe deer collected in the proximity of active foci of this disease and represented over

Table 2
Summary of the real-time and nested PCR results (positive samples only).

ID	Species	Real-Time PCR results[b]				*cpb* ID[c]
		Earlobe Skin	Spleen	Liver	lymph Node	
24	Roe deer	32.62 (92)	–	N.A.	N.A.	L
25	Roe deer	29.48 (771)	29.97 (553)	N.A.	N.A.	S
28	Roe deer	36.07 (9)	–	N.A.	N.A.	L
29	Roe deer	32.02 (138)	–	N.A.	N.A.	L
30	Roe deer	38.27 (11)	–	N.A.	N.A.	L
34	Roe deer	28.72 (1290)	–	N.A.	N.A.	L
35	Roe deer	38.07 (2)	–	N.A.	N.A.	L
36	Roe deer	27.29 (3399)	–	N.A.	N.A.	L
123	Roe deer	33.24 (51)	–	N.A.	N.A.	L
126	Roe deer	30.6 (361)	–	N.A.	N.A.	L
131	Roe deer	33.5 (61)	–	N.A.	N.A.	L
165	Hedgehog	36.78 (6)	–	–	–	S
181	Hedgehog	–	35.66 (12)	–	–	L
182	Hedgehog	–	36.65 (6)	–	–	L
183	Hedgehog	–	34.04 (33)	–	–	L
192	Bank vole	–	35.26 (15)	33.69 (45)	–	–
193	Badger	–	–	34.144 (33)	–	–
57[a]	Mouse	32.7 (87)	29.68 (676)	–	–	L
59[a]	Mouse	–	30.77 (316)	31.97 (143)	–	–
67[a]	Mouse	–	–	–	33.61 (47)	–
98[a]	Mouse	–	36.71 (5.8)	–	37.07 (4.5)	S
111[a]	Mouse	–	–	35.9 (10)	–	L
4[a]	Brown rat	34.25 (30.9)	–	–	–	–
86[a]	Brown rat	–	–	36.47 (6.8)	–	S
141[a]	Brown rat	37.75 (2.9)	–	–	–	S
175[a]	Brown rat	–	–	36.27 (7.8)	–	–
178[a]	Brown rat	–	–	36.67 (5.8)	–	S
37[a]	Black rat	–	36.47 (6.8)	–	–	S
60[a]	Black rat	36.86 (6.2)	–	–	–	S
95[a]	Black rat	–	37.44 (6.2)	–	–	S
179[a]	Black rat	–	–	–	36.63 (6.3)	L
206[a]	Black rat	37.89 (2.6)	–	–	–	–

[a] Samples analyzed by real-time PCR in the previous study (Magri et al., 2022).
[b] Real-time PCR results are reported as Ct values averaged for triplicates with the estimated quantity of parasites/ml in parentheses; dash, amplification failure; N.A., specimen not available.
[c] Strains are classified by *cpb* (gene encoding cysteine peptidase B) PCR product length: S, short; L, long.

90% of *L. infantum* infections in these animals. This, together with other facts (predominant parasite localization in the skin facilitating transmission and the preference for roe deer by sand flies in the analyzed area) implies that roe deer can serve as a reservoir of HVL. The role of other potential wildlife reservoir species remains to be investigated further.

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References

Abdalmaula, G.H., Barbadoro, P., Marigliano, A., Illuminati, D., Di Stanislao, F., D'Errico, M.M., Prospero, E., 2013. Human visceral leishmaniasis: a picture from Italy. J. Infect. Public Health 6, 465–472.
Alemayehu, B., Alemayehu, M., 2017. Leishmaniasis: a review on parasite, vector and reservoir host. Health Sci. J. 11, 519.
Bruschi, F., Gradoni, L., 2018. The Leishmaniases: Old Neglected Tropical Diseases. Springer, Cham, Switzerland.
Calzolari, M., Romeo, G., Bergamini, F., Dottori, M., Rugna, G., Carra, E., 2022. Host preference and *Leishmania infantum* natural infection of the sand fly *Phlebotomus perfiliewi* in northern Italy. Acta Trop. 226, 106246.
Cardoso, L., Schallig, H., Persichetti, M.F., Pennisi, M.G., 2021. New epidemiological aspects of animal leishmaniosis in Europe: the role of vertebrate hosts other than dogs. Pathogens 10, 307.
Cesinaro, A.M., Nosseir, S., Mataca, E., Mengoli, M.C., Cavatorta, C., Gennari, W., 2017. An outbreak of cutaneous leishmaniasis in Modena province (Northern Italy): report of 35 cases. Pathologica 109, 363–367.
Chaouch, M., Fathallah-Mili, A., Driss, M., Lahmadi, R., Ayari, C., Guizani, I., Ben Said, M., Benabderrazak, S., 2013. Identification of Tunisian *Leishmania* spp. by PCR amplification of cysteine proteinase B (*cpb*) genes and phylogenetic analysis. Acta Trop. 125, 357–365.
Courtenay, O., Carson, C., Calvo-Bado, L., Garcez, L.M., Quinnell, R.J., 2014. Heterogeneities in *Leishmania infantum* infection: using skin parasite burdens to identify highly infectious dogs. PLoS Negl. Trop. Dis. 8, e2583.
De Colmenares, M., Portús, M., Botet, J., Dobaño, C., Gállego, M., Wolff, M., Seguí, G., 1995. Identification of blood meals of *Phlebotomus perniciosus* (Diptera: Psychodidae) in Spain by a competitive enzyme-linked immunosorbent assay biotin/avidin method. J. Med. Entomol. 32, 229–233.
Ferroglio, E., Battisti, E., Zanet, S., Bolla, C., Concialdi, E., Trisciuoglio, A., Khalili, S., Biglino, A., 2018. Epidemiological evaluation of *Leishmania infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy. Zoonoses Public Health 65, 675–682.
Helhazar, M., Leitao, J., Duarte, A., Tavares, L., da Fonseca, I.P., 2013. Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra Helhazar – Portugal. Parasit. Vectors 6, 88.
Hide, M., Bañuls, A.L., 2006. Species-specific PCR assay for *L. infantum*/*L. donovani* discrimination. Acta Trop. 100, 241–245.
Kostygov, A.Y., Karnkowska, A., Votýpka, J., Tashyreva, D., Maciszewski, K., Yurchenko, V., Lukeš, J., 2021. Euglenozoa: taxonomy, diversity and ecology, symbioses and viruses. Open Biol. 11, 200407.
Lukeš, J., Mauricio, I.L., Schönian, G., Dujardin, J.C., Soteriadou, K., Dedet, J.P., Kuhls, K., Tintaya, K.W., Jirků, M., Chocholová, E., Haralambous, C., Pratlong, F., Oborník, M., Horák, A., Ayala, F.J., Miles, M.A., 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proc. Natl. Acad. Sci. U. S. A. 104, 9375–9380.

Magri, A., Galuppi, R., Fioravanti, M., Caffara, M., 2022. Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy). Vet. Res. Commun. https://doi.org/10.1007/s11259-11022-09925-11254.

Maroli, M., Rossi, L., Baldelli, R., Capelli, G., Ferroglio, E., Genchi, C., Gramiccia, M., Mortarino, M., Pietrobelli, M., Gradoni, L., 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop. Med. Int. Health 13, 256–264.

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet. Parasitol. 190, 268–271.

Ortalli, M., De Pascali, A.M., Longo, S., Pascarelli, N., Porcellini, A., Ruggeri, D., Randi, V., Procopio, A., Re, M.C., Varani, S., 2020. Asymptomatic *Leishmania infantum* infection in blood donors living in an endemic area, northeastern Italy. J. Infect. 80, 116–120.

Pampiglione, S., 1975. Visceral leishmaniasis in Emilia-Romagna. Ann. Sanita. Pubblica 35, 1021–1028. in Italian.

Pampiglione, S., La Placa, M., Schlick, G., 1974. Studies on mediterranean leishmaniasis. I. An outbreak of visceral leishmaniasis in Northern Italy. Trans. R. Soc. Trop. Med. Hyg. 68, 349–359.

Roque, A.L., Jansen, A.M., 2014. Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int. J. Parasitol. Parasites Wildl. 3, 251–262.

Rugna, G., Carra, E., Corpus, F., Calzolari, M., Salvatore, D., Bellini, R., Di Francesco, A., Franceschini, E., Bruno, A., Poglayen, G., Varani, S., Vitale, F., Merialdi, G., 2017. Distinct *Leishmania infantum* strains circulate in humans and dogs in the Emilia-Romagna region, Northeastern Italy. Vector Borne Zoonotic Dis. 17, 409–415.

Rugna, G., Carra, E., Bergamini, F., Calzolari, M., Salvatore, D., Corpus, F., Gennari, W., Baldelli, R., Fabbi, M., Natalini, S., Vitale, F., Varani, S., Merialdi, G., 2018. Multilocus microsatellite typing (MLMT) reveals host-related population structure in *Leishmania infantum* from northeastern Italy. PLoS Negl. Trop. Dis. 12, e0006595.

Santi, A., Rossi, A., Rocca, R., Galletti, G., Casadei, G., Tamba, M., 2022. [Epidemiological surveillance. Emilia-Romagna region. Regional leishmaniasis control plan - results for the year 2021], Sorveglianza Epidemiologica Emilia Romagna (SEER). Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Bologna, Italy. (in Italian).

Tomassone, L., Berriatua, E., De Sousa, R., Duscher, G.G., Mihalca, A.D., Silaghi, C., Sprong, H., Zintl, A., 2018. Neglected vector-borne zoonoses in Europe: into the wild. Vet. Parasitol. 251, 17–26.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J., Arenas, R., 2017. Leishmaniasis: a review. F1000Res 6, 750.

Tsakmakidis, I., Angelopoulou, K., Dovas, C.I., Dokianakis, E., Tamvakis, A., Symeonidou, I., Antoniou, M., Diakou, A., 2017. *Leishmania* infection in rodents in Greece. Trop. Med. Int. Health 22, 1523–1532.

Varani, S., Cagarelli, R., Melchionda, F., Attard, L., Salvadori, C., Finarelli, A.C., Gentilomi, G.A., Tigani, R., Rangoni, R., Todeschini, R., Scalone, A., Di Muccio, T., Gramiccia, M., Gradoni, L., Viale, P., Landini, M.P., 2013. Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. Euro. Surveill. 18, 20530.

Zackay, A., Nasereddin, A., Takele, Y., Tadesse, D., Hailu, W., Hurissa, Z., Yifru, S., Weldegebreal, T., Diro, E., Kassahun, A., Hailu, A., Jaffe, C.L., 2013. Polymorphism in the HASPB repeat region of East African *Leishmania donovani* strains. PLoS Negl. Trop. Dis. 7, e2031.

General Discussion

This PhD project aimed to give new insights into a question that was raised from the first report of human visceral leishmaniasis (HVL) in Emilia-Romagna region: is the dog really the only reservoir of *Leishmania infantum* in this region? Therefore, in concert with other Italian institutions, such as CREEM (Centro di Riferimento Regionale per Emergenze Microbiologiche), and regional Istituti Zooprofilattici Sperimentali (State Veterinary Institutes of the Italian Ministry of Health), this project aimed to understand the role of wild and synanthropic mammals in the epidemiology of *L. infantum*, also taking into account the results of studies on blood meal preferences of local phlebotomine populations conducted by the colleagues of the IZS of Reggio Emilia which indicated a preference for roe deer in sandflies collected in areas with active HVL (Calzolari *et al*., 2022).

To achieve this aim, several critical points had to be overcome.

A first relevant critical issue was to obtain biological material suitable for the analyses from a significant number of different wild and synanthropic mammals, even if several meetings were held with pest control services, selection hunters and volunteer park rangers to arrange the collection of carcasses and organs suitable for the studies. Concerning synanthropic rodents a huge effort was made at the beginning of the study to identify pest control services collecting carcasses using snap-traps, because of the common use of rodenticides that does not allow the later collection of the remains. For this reason, unfortunately, the only available material for our analyses were carcasses and organs collected and frozen by the pest control services. In view of this, it was not possible to isolate and establish a laboratory culture for further analysis. This made necessary to select a sensitive assay for the detection of the protozoa to overcome the problems arising from the state of conservation of the biological material. So, basing on literature (Tsakmakidis *et al.*, 2017), a very sensitive real-time PCR assay was optimized and applied to the collected samples also when showing a poor state of conservation due to phenomena of initial decomposition.

As it is known that in the ER region two different *L. infantum* strains are currently circulating in human and canine populations (Rugna *et al.*, 2017), to discriminate the strains of positive samples, a collaboration was undertaken with the Life Science Research Centre (Faculty of Science – University of Ostrava, Czech Republic), specialized in the research field on Trypanosomatids. Here, basing on the molecular studies previously conducted on the two strains present in ER (Rugna *et al*., 2017), a new nested PCR to discriminate the two strains even in biological samples (not only in parasite cultures) was developed.

Concerning the results, of particular interest is the positivity for *L. infantum* of about a third of the examined roe deer at skin level; furthermore, most of them (10/11) harbored the strain (L) associated with HVL in the Emilia-Romagna region. This suggests that roe deer may represent a sylvatic reservoir of *L. infantum* in the region of interest, and in general for HVL, even if further data from surveys performed in other regions or Countries is required. This consideration is based on the fact that: (*i*) the parasite load, especially at skin level can reflect infectiousness in the natural life cycle (Courtenay *et al*., 2014); (*ii*) to serve as a reservoir, an animal species should have an infection prevalence over 20% (Roque and Jansen, 2014; Alemayehu and Alemayehu, 2017) - in our study 33% of roe deer tested positive (Magri *et al.*, 2022); (*iii*) the frequency of contacts with the vector has to be demonstrated (WHO, 2022) - and in ER a relevant presence of roe deer's blood was found in the phlebotomine population (Calzolari *et al*., 2022). Taken together, all these findings suggest a possible role of roe deer in the epidemiology of *L. infantum* in the study area, even if probative evidence should be based on xenodiagnosis (Quinnell and Courtenay, 2009).

The presence of L-strains was also observed in hedgehogs (3/4) and badgers (1/4). However, given the low number of samples examined, further studies will have to be conducted to establish whether these animal species can also represent possible reservoirs of *L. infantum*.

The role of synanthropic rodents, such as black rats, brown rats, and mice in the life cycle of *L. infantum* in Emilia-Romagna should be carefully evaluated, since they tested

positive for both strains. In Greece and in Portugal, mice have been suggested as a potential reservoir of *L. infantum* (Helhazar *et al*., 2013; Tsakmakidis *et al*., 2017), and in Italy brown rats from Montecristo Island, where no wild or domestic carnivores are present, were found positive for *L. infantum* (Zanet *et al*., 2014). The present study reports the presence of *L. infantum* in 11% of the rodents examined, even in two species never tested before in Italy, namely *M. musculus* and *R. norvegicus*. Based on our findings, the rodents tested were positive for both *L. infantum* strains. Even if their role as reservoir of *L. infantum* in Emilia-Romagna is not sufficiently supported by the results of these researches, the finding of specimens positive to the L strain in the province of Ravenna, where autochthonous HVL cases have not lately been reported (Santi *et al*., 2021), could suggest their possible involvement in the epidemiology of leishmaniasis given their abundance and synanthropic relations with humans.

Closing Remarks

Although the dog is considered the main reservoir of *L. infantum* causing HVL, different Countries are evaluating the potential role of other species in the epidemiology of this infection. In Europe the treatment of dogs and the use of prophylactic tools reduce the risk of transmission from dogs to humans, therefore the role of wild fauna should be carefully evaluated.

Considering the objectives of the current project, the following results were achieved, while taking into due consideration the critical issues already highlighted in General Discussion.

- The presence of *Leishmania* spp. was assessed in wild and synanthropic mammals collected nearby notified HVL cases or foci. The parasite was reported even in species never tested before in Italy, like *R. norvegicus* and *M. musculus*, or worldwide, like *C. capreolus*. Concerning roe deer, most of the positive specimens revealed the strain associated with HVL in Emilia-Romagna region. This, with the evidence of roe deer blood preferences of the sand flies from the same area is suggestive of their potential reservoir role, even though further studies should be aimed to assess if they can constitute a primary reservoir in the transmission to humans, to solve the conundrum of HVL in ER. The present study demonstrated the presence of *L. infantum* also in other wild and synanthropic mammals that could play a role in the transmission of the parasite in the ER. Also in this case, further studies are necessary to understand whether they can act as primary or secondary reservoir and give new insights on the epidemiological scenario of the ER (Magri *et al*., 2022a).
- Concerning synanthropic rodents, even though the prevalence of infection here observed (Magri *et al*., 2022b) is not suggestive of a reservoir role, its value is comparable to the one reported in other Italian areas, also in absence of carnivores. Due to the close relationship of rodents with humans and their ability to colonize new environments, making not negligible their potential role in the transmission of

leishmaniasis, especially in urban and peri-urban areas, they should be monitored even in non-endemic areas.

- During this PhD project a new nested PCR was developed thanks to the cooperation with the Life Science Research Centre of the University of Ostrava (CZ). This new assay is of great value because it can discriminate the two different strains L and S thanks to an indel region, without gene sequencing or parasite isolation, which can represent critical step especially in biological samples in not-optimal state of conservation collected from the territory (Magri *et al*., 2022a).
- Epidemiological data concerning the spread of non-pathogenic trypanosomes were collected and reviewed. On a general note, due to their scant pathogenic effects on the host, these species are more frequently reported as occasional findings during parasitological surveys not specifically focused on trypanosomes and/or during the molecular diagnosis of other strictly related pathogens, such as *Leishmania* spp. Although accidental, such findings can provide useful information on the current epidemiological distribution of trypanosomatids in different geographical areas and hosts, with relevant implications also for the improvement of diagnostics (Magri *et al*., 2021).
- Concerning the possible *Leishmania* spp. vectors other than sand flies, the current project reports the first finding of the protozoa in questing *Ixodes ricinus* ticks collected from rural environment. kDNA was found in unfed larvae, nymphs and males supporting the hypothesis that, in ticks, *Leishmania* spp. could have transstadial and transovarian transmission. Obviously, the percentage of positive specimens observed in this study is lower than the one reported in brown ticks collected from infected dogs (Dantas-Torres, 2011), that showed higher values than sand flies (Latrofa *et al*., 2018). Albeit phlebotomines are the only proven *Leishmania* efficient vectors, *I. ricinus* could have a minor role in the transmission of *L. infantum* in the Emilia-Romagna region, especially in a sylvatic or rural cycle (Magri *et al*., 2022c), although further and wider studies will be necessary to confirm this hypothesis.

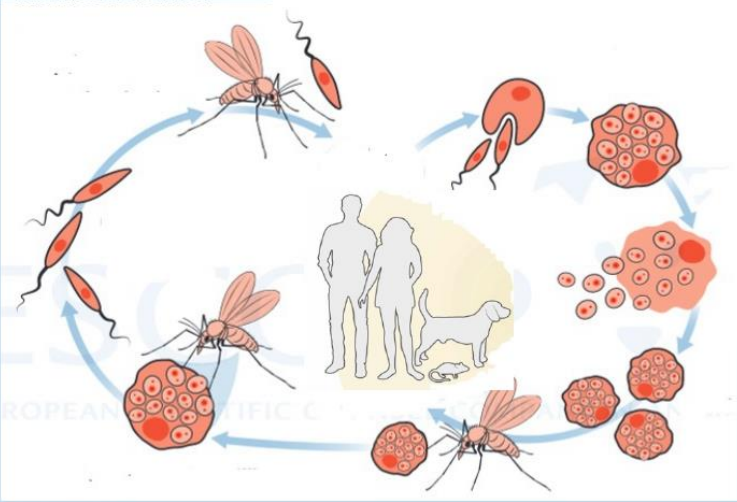
Despite this three-years project has been developed in a difficult period, due to the COVID pandemic, several attainments were achieved. The initial difficulties were overcome, developing new useful molecular tools, and giving new insights into the role of wild and peridomestic mammals in the epidemiology of *L. infantum*, likewise to other European and Italian studies. To finally solve the conundrum of the epidemiology of HVL in the Emilia-Romagna region, further studies should be aimed to test a more significant number of animal specimens, to understand the circulation of different strains of *L. infantum*, and to perform other tests, such as xenodiagnoses, aimed at determining the infectiousness of positive wild and peridomestic mammals to vector sand flies, assessing their role as a potential reservoir in the ecology of *L. infantum* transmission in the Emilia-Romagna region.

Appendix 1

Informative Flyer for Volunteer Park Rangers

Cos'è la leishmaniosi...

La Leishmaniosi è una malattia parassitaria sostenuta, in Italia, da *Leishmania infantum* che ha come ospite principale il cane, nel quale determina una malattia cronica ingravescente parassitologicamente incurabile, ma può colpire vari mammiferi incluso l'uomo.



Il parassita viene trasmesso tramite la puntura di flebotomi (pappataci) e, nell'ospite mammifero, si localizza nelle cellule del sistema immunitario.

I pappataci sono piccoli insetti (1-2 mm) che volano silenziosamente; solo le femmine si nutrono di sangue per poter deporre le uova, e pungono soprattutto al crepuscolo e durante le ore notturne. A differenza delle zanzare non depongono le uova in acqua stagnante, ma in crepe dei muri, del terreno, tra foglie o legni secchi.

Nel cane la Leishmaniosi è una malattia grave che determina sintomi generali molto variabili. Frequentemente è presente dermatite.

In Italia nell'uomo si parla di Leishmaniosi canina zoonotica (LCZ) in quanto nelle zone endemiche (Italia meridionale e isole) il cane è conosciuto come serbatoio (cioè la specie in grado di mantenere la malattia sul territorio per un lungo periodo di tempo), e può manifestarsi con forme cliniche differenti (viscerale o cutanea).

in Emilia-Romagna il cane non sembra essere l'unico responsabile del mantenimento della malattia sul territorio.

A tal proposito si sta indagando quale possa essere il ruolo delle specie selvatiche e peridomestiche nel ciclo di questo parassita. Le specie di interesse nello studio corrente sono in particolare i piccoli mammiferi come lepri, ratti, topi, scoiattoli, topi ragno o arvicole.

Il conferimento di carcasse da parte delle Guardie Ecologiche Volontarie presso il Dipartimento di Scienze Mediche Veterinarie potrà aiutare nella riuscita di questo progetto, per cercare di chiarire gli aspetti epidemiologici di questa complessa malattia nella nostra area geografica.

Di seguito alcune istruzioni mirate a raccogliere i campioni con attenzione ma senza intralciare eccessivamente il vostro lavoro.

- Compilare la scheda di rilevamento, ponendo particolare attenzione al luogo di ritrovamento della carcassa (se possibile inviare foto geolocalizzate o in alternativa segnare le coordinate del ritrovamento)
- A seconda delle dimensioni della carcassa rinvenuta, scegliere un sacchetto di dimensioni adeguate dove porre l'animale
- Una volta raccolta la carcassa chiudere con attenzione il sacchetto e unirlo alla scheda di rilevamento in modo che sia sempre possibile identificarla
- Riporre la carcassa in un congelatore avendo cura di accompagnarla alla scheda identificativa.

Il conferimento delle carcasse può essere eseguito dal lunedì al venerdì (dalle 9 alle 18) presso il Servizio di Malattie Trasmissibili e Sanità Pubblica Veterinaria, Dipartimento di Scienze Mediche Veterinarie, Via Tolara di Sopra 50, Ozzano Emilia (BO), avvisandoci cortesemente per via telefonica o per email; in alternativa potremo venire noi presso la sede GEV di San Lazzaro di Savena a ritirare le carcasse rinvenute previ accordi con le stesse modalità.

Per ulteriori chiarimenti o per il conferimento delle carcasse è possibile utilizzare questi contatti:

Laboratorio di Parassitologia: 0512097056

Alice Magri 3207770784
alice.magri3@unibo.it

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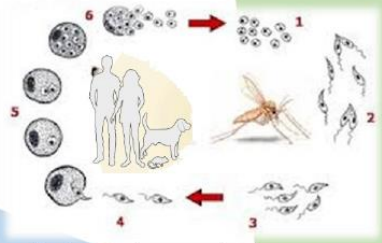
benedetto.morandi2@unibo.it

Appendix 2

Informative Flyer for Ungulate Selection Hunters

Dipartimento di Scienze Mediche Veterinarie – Università di Bologna
Servizio di Malattie Trasmissibili e Sanità Pubblica Veterinaria

Cos'è la leishmaniosi...



La Leishmaniosi è una malattia parassitaria degli animali e dell'uomo sostenuta, in Italia, dal protozoo *Leishmania infantum* che viene trasmesso da insetti vettori (flebotomi o pappataci).

I pappataci sono piccoli insetti (1-2 mm) che volano silenziosamente; solo le femmine si nutrono di sangue per poter deporre le uova, e pungono soprattutto al crepuscolo e durante le ore notturne. A differenza delle zanzare non depongono le uova in acqua stagnante, ma in crepe dei muri, del terreno, tra foglie o legni secchi.

La malattia si presenta nell'uomo con due forme cliniche principali: la leishmaniosi viscerale (LV), una malattia grave che porta a dimagrimento, ingrossamento di milza, fegato e linfonodi e che può essere fatale se non curata adeguatamente e la leishmaniosi cutanea (LC), una forma benigna, talvolta deturpante, con tendenza spontanea alla guarigione.

Nelle zone endemiche dell'Italia (Italia meridionale e isole) il cane è riconosciuto come serbatoio (cioè la specie in grado di mantenere la il prassita sul territorio) e in questo animale la malattia può manifestarsi contemporaneamente con forme cliniche differenti (viscerale e cutanea).

Nella nostra regione, dopo il focolaio descritto negli anni '70 nella zona pedecollinare tra Imola e Bologna, si è assistito ad un allarmante incremento dei casi e dell'areale di distribuzione; recenti indagini condotte in Emilia-Romagna hanno evidenziato differenze genetiche tra i ceppi parassitari isolati dall'uomo rispetto a quelli isolati dal cane nella stessa zona rendendo necessari ulteriori studi sulla fauna del territorio per comprendere quale specie possa fungere da serbatoio.

In Emilia-Romagna il cane quindi non sembra essere l'unico responsabile del mantenimento della malattia sul territorio.

Analisi sui pasti di sangue eseguiti dai flebotomi hanno rilevato la forte presenza di DNA di ruminanti selvatici, come ad esempio caprioli e cervi suscitando un forte interesse per cercare di comprendere quale ruolo possano svolgere queste specie nel ciclo del parassita *Leishmania infantum*.

COSA FACCIAMO

Nell'ambito di una più ampia indagine sui possibili serbatoi alternativi al cane, si sta indagando quale possa essere il ruolo svolto anche dagli ungulati selvatici nel ciclo di questo parassita.

COSA FARE PER AIUTARCI

Il conferimento di campioni di organi o tessuti di ungulati selvatici da parte dei Biorilevatori dell'ATC presso il Dipartimento di Scienze Mediche Veterinarie potrà aiutare nella riuscita di questo progetto, per cercare di chiarire gli aspetti epidemiologici di questa complessa malattia nella nostra area geografica.

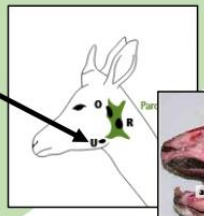
COME

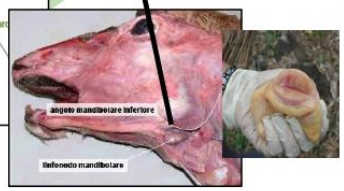
1) - Annotare luogo di abbattimento sulla scheda di rilevamento;

2) - Rimuovere:

- un orecchio
- una porzione di milza
- una porzione di fegato
- un pezzetto dei muscoli della guancia e un linfonodo della testa

....e riporli, ciascuno separatamente, negli appositi sacchetti che vi verranno forniti;





3) - rimuovere il coagulo di sangue dal cuore e riporlo nel provettone dato in dotazione;

4) - Tutti i singoli campioni potranno poi essere messi, assieme alla scheda di rilevamento, in un unico sacchetto e il tutto dovrà essere conservato in congelatore fino al conferimento.

Per il conferimento dei campioni o per dubbi/chiarimenti contattare:
Laboratorio di Parassitologia 051 2097056
Dott.ssa Alice Magri 320 7770784

General References

Abbate J.M., Arfuso F., Napoli E., Gaglio G., Giannetto S., Latrofa M.S., Otranto D., Brianti E. (2019). *Leishmania infantum* in wild animals in endemic areas of southern Italy. Comp Immunol Microbiol Infect Dis, 67: 101374. https://doi.org/10.1016/j.cimid.2019.101374.

Agostinoni C., Dorigoni N., Malfitano A., Caggese L., Marchetti G., Corona S., Gatti S., Scaglia M. (1997). Mediterranean Leishmaniasis in HIV-infected patients: epidemiological, clinical, and diagnostic features of 22 cases. Infections, 26(2): 93-99.

Akhoundi M., Kuhls K., Cannet A., Votýpka J., Marty P., Delaunay P, Sereno D. (2016). A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis, 10(3): e0004349. https://doi.org/10.1371/journal.pntd.0004349.

Akhoundi M., Downing T., Votýpka J., Kuhls K., Lukeš J., Cannet A., Ravel C., Marty P., Delaunay P., Kasbari M., Granouillac B., Gradoni L., Sereno D. (2017). *Leishmania* infection: Molecular targets and Diagnosis. Mol Aspects Med, 57: 1-29. http://dx.doi.org/10.1016/j.mam.2016.11.012.

Alam M. Z., Haralambous C., Kuhls K., Gouzelou E., Sgouras D., Soteriadou K., Schnur L., Pratlong F., Schönian G. (2009). The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes infect, 11: 707–715. https://doi.org/10.1016/j.micinf.2009.04.009.

Alvar J., Cañavate C., Molina R., Moreno J., Nieto J. (2004). Canine leishmaniasis. Adv Parasitol, 57: 1-88. https://doi.org/10.1016/S0065-308X(04)57001-X.

Alvar J., Yactayo S., Bern C. (2006). Leishmaniasis and poverty. TRENDS Parasitol, 22(12): 552-557. https://doi.org/10.1016/j.pt.2006.09.004.

Aluru S., Hide M., Michel G., Bañuls A.L., Marty P., Pomares C. (2015). Multilocus microsatellite typing of *Leishmania* and clinical applications: a review. Parasite, 22(16). https://doi.org/110.1051/parasite/2015016.

Amro A., Al-Dwibe H., Gashout A. (2017). Spatiotemporal and molecular epidemiology of cutaneous leishmaniasis in Libya PLoS Negl Trop Dis, 11: e0005873. https://doi.org/10.1371/journal.pntd.0005873.

Antinori S., Calattini S., Longhi E., Bestetti G., Piolini R., Magni C., Orlando G., Gramiccia M., Acquaviva V., Foschi A., Corvasce S., Colomba C., Titone L., Parravicini C., Cascio A., Corbellino M. (2007). Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. Clin Infect Dis, 44(12): 1602-10. https://doi.org/10.1086/518167.

Ashford R.W. (1996). The leishmaniasis reservoirs and their significance in control. Int J Parasitol, 30: 1269-1281. https://doi.org/10.1016/0738-081x(96)00041-7.

Artusi T., Grossi G. (1962). Kala-azar. Osservazioni diagnostico differenziali e terapeutiche in un caso di leishmaniosi viscerale dell'adulto. Minerva Medica, 53: 3593-3596. [Article in Italian]

Aureli S., Galuppi R., Ostanello F., Foley J.E., Bonoli C., Rejmanek D., Rocchi G., Orlandi E., Tampieri M.P. (2015) Abundance of questing ticks and molecular evidence for pathogens in ticks in three parks of Emilia-Romagna region of Northern Italy. Ann Agric Environ Med, 22(3): 459–466. https://doi.org/10.5604/12321 966.1167714.

Baldelli R., Piva S., Salvatore D., Parigi M., Melloni O., Tamba M., Bellini R., Poglayen G. (2011). Canine leishmaniasis surveillance in a northern Italy kennel. Vet Parasitol, 179: 57-61. https://doi.org/10.1016/j.vetpar.2011.01.052.

Baneth G., Koutinas A.F., Solano-Galego L., Bourdeau P., Ferrer L. (2008). Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part one. Trends Parasitol, 24(7): 324-330. https://doi.org/10.1016/j.pt.2008.04.001.

Bangert M., Flores-Chávez M.D., Llanes-Acevedo I.P., Arcones C. Chicharro C. García E., Ortega S., Nieto J., Cruz I. (2018). Validation of rK39 immunochromatographic test and direct agglutination test for the diagnosis of Mediterranean visceral

leishmaniasis in Spain. PLoS Negl Trop Dis, 12: e0006277. https://doi.org/10.1371/journal.pntd.0006277.

Bañuls A.L., Hide M., Prugnolle F. (2007). *Leishmania* and the leishmaniasis: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol, 64: 1-109. https://doi.org/10.1016/S0065-308X(06)64001-3.

Barroso-Freitas A.P., Passos, S.R., Mouta-Confort, E., Madeira, M.F., Schubach, A.O., Santos, G.P., Nascimento, L.D., Marzochi, M.C., Marzochi, K.B. (2009) Accuracy of an ELISA and indirect immunofluorescence for the laboratory diagnosis of American tegumentary leishmaniasis. Trans R Soc Tro Med Hyg, 103: 383–389. https://doi.org/10.1016/j.trstmh.2008.12.019.

Battisti E., Zanet S., Khalil S., Trisciuoglio A., Hertel B., Ferroglio E. (2020). Molecular survey on vector-borne pathogens in alpine wild carnivorans. Front Vet Sci, 7:1. https://doi.org/10.3389/fvets.2020.00001.

BAYER (2020). La Leishmaniosi sempre più diffusa. (Flyer). [Article in Italian]

Becker D.J., Seifert S.N., Carlson C.J. (2020). Beyond infection: integrating competence into reservoir host prediction. Trends Ecol Evol, 35(12): 1062-1065. https://doi.org/10.1016/j.tree.2020.08.014.

Bern C., Maguire J.H., Alvar J. (2008). Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis, 2: e313. https://doi.org/10.1371/journal.pntd.0000313.

Bettini S., Pozio E., Gradoni L. (1980). Leishmaniasis in Tuscany (Italy): (II) *Leishmania* form wild Rodentia and Carnivora in a human and canine leishmaniasis focus. Trans R Soc Trop Med Hyg, 74(1): 77-83. https://doi.org/10.1016/0035-9203(80)90015-2.

Bevere L., Tobia A. (1947). La Leishmaniosi in Italia. Not Amm San, 3(1): 160-180. [Article in Italian]

Brittingham A., Morrison C.J., McMaster W.R., McGwire B.S., Chang K.P., Mosser D.M. (1995). Role of the *Leishmania* surface protease gp63 in complement fixation,

cell adhesion, and resistance to complement-mediated lysis. J Immunol, 155: 3102-3111.

Burnham A., Ordeix L., Alcover M.M., Martínez-Orellana P., Montserrat-Sangrà S., Willem L., Spitzova T., Volf P., Solano-Gallego L. (2020). Exploring the relationship between susceptibility to canine leishmaniosis and anti-*Phlebotomus perniciosus* saliva antibodies in Ibizan hounds and dogs of other breeds in Mallorca, Spain. Parasites Vectors, 13: 129. https://doi.org/10.1186/s13071-020-3992-8.

Calzolari M., Rugna G., Clementi E., Carra E., Pinna M., Bergamini F., Fabbi M., Dottori M., Sacchi L., Votýpka J. (2018) Isolation of a Trypanosome Related to *Trypanosoma theileri* (Kinetoplastea: Trypanosomadidae) from *Phlebotomus perfiliewi* (Diptera: Pychodidae). BioMed Res Int: 8p. https://doi.org/10.1155/2018/2597074.

Calzolari M., Romeo G., Bergamini F., Dottori M., Rugna G., Carra E. (2022). Host preference and *Leishmania infantum* natural infection of the sand fly *Phlebotomus perfiliewi* in northern Italy. Acta Trop, 226: 106246. https://doi.org/10.1016/j.actatropica.2021.106246.

Capelli G., Baldelli R., Ferroglio E., Genchi C., Gradoni L., Gramiccia M., Maroli M., Mortarino M., Pietrobelli M., Rossi L., Ruggiero M. (2004). Monitoring of canine leishmaniasis in northern Italy: an update from a scientific network. Parassitologia, 46(1-2):193-7. [Article in Italian]

Cardoso L., Schallig H., Persichetti M.F., Pennisi M.G. (2021). New epidemiological aspects of animal leishmaniosis in Europe: the role of vertebrate hosts other than dogs. Pathogens, 10: 307. https://doi.org/10.3390/pathogens10030307.

Casolari C., Guaraldi G., Pecorari M., Tamassia G., Cappi C., Fabio G., Cesinaro A.M., Piolini R., Rumpianesi F., Presutti L. (2005). A rare case of localized mucosal leishmaniasis due to *Leishmania infantum* in an immunocompetent Italian host. Eur J Epidemiol, 20: 559-561. https://doi.org/10.1007/s10654-005-1249-7.

Castelli G., Galante A., Lo Verde V., Migliazzo A., Reale S., Lupo T., Piazza M., Vitale F., Bruno F. (2014). Evaluation of two modified culture media for *Leishmania*

infantum cultivation versus different culture media. J Parasitol, 100(2): 228-230. https://doi.org/ 10.1645/13-2.

Castelli G., Bruno F., Caputo V., Fiorella S., Sammarco I., Lupo T., Migliazzo A., Vitale F., Reale S. (2020). Genetic tools discriminate strains of *Leishmania infantum* isolated from humans and dogs in Sicily, Italy. PLoS Negl Trop Dis, 14(7): e0008465. https://doi.org/10.1371/journal.pntd.0008465.

Cavalcanti D.P., de Souza W. (2018). The kinetoplast of Tripanosomatids: from early studies of electron microscopy to recent advances in atomic force microscopy. Hindawi, 2018: 960351. https://doi.org/10.1155/2018/9603051.

Ceccarelli M., Galluzzi L., Migliazzo A., Magnani M. (2014). Detection and characterization of *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green based real-time PCR and high-resolution melt analysis targeting kinetoplast minicircle DNA. PLoS One, 9: e88845. https://doi.org/10.1371/journal.pone.0088845.

Ceccarelli M., Galluzzi L., Diotallevi A., Gasparini E., Migliazzo A., Magnani M. (2016) The relevance of molecular diagnosis in a dog vaccinated against leishmaniasis. Vet Med Anim Sci: 4. https://doi.org/10.7243/2054-3425-4-4.

Center for Disease Control and Prevention (CDC). Leishmaniasis. https://www.cdc.gov/parasites/leishmaniasis/biology.html. (accessed online 06th September 2022).

Cesinaro A.M., Nossier S., Mataca E., Mengoli M.C., Cavatorta C., Gennari W. (2017). An outbreak of cutaneous leishmaniasis in Modena province (Northern Italy): report of 35 cases. Pathologica, 109: 363-367.

Chakravarty J., Hasker E., Kansai S., Singh O.P., Malaviya P., Singh A.K., Chourasia A., Singh T., Sudarshan M., Singh A.P., Singh B., Singh R.P., Ostyn B., Fakiola M., Picado A., Menten J., Blackwell J.M., Wilson M.E., Sacks D., Boelaert M., Sundar S. (2019). Determinants for progression from asymptomatic infection to symptomatic visceral leishmaniasis: a cohort study. PLoS Negl Trop Dis, 13(3): e0007216. https://doi.org/10.1371/journal.pntd.0007216.

Christodoulou V., Antoniou M., Ntais P., Messaritakis I., Ivovic V., Dedet J.P., Pratlong F., Dvorak V., Tselentis Y. (2012). Re-emergence of visceral and cutaneous leishmaniasis on the Greek island of Crete. Vector Borne Zoonotic Dis, 12(3): 214-22. https://www.doi.org/10.1089/vbz.2011.0004.

Corradetti A. (1960). I Focolai di italiani di Kala Azar e il problema della leishmaniosi nel sud Europa. Parassitologia. 2(1-2): 95-98. [Article in Italian]

Courtenay O., Carson C., Calvo-Bado L., Garcez L.M., Quinnell R.J. (2014). Heterogeneities in *Leishmania infantum* infection: using skin parasite burdens to identify highly infectious dogs. PLoS Negl Trop Dis, 8: e2583. https://doi.org/10.1371/journal.pntd.0002583.

Dantas-Torres (2007). The role of dogs as reservoir of *Leishmania* parasites with emphasis of *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *braziliensis*. Vet Parasitol, 149: 139-146. https://www.doi.org/10.1016/j.vetpar.2007.07.007.

Dantas-Torres (2011). Ticks as vectors of *Leishmania* parasites. Trends Parasitol, 27(4): 155-159. https://doi.org/10.1016/j.pt.2010.12.006.

David C.V., Craft N. (2009). Cutaneous and mucocutaneous leishmaniasis. Dermatol Ther, 22(6): 491-502. https://doi.org/10.1111/j.1529-8019.2009.01272.x.

Denise H., McNeil K., Brooke D.R., Alexander J., Coombs G.H., Mottram J.C. (2003). Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence in vivo. Infect Immun, 71(6): 3190-3195. https://www.doi.org/10.1128/IAI.71.6.3190–3195.2003.

Deplazes P., Eckert J., Mathis A., von Samson-Himmelstierna G., Zahner H. (2016). Genus Leishmania. In Parasitology in Veterinary Medicine, 1st edition, Wageningen Academic Pub, The Netherlands. https://doi.org/10.3920/978-90-8686-274-0.

Di Cristina G., Caronia G. (1915). Sulla terapia della leishmaniosi interna. Pathologica, 7: 82-83. [Article in Italian]

Di Muccio T., Scalone A., Bruno A., Marangi M., Grande R., Armignaccio O., Gradoni L., Gramiccia M. (2015). Epidemiology of imported Leishmaniasis in Italy: implications for a European Endemic Country. PLoS ONE, 10(6): e0129418. https://doi.org/10.1371/journal.pone.0129418.

di Pietro S., Bosco V.R.F., Crinò C., Francaviglia F., Giudice E. (2016). Prevalence, type, and prognosis of ocular lesions in shelter and owns-client dogs naturally infected by *Leishmania infantum*. Vet World, 9(6): 633-637. https://doi.org/10.14202/vetworld.2016.633-637.

Díaz-Sáez V., Merino-Espinosa G., Morales-Yuste M., Corpas-López V., Pratlong F., Morillas-Márquez F., Martín-Sánchez J. (2014). High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: Epidemiological consequences. Vet Parasitol, 202: 119–127.

Dipineto L., Manna L., Baiano A., Gala M., Fioretti A., Gravino A.E., Menna L.F. (2007). Presence of *Leishmania infantum* in red foxes (*Vulpes vulpes*) in southern Italy. J Wildl Dis, 43(3): 518-520. https://doi.org/10.7589/0090-3558-43.3.518.

Donovan C. (1903). On the possibility of the occurrence of trypanosomiasis in India. Natl Med J India, 7(4): 201-202.

Dvorak V., Shaw J., Volf P. (2018). Parasite Biology: The Vector. In: Bruschi F, Gradoni L, (eds.) The leishmaniases: old neglected tropical diseases. Springer, Berlin, Germany, pp. 31-77. https://doi.org/10.1007/978-3-319-72386-0.

Elmahallawy E.K., Sampedro Martinez A., Rodrigues-Granger J., Hoyos- Mallecot Y. Agil A., Navarro Mari J.M., Gutierrez Fernandez J. (2014) Diagnosis of leishmaniasis. J Infect Dev Ctries, 8: 961–972. https://doi.org/10.3855/jidc.4310.

European Center for Disease Control and Prevention (2022). Surveillance, prevention and control of leishmaniases in the European Union and its neighbouring countries. Stockholm: ECDC. https://doi.org/10.2900/823484.

Europen Medicines Agency (2016). CaniLeish © - EMEA/V/C/002232 - R/0004. www.ema.europa.eu.

Europen Medicines Agency (2016). Letifend © - EMEA/V/C/003865 - IB/0027. www.ema.europa.eu.

Feliciangeli M.D. (2004). Natural breeding places of phlebotomine sandflies. Med Vet Entomol, 18: 71-81. https://doi.org/10.1111/j.0269-283X.2004.0487.x.

European Scientific Counsel Companion Animal Parasites – ESCCAP (2016). Leishmaniasis. In: Control of vector borne diseases in dog and cat. Malvern Worcestershire, United Kingdom. Third Edition; pp: 9-18.

Fenwick A. (2012). The global burden of neglected tropical diseases. Public Health, 126: 233-236. https://doi.org/10.1016/j.puhe.2011.11.015.

Fernandes O., Murthy V.K., Kurath U., Degrave W.M., Campbell D.A. (1994). Mini-exon gene variation in human pathogenic *Leishmania* species. Mol Biochem Parasitol, 66: 261-271. https://doi.org/10.1016/0166-6851(94)90153-8.

Fernández-Arévalo A., El Baidouri F., Ravel C., Ballart C., Abras A., Lachaud L., Tebar S., Lami P., Pratlong F., Gállego M., Muñoz C. (2020). The *Leishmania donovani* species complex: A new insight into taxonomy. Int J Parasitol, 50: 1079-1088. https://doi.org/10.1016/j.ijpara.2020.06.013.

Franceschini E., Puzzolante C., Menozzi M., Rossi L., Bedini A., Orlando G., Gennari W., Meacci M., Rugna G., Carra E., Codeluppi M., Mussini C. (2016). Clinical and microbiological characteristics of visceral leishmaniasis outbreak in a northern Italian Nonendemic area: A retrospective observational study. Biomed Res Int, 6481028. http://dx.doi.org/10.1155/2016/6481028.

Fuehrer H.P., Savić S. (2017). Editorial: Vectors and VBDs. Mol Cell Probes, 3(1). https://doi.org/10.1016/j.mcp.2017.01.001.

Fusaroli A. (1952). Primo caso autoctono di leishmaniosi viscerale verificatosi nel comune di Forlì. Comunicazione a Convegno Interregionale dell'Associazione Italiana per l'Igiene, Bologna 20/07/1952: 6-17. [Article in Italian]

Gabbi U. (1911). Malattie tropicali dell'Italia meridionale e delle isole, 2 vol. Messina. [Article in Italian]

Galluzzi L., Ceccarelli M., Diotallevi A., Menotta M., Magnani M (2018). Real-time PCR applications for diagnosis of leishmaniasis. Parasites vectors, 11: 273. https://doi.org/10.1186/s13071-018-2859-8.

Gasparri V., Ortalli M., Foschi M.P., Baldovi C., Lanzoni A., Cagarelli R., Gaibani P., Rossini G., Vocale C., Tigani R., Gentilomi G.A., Misciali C., Pesci S., Patrizi A., Landini M.P., Varani S. (2017). New evidence of cutaneous leishmaniasis in north-eastern Italy. J Eur Acad Dermatol Venereol, 31: 1534-1540. https://doi.org/10.1111/jdv.14309.

Geoportale Emilia-Romagna. https://geoportale.regione.emilia-romagna.it. (accessed online 30th August 2022).

Girolami M. (1948). La Leishmaniosi Viscerale in Italia. Comunicazione a Congresso Italiano di Medicina ed Igiene Tropicale e Subtropicale, 37-38: 528-530. [Article in Italian]

Giungi F. (1954). Primo caso di leishmaniosi viscerale dell'adulto autoctona in Bologna. Parassitologia, XIV(4): 2-9. [Article in Italian]

Gizzarelli M., Fiorentino E., Ben Fayala N.E.H, Montagnaro S., Torras R., Gradoni L., Oliva G. Foglia Manzillo V. (2020). Assessment of Circulating Immune Complexes During Natural and Experimental Canine Leishmaniasis. Front Vet Sci, 7: 273. https://doi.org/10.3389/fvets.2020.00273.

Gradoni L. (2018). A brief introduction to Leishmaniasis epidemiology. In: Bruschi F, Gradoni L, (eds.) The leishmaniases: old neglected tropical diseases. Springer, Berlin, Germany, pp. 1-13. https://doi.org/10.1007/978-3-319-72386-0.

Gradoni L., Gramiccia M. (2004). Sarcomastigophora. In: de Carneri (ed.) Parassitologia generale ed umana. Casa Editrice Ambrosiana, Milano, Italy, pp: 158-180.

Gradoni L., Ferroglio E., Zanet S., Mingone W., Venco L., Bongiorno G., Fiorentino E., Cassini R., Grillini M., Simonato G., Michelutti A., Montarsi F., Natale A., Gizzarelli M., Foglia Manzillo V., Solari Basano F., Nazzari R., Melideo O., Gatti D., Olivia G. (2022). Monitoring and detection of new endemic foci of canine leishmaniosis in

northern continental Italy: An update from a study involving five regions (2018–2019). Vet Parasitol: Reg Stud Rep, 27: 100676. https://doi.org/10.1016/j.vprsr.2021.100676.

Gramiccia M., Scalone A., Di Muccio T., Orsini S., Fiorentino E., Gradoni L. (2013). The burden of visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual epidemic that occurred from 1989 to 2009. Euro Surveill, 18(29): 20535. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20535.

Gramiccia M., di Muccio T. (2018). Diagnosis. In: Bruschi F, Gradoni L, (eds.) The leishmaniases: old neglected tropical diseases. Springer, Berlin, Germany, pp. 137-168. https://doi.org/10.1007/978-3-319-72386-0.

Garcia L., Kindt A., Bermudez H., Llanos-Cuentas A., De Doncker S., Arévalo J., Quispe Tintaya K. W., Dujardin J. C. (2004). Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J Clin Microbiol, 42: 2294-2297. https://doi.org/10.1128/JCM.42.5.2294–2297.2004.

Guerbouj S., Victoir K., Guizani I., Seridi N., Nuwayri-Salti N., Belkaid M., Ben Ismail R., le Ray D., Dujardin J.C. (2001). Gp63 gene polymorphism and population structure of *Leishmania donovani* complex: influence of the host selection pressure? Parasitology, 122: 25-35. https://doi.org/10.1017/s0031182000007125.

Haydon D.T., Cleaveland S., Taylor L.H., Laurenson M.K. (2002). Identify reservoirs of infection: A conceptual and practical challenge. Emerg Infect Dis, 8(12): 1468-1473. https://doi.org/10.3201/eid0812.010317.

Hassan M.D.Q., Das S., Adhya S. (1992). Mini-exon derived RNA gene of *Leishmania donovani*: structure, organization and expression. J Biosci, 17(1): 55-66. https://doi.org/10.1007/BF02716774.

Helhazar M., Leitão J., Duarte A., Tavares L., da Fonseca I.P. (2013). Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania*

infantum in Sesimbra and Sintra – Portugal. Parasites Vectors, 6: 88. https://doi.org/10.1186/1756-3305-6-88.

Hide M., Bañuls A.L. (2006). Species-specific PCR assay for *L. infantum*/*L. donovani* discrimination. Acta Trop, 100: 241-245. https://doi.org/10.1016/j.actatropica.2006.10.012.

Hide M., Bras-Gonçalves R., Bañuls A.L. (2007). Specific *cpb* copies within the *Leishmania donovani* complex: evolutionary interpretation and potential clinical implications in humans. Parasitol, 134: 379-389. https://doi.org/10.1017/S0031182006001600.

Inceboz T. (2019). Epidemiology and Ecology of Leishmaniasis. In: Rodriguez-Morales A.J. (eds.) Current Topics in Neglected Tropical Diseases. IntechOpen, London. https://www.intechopen.com/chapters/67175.

Iori A., Di Giulio A., De Felici S. (2005). Zecche d'Italia. In: Cringoli G., Iori A., Rinaldi L., Veneziano V., Genchi C. (eds.). Mappe parassitologiche: Zecche. Rolando Editore, Napoli, pp: 52–163.

Killick-Kendrick R. (1999). The Biology and Control of phlebotomine sand flies. Clin Dermatol, 17(3): 279-89. https://doi.org/10.1016/s0738-081x(99)00046-2.

Kumar R., Nylén S. (2012). Immunobiology of visceral leishmaniasis. Front Immunol, 6: 251. https://doi.org/10.3389/fimmu.2012.00251.

Jarne P., Lagoda P.J. (1996). Microsatellites, from molecules to populations and back. Trends Ecol Evol, 11: 424–429. https://doi.org/10.1016/0169-5347(96)10049-5.

Latrofa M.S., Iatta R., Dantas-Torres F., Annoscia G., Gabrielli S., Pombi M., Gradoni L., Otranto D. (2018). Detection of *Leishmania infantum* DNA in phlebotomine sand flies from ad area where canine leishmaniosis is endemic in southern Italy. Vet Parasitol, 253: 39–42. https://doi.org/10.1016/j.vetpar.2018.02.006.

Laveran A., Mensil F. (1904). On a new protozoon (*Piroplasma donovani*, Laveran and Mensil), the parasite on an Indian Fever. BMJ Military Health, 2(2): 216-218.

Leishman W.B. (1903). On the possibility of the occurrence trypanosomiasis in India. Br Med J, 1(2213): 1252–1254.

Lieke T., Nylén S., Eidsmo L., McMaster W.R., Mohammadi A.M., Khamesipour A., Berg L., Akuffo H. (2008). *Leishmania* surface protein gp63 binds directly to human natural killer cells and inhibits proliferation. Clin Exp Immunol, 153: 221-230. https://doi.org/10.1111/j.1365-2249.2008.03687.x.

Lindoso J.A.L., Moreira C.H.V., Cunha M.A., Queiroz I.T. (2018). Visceral leishmaniasis and HIV coinfection: current perspectives. HIV AIDS (Auckl). 10: 193-201. https://doi.org/10.2147/HIV.S143929.

Llanes A, Restrepo CM, Lleonart R. (2018). ViannaTopes: a database of predicted immunogenic peptides for *Leishmania* (*Viannia*) species. Database (Oxford), bay111. https://doi.org/10.1093/database/bay111.

Lukeš J., Guilbride D.L., Votýpka J., Zíková, Benne R., Englund P.T. (2002). Kinetoplast DNA network: Evolution of an improbable structure. Eukaryot Cell, 1(4): 495-502. https://doi.org/10.1128/EC.1.4.495–502.2002.

Lukeš J., Hashimi H., Ziková A. (2005). Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. Curr Genet, 48: 277-299. https://doi.org/10.1007/s00294-005-0027-0.

Machado G.U., Prates F.V., Machado P.R.L. (2019). Disseminated leishmaniasis: clinical, pathogenic, and therapeutic aspects. An Bras Dermatol, 94(1): 9-16. http://dx.doi.org/10.1590/abd1806-4841.20198775.

Madeddu G., Fiori M.L., Ena P., Riu F., Lovigu C., Nunnari G., Bagella P., Maida I., Badudieri S., Mura M.S. (2014). Mucocutaneous leishmaniasis as presentation of HIV infection in Sardinia, insular Italy. Parasitol Int, 63: 35-36. http://dx.doi.org/10.1016/j.parint.2013.10.002.

Magri A., Galuppi R., Fioravanti M. (2021). Autochthonous *Trypanosoma* spp. in European Mammals: A Brief Journey amongst the Neglected Trypanosomes. Pathogens, 10(3): 334. https://doi.org/10.3390/pathogens10030334.

Magri, A., Bianchi, C., Chmelová, L., Caffara, M., Galuppi, R., Fioravanti, M., Yurchenko, V., Kostygov, A.Y. (2022a). Roe deer (*Capreolus capreolus*) are a novel

potential reservoir for human visceral leishmaniasis in the Emilia-Romagna region of northeastern Italy. Int J Parasitol, https://doi.org/10.1016/j.ijpara.2022.09.002.

Magri A., Galuppi R., Fioravanti M., Caffara M. (2022b). Survey on the presence of *Leishmania* sp. in peridomestic rodents from Emilia-Romagna region (North-Eastern Italy). Vet Res Commun, 2022, https://doi.org/10.1007/s11259-022-09925-4.

Magri A., Caffara M., Fioravanti M., Galuppi R. (2022c) Detection of *Leishmania* sp. kDNA in questing *Ixodes ricinus* (Acari, Ixodidae) from the Emilia-Romagna Region in northeastern Italy. Parasitol Res, 121(11): 3331-3336. https://doi.org/10.1007/s00436-022-07655-9.

Manilla G. (1998). Fauna d'Italia. Acari Ixodida. Edizioni Calderini, Bologna, Italy. [Book in Italian]

Mantovani M. (1915). La leishmaniosi cutanea a Ravenna. Pathologica, 7(150): 57-60. [Article in Italian]

Marcondes M., Biondo A.W., Gomes A.A.D., Silva A.R.S., Vieira R.F.C., Camacho A.A., Quinn J., Chandrashekar R. (2011). Validation of a *Leishmania infantum* ELISA rapid test for serological diagnosis of *Leishmania chagasi* in dogs. Vet Parasitol, 175: 15-19. https://doi.org/10.1016/j.vetpar.2010.09.036.

Maroli M., Khoury C., Bianchi R., Ferroglio E., Natale A. (2002). Recent findings of *Phlebotomus neglectus* Tonnoir, 1921 in Italy and its western limit of distribution. Parassitologia, 44(1-2): 103-9.

Maroli M., Gradoni L., Oliva G., Castagnaro M., Crotti A., Lubas G., Paltrinieri S., Roura X., Zatelli A., Zini E. (2009): Leishmaniosi canina: linee guida su diagnosi, stadiazione, terapia, monitoraggio e prevenzione. Parte III: Prevenzione. Veterinaria, 23(4): 19-26.

Maroli M., Pennisi M.G., di Muccio T., Khoury C., Gradoni L., Gramiccia M. (2007). Infection of sandflies by a cat naturally infected with *Leishmania infantum*. Vet Parasitol, 145: 357-360. https://doi.org/10.1016/j.vetpar.2006.11.009.

Maroli M., Rossi L., Baldelli R., Capelli G., Ferroglio E., Genchi C., Gramiccia M., Mortarino M., Pietrobelli M., Gradoni L. (2008). The northward spread of

leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health, 13(2): 256-264. https://doi.org/10.1111/j.1365-3156.2007.01998.x.

Marquardt W.C., Demaree R.S., Grieve R.B. (2000). Parasitology & Vector Biology. Academic Press, San Diego, Second Edition, pp: 39-59.

Martinotti L. (1952). Problemi attinenti alla Leishmaniosi cutanea. Accademia delle Scienze, pp: 58-63.

Maurício I.L. (2018). *Leishmania* taxonomy. In: Bruschi F, Gradoni L, (eds.) The leishmaniases: old neglected tropical diseases. Springer, Berlin, Germany, pp. 15-30. https://doi.org/10.1007/978-3-319-72386-0.

Maurício I.L., Stothard J.R., Miles M.A. (2000). The strange case of *Leishmania chagasi*. Parasitol Today, 16(5): 188-189. https://doi.org/10.1016/s0169-4758(00)01637-9.

McGwire B.S., Satoskar A.R. (2014). Leishmaniasis: clinical syndromes and treatment. Q J Med, 107: 7-14. https://doi.org/10.1093/qjmed/hct116.

Mendoza-Roldan J., Benelli G., Panarese R., Iatta R., Furlanello T. Beugnet F., Zatelli A., Otranto D. (2020). *Leishmania infantum* and *Diofilaria immitis* infections in Italy, 2009-2019: changing distribution patterns. Parasites Vectors, 13: 193. https://doi.org/10.1186/s13071-020-04063-9.

Merino-Espinosa G., Corpas-López V., Morillas-Márquez F., Díaz-Sáez V., Martín-Sánchez J. (2016). Genetic variability and infective ability of the rabbit trypanosome, *Trypanosoma nabiasi* Railliet 1895, in southern Spain. Infect Genet Evol, 45: 98–104. https://doi.org/10.1016/j.meegid.2016.08.028.

Messner M., Kayikci F.N., Shahi-Barogh B., Harl J., Messner C., Fuehrer H.P. (2019). Screening of wild ruminants from the Kaunertal and other alpine regions of Tyrol (Austria) for vector-borne pathogens. Parasitol Res. 118:2735-2740. https://doi.org/10.1007/s00436-019-06412-9.

Michelutti A., Toniolo F., Bertola M., Grillini M., Simonato G., Ravagnan S., Montarsi F. (2021). Occurrence of Phlebotomine sand flies (Diptera: Psychodidae) in the

northeastern plain of Italy. Parasites Vectors, 14: 164. https://doi.org/10.1186/s13071-021-04652-2.

Minodier P., Piarroux R., Gambarelli F., Joblet C., Dumon H. (1997). Rapid identification of causative species in patients with Old World Leishmaniasis. J Clin Microbiol, 35(10): 2551-2555. https://doi.org/10.1128/jcm.35.10.2551-2555.1997.

Molina R., Jiménez M.I., Cruz I., Iriso A., Martìn-Martìn I., Sevillano O., Melero S. Bernal J. (2012). The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet Parasitol,190: 268-271.

Mollicone E, Battelli G, Gramiccia M, Maroli M, Baldelli R. A stable focus of canine leishmaniosis in the Bologna Province, Italy. Parassitologia, 45(2): 85-8.

Monacelli M. (1934). Il primo caso di leishmaniosi cutanea autoctona nella provincia di Forlì. Il policlinico, XLI(21): 813-816. [Article in Italian]

Montalvo A.M., Fraga J., Monzote L., Montano I., De Doncker S., Dujardin J. C., Van der Auwera G. (2010). Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. Parasitology, 137: 1159-1168. https://doi.org/10.1017/S0031182010000089.

Montalvo A.M., Fraga J., Maes I., Dujardin J. C., Van der Auwera G. (2012). Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. Eur J Clin Microbiol Infect Dis, 31: 1453-1461. https://doi.org/10.1007/s10096-011-1463-z.

Moreira O.C., Yadon Z.E., Cupolillo E. (2018) The applicability of real-time PCR in the diagnostic of cutaneous leishmaniasis and parasite quantification for clinical management: Current status and perspectives. Acta Trop, 184: 29–37. https://doi.org/10.1016/j.actatropica.2017.09.020.

Mouri O., Morizot G., van der Auwera G., Ravel C., Passet M., Chartrel N., Joly I., Thellier M., Jauréguiberry S., Caumes E., Mazier D., Marinach-Patrice C., Buffet P. (2014) Easy identification of *Leishmania* species by mass spectrometry. PLoS Negl Trop Dis, 8: e2841. http://dx.doi.org/10.1371/journal.pntd.0002841.

Mundodi V., Somana A., Farrel P.J., Gademu L. (2002). Genomic organization and functional expression of differentially regulated *cysteine protease* genes of *Leishmania donovani* complex. Gene, 282: 257-265. https://doi.org/10.1016/s0378-1119(01)00851-4.

Nicolle C. (1908). Sur trois cas d'infection splénique infantile à corps de Leishman observés en Tunisie. Arch Inst Pasteur Tunis, 3–26.

OIE- International Office of Epizootics (2021). Chapter 3.1.11 Leishmaniosis in: OIE Terrestrial manual, pp: 1-13.

Pace D. (2014). Leishmaniasis. J Inf, 69(S): 10-18. http://dx.doi.org/10.1016/j.jinf.2014.07.016.

Pampiglione S. (1975): La leishmaniosi viscerale in Emilia-Romagna. Ann San Pubbl. 35(6): 1021–1028. [Article in Italian]

Pampiglione S., La Placa M., Schlick G. (1974). Studies on Mediterranean Leishmaniasis I. An Outbreak of Visceral Leishmaniasis in Northern Italy. Trans R Soc Trop Med Hyg, 68(5): 349-359. http://dx.doi.org/10.1016/0035-9203(74)90148-5.

Patil R.R., Muliyil J.P., Nandy A., Addy A., Maji A.K., Chatterjee P. (2012) Dynamics of the antibodies in cohorts of cured cases of visceral leishmaniasis: Its implication on the validity of serological test, value in prognosis and in post therapeutic assessment. Hum Vaccines Immunother, 8: 725–730. https://doi.org/10.4161/hv.19542.

Piergili Fioretti D., Moretti A. (2020). Parassitologia e Malattie parassitarie in Medicina Veterinaria. Bononia University Press, Bologna, Italy. First Edition, p: 46-54. [Article in Italian]

Piredda A., Gasparri G. (1951). Rilievi e considerazioni sulla leishmaniosi cutanea nella provincia di Forlì. Arcispedale Sant'Anna, 14:139-158. [Article in Italian]

Pratlong F., Balard Y., Lami P., Taliagnani L., Ravel C., Dereure J., Lefebvre M., Serres G., Bastien P., Dedet J.P. (2016). The Montpellier *Leishmania* collection, from a laboratory collection to a biological resource center: a 39-year-long story. Biopreserv Biobank, 14(6): 470-479. http://dx.doi.org/10.1089/bio.2015.0101.

Pullè F. (1951). La leishmaniosi cutanea in Italia. La rassegna di clinica, terapia e scienze affini, 50(4): 161-206. [Article in Italian]

Quinnell R., Courtenay O. (2009). Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology, 136(14), 1915-1934. https://doi.org/10.1017/S0031182009991156.

Ratcliffe N.A., Frutado Pacheco J.P., Dyson P., Castro H.C., Gonzales M.S., Azambuja P., Mello C.B. (2022). Overview of paratransgenesis as a strategy to control pathogen transmission by insect vectors. Parasites Vectors, 15: 112. https://doi.org/10.1186/s13071-021-05132-3.

Reguera R.M., Morán M., Pérez-Pertejo Y., García-Estrada C., Balaña-Fouce R. (2016). Current status on prevention and treatment of canine leishmaniasis. Vet Parasitol, 227: 98-114. http://dx.doi.org/10.1016/j.vetpar.2016.07.011.

Reimão J.Q., Coser E.M. Lee M.R., Coelho A.C. (2020). Laboratory diagnosis of Cutaneous and Visceral Leishmaniasis: current and future methods. Microorganisms, 8: 1632. https://doi.org/10.3390/microorganisms8111632.

Ribeiro R.R., Marquez Michalick M.S., da Silva M.E., dos Santos C.C.P, Frézard F.J.G., da Silva S.M. (2018). Canine leishmaniasis: an overview of the current status and strategies for control. Biomed Res Int, 2018: 3296893. https://doi.org/10.1155/2018/3296893.

Richter J., Hanus I., Häussinger D., Löscher T., Harms G. (2011). Mucosal *Leishmania infantum* infection. Parasitol Res, 109: 959-962. https://doi.org/10.1007/s00436-011-2356-x.

Rogers M.B., Downing T., Smith B.A., Imamura H., Sanders M., Svobodova M., Volf P., Berriman M., Cotton J.A., Smith D.F. (2014). Genomic confirmation of hybridization and recent inbreeding in a vector-isolated *Leishmania* population. PLoS Genet, 10(1): e1004092. https://doi.org/10.1371/journal.pgen.1004092.

Romano A., Inbar E., Debrabant A., Charmoy M., Lawyer P., Ribeiro-Gomes F. (2014). Cross-species genetic exchange between visceral and cutaneous strains of

Leishmania in the sand fly vector. Proc Natl Acad Sci USA, 111: 16808–13. https://doi.org/10.1073/pnas.1415109111.

Roque A.L., Jansen A.M. (2014). Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int J Parasitol Parasites Wildl, 3: 251-62. http://dx.doi.org/10.1016/j.ijppaw.2014.08.004.

Ross R. (1904). Further notes of Leishman's bodies. Br Med J, 2: 1401.

Rossi M., Fasel N. (2017). How to master the host immune system? *Leishmania* parasites have the solutions! Int Immunol. 3(30): 103-111. https://doi.org/10.1093/intimm/dxx075.

Rougeron V, De Meeûs T, Kako Ouraga S, Hide M, Bañuls AL. (2010). "Everything you always wanted to know about sex (but were afraid to ask)" in *Leishmania* after two decades of laboratory and field analyses. PLoS Pathog, 6: e1001004. https://doi.org/10.1371/journal.ppat.1001004.

Roura X., Cortadellas O., Day M.J., Benali S.L., Canine Leishmaniosis Working Group and Zatelli A. (2021). Canine leishmaniosis and kidney disease: Q&A for an overall management in clinical practice. J Small Anim Pract, 62: E1-E19. https://doi.org/10.1111/jsap.13237.

Rugna G., Carra E., Corpus F., Calzolari M., Salvatore D., Bellini R., Di Francesco A., Franceschini E., Bruno A., Poglayen G., Varani S., Vitale F., e Merialdi G. (2017). Distinct *Leishmania infantum* strains circulate in human and dogs in the Emilia-Romagna region Northeastern Italy. Vector-Borne Zoonotic Dis, 17(6): 409-415. http://dx.doi.org/10.1089/vbz.2016.2052.

Rugna G., Carra E., Bergamini F., Calzolari M., Salvatore D., Corpus F., Gennari W., Baldelli R., Fabbi M., Natalini S., Vitale F., Varani S., Merialdi G. (2018). Multilocus microsatellite typing (MLMT) reveals host-related population structure in *Leishmania infantum* from northeastern Italy. PLoS negl Trop Dis, 12(7): e0006595. https://doi.org/10.1371/journal.pntd.0006595.

Sadeghian G., Ziaei H., Bidabadi L.S., Nilforoushzadeh M.A. (2013). Evaluation of *Leishmania* skin test reaction in different variants of cutaneous leishmaniasis. Indian J Dermatol, 58: 239. https://doi.org/10.4103/0019-5154.110838.

Sagols E., Ferraz F., Claret E., McGahie D. (2013). Evaluation of the humoral immune response after the first annual CaniLeish booster vaccination. Proceedings of the 79th SCIVAC National Congress 2013: 155–156.

Shah K., Maghsoudlou P. (2016). Enzyme-linked immunosorbent assay (ELISA): the basics. Br J Hosp Med, 77(7) C: 98-101.

Salvatore D., Di Francesco A., Parigi M., Poglayen G., Battistini M., Baldelli R. (2013). Canine leishmaniasis surveillance program in a San Marino Republic kennel. Vet ital, 49(4):341-346. https://doi.org/10.12834/VetIt.1302.01.

Salvatore D., Di Francesco A., Poglayen G., Rugna G., Santi A., Morandi B., Baldelli R. (2016). Molecular characterization of *Leishmania infantum* strains by kinetoplast DNA RFLP-PCR. Vet ital, 52(1): 71-75. https://doi.org/10.12834/VetIt.554.2623.3.

Santi A., Rossi A., Rocca R., Galletti G., Casadei G., Tamba M. (2022). Piano regionale di controllo della leishmaniosi – Risultati anno 2021. https://www.mediciveterinari.bo.it/userfiles/file/news/Relazione_Piano_Leishmania_2021.pdf.

Santos J.B., Lauand L., Santos de Souza G., Macêdo V.O. (2005). Socioeconomic factors and attitudes towards household prevention of American cutaneous leishmaniasis in an endemic area in Southern Bahia, Brazil. Cad Saude Publica, 16(3): 701-708. https://doi.org/10.1590/s0102-311x2000000300018.

Schönian G., Nasereddin A., Dinse N., Schweynoch C., Schallig H.D.F.H., Presber W., Jaffe C.L. (2003). PCR diagnosis and characterization of *Leishmania* in local and importet clinical samples. Diagn. Microbiol. Infect. Dis, 47: 349-358. https://doi.org/10.1016/S0732-8893(03)00093-2.

Schönian G., Mauricio I., Gramiccia M., Cañavate C., Boelaert M., Dujardin J.-C. (2008). Leishmaniasis in the Mediterranean in the era of molecular epidemiology. Trends Parasitol, 24(3): 135-142. https://doi.org/10.1016/j.pt.2007.12.006.

Schönian G., Kuhls K., Maurício I.L. (2011). Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. Parasitology, 138: 405-425. https://doi.org/10.1017/S0031182010001538.

Serafim T.D., Iniguez E., Oliveira F. (2020). *Leishmania infantum*. Trends in Parasitol, 36(1): 80-81. https://doi.org/10.1016/j.pt.2019.10.006.

Silva E.S., Gontijo C.M., Melo M.N. (2005). Contribution of molecular techniques to the epidemiology of neotropical *Leishmania* species. Trends Parasitol, 21: 550–552. https://doi.org/ 10.1016/j.pt.2005.09.008.

Silva A.R.S, Oliveira H.S., Gomes A.A.D., Beserra H.E.O., Silva J.P., Santos-Doni T.R., Tsunemi M.H., Marcondes M., Rahal S.C., Mamprim M.J. (2021). Joint involvement in canine visceral leishmaniasis: orthopedic physical examination, radiographic and computed tomographic findings. Vet Parasitol, 299: 109569. https://doi.org/10.1016/j.vetpar.2021.109569.

Silvestrini P. (2021). Leishmaniosis in dogs and cats. In practice, 41: 5-14. https://doi.org/10.1136/inp.k5122.

Simonato G., Marchiori E., Marcer F., Ravagnan S., Danesi P., Montarsi F., Bononi C., Capelli G., Pietrobelli M., Cassini R. (2020). Canine Leishmaniosis Control through the Promotion of Preventive Measures Appropriately Adopted by Citizens. J Parasitol Res, 2020: 8837367. https://doi.org/10.1155/2020/8837367.

Singh N., Curran M.D., Middleton D. Rastogi A.K. (1999) Characterization of kinetoplast DNA minicircles of an Indian isolate of *Leishmania donovani*. Acta Trop, 73: 313–319. https://doi.org/10.1016/S0001-706X(99)00036-4.

Singh S., Sivakumar R. (2003). Recent advances in the diagnosis of leishmaniasis. J Postgrad Med, 49(1): 55-60. https://doi.org/10.4103/0022-3859.927.

Singh O.P., Hasker E., Boelaert M., Sacks D., Sundar S. (2020). Xenodiagnosis to address key questions in visceral leishmaniasis control and elimination. PLoS Negl Trop Dis, 14: e0008363. https://doi.org/10.1371/journal.pntd.0008363.

Sirrotti R. (1954). Leishmaniasis in inhabitants of Modena: epidemiological and clinical study. La clinica pediatrica. 36(3): 219-233. [Article in Italian]

Soares M.J.V., Morares J.R.E., Borges P.V., Miyazato L.G., Moraes F.R. (2005). Renal involvement in visceral leishmaniasis in dogs. J Venom Anim Toxins incl Trop Dis, 11(4): 579-593. https://doi.org/10.1590/S1678-91992005000400014.

Socha W., Kwasnik M., Larska M., Rola J., Rozek W. (2022). Vector-Borne Viral Diseases as a Current Threat for Human and Animal Health—One Health Perspective. J Clin Med, 11: 3026. https://doi.org/10.3390/jcm11113026.

Solano-Gallego L., Koutinas A., Miró G., Cardoso L., Pennisi M.G., Ferrer L., Bourdeau P., Oliva G., Baneth G. (2009). Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol, 165(1-2): 1-18. https://doi.org/10.1016/j.vetpar.2009.05.022.

Soroka M, Wasowicz B., Rymaszewska A. (2021). Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? Cells, 10: 1931. https://doi.org/10.3390/cells10081931.

Soto M., Iborra S., Quijada L., Folgueira C., Alonso C., Requena J.M. (2004). Cell-cycle-dependent translation of histone mRNAs is the key control point for regulation of histone biosynthesis in *Leishmania infantum*. Biochem J, 379: 617e625. https://doi.org/10.1042/BJ20031522.

Späth G.F., Garraway L.A., Turco S.J., Beverley S.M. (2003). The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc Natl Acad Sci USA, 100(16): 9536-9541. https://doi.org/10.1073/pnas.1530604100.

Sundar S., Rai M. (2002). Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol, 9: 951–958. https://doi.org/10.1128/CDLI.9.5.951-958.2002.

Suzzi Valli E., Dominci L. (1953). Primo caso di Leishmaniosi viscerale autoctona in Romagna. Minerva Medica, 44: 280-285. [Article in Italian]

Thakur S., Joshi J., Kaur S. (2020). Leishmaniasis diagnosis: an update on the use of parasitological immunological and molecular methods. J Parasit Dis, 44(2): 253-272. https://doi.org/10.1007/s12639-020-01212-w.

Tomassone L., Berriatua E., de Sousa R., Duscher G.G., Mihalca A.D., Silaghi C., Sprog H., Zinti A. (2018). Neglected vector-borne zoonoses in Europe: into the wild. Vet Parasitol, 251: 17-26. https://doi.org/10.1016/j.vetpar.2017.12.018.

Tsakmakidis I., Angelopoulou K., Dovas C.I., Dokianakis E., Tamvakis A., Symeonidou I., Antoniou M., Diakou A. (2017). *Leishmania* infection in rodents in Greece. Trop Med Int Health, 22(12): 1523-1532. https://doi.org/10.1111/tmi.12982.

Van der Auwera G., Ravel C., Verweij J.J., Bart A., Schönian G., Felger I. (2014). Evaluation of four single-locus markers for *Leishmania* species discrimination by sequencing. J Clin Microbiol, 52: 1098–1104. https://doi.org/10.1128/JCM.02936-13.

Van der Auwera G., Dujardin J.C. (2015). Species typing in dermal leishmaniasis. Clin Microbiol Rev, 28: 265-294. https://doi.org/10.1128/CMR.00104-14.

Van Eys G.J.J.M., Schoone G.J., Kroon N.C.M., Ebeling S.B. (1992). Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol, 51: 133-142. https://doi.org/10.1016/0166-6851(92)90208-2.

Vannier-Santos M.A., Martiny A., de Souza W. (2002). Cell Biology of *Leishmania* spp.; Invading and Evading. Curr Pharm Des, 8: 297-318.

Varani S. Cagarelli R., Melchionda F., Attard L., Salvadori C., Finarelli A.C., Gentilomi G.A., Tigani R., Rangoni R., Todescihni R., Scalone A., di Muccio T., Gramiccia M., Gradoni L., Viale P., Landini M.P. Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. Euro Surveill, 18(29): 20530. https://www.eurosurveillance.org/content/10.2807/1560-7917.ES2013.18.28.20530.

Velez R., Gállego M. (2020). Commercially approved vaccines for canine leishmaniosis: a review of available data on their safety and efficacy. Trop Med Int Health, 25(5): 540-557. https://doi.org/10.1111/tmi.13382.

Venturi L., Angelini P., Baldelli R., Calzolari M., Borrini B.M., Dottori M., Poglayen G., Rugna G., Venturelli E., Martini E., Tamba M. (2009). Surveillance on vector-borne diseases in Emilia-Romagna region, Italy. Trop Med Int Health, 14(S2): 49. https://doi.org/10.1111/j.1365-3156.2009.02353.x.

Verin R., Poli A., Ariti G., Nardoni S., Bertuccelli Fanucchi M., Mancianti F. (2010). Detection of *Leishmania infantum* DNA in tissues of free-ranging red foxes (*Vulpes vulpes*) in Central Italy. Eur J Wild Res, 56: 689-692. https://doi.org/10.1007/s10344-010-0395-8.

Victoir K., de Doncker S., Cabrera L., Alvarez E., Arevalo J., Llanos.Cuentas A., Le Ray D., Dujardin J.-C. (2003). Direct identification of *Leishmania* species in biopsies from patients with American tegumentary leishmaniasis. Trans R Soc Trop Med Hyg, 97: 80-87. https://doi.org/10.1016/S0035-9203(03)90031-9.

Volf P., Benkova I., Myskova J., Sadlova J., Campino L., Ravel C. (2007). Increased transmission potential of *Leishmania major/Leishmania infantum* hybrids. Int J Parasitol, 37: 589–93. https://doi.org/10.1016/j.ijpara.2007.02.002.

Wickstead B., Ersfeld K., Gull K. (2003). Repetitive elements in genomes of parasitic protozoa. Microbiol Mol, 67(3): 360-375. https://doi.org/10.1228/MMBR.67.3.360–375.2003.

Woolhouse M.E.J., Dye C., Etard J.F., Smith T., Charlwood J.D., Garnett G.P., Hagan P., Hii J.L.K., Ndhlovu P.D., Quinnell R.J., Watts D.H., Chandiwana S.K., Anderson R.M. (1997). Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc Natl Acad Sci USA, 94: 338-342. https://doi.org/10.1073/pnas.94.1.338.

World Health Organization. (1990). Control of Leishmaniases. Geneva: World Health Organization.

World Health Organization. Global Health Observatory data repository (GHRD), Leishmaniasis. https://apps.who.int/gho/data/node.main.NTDLEISH?lang=en.

World Health Organization, Leishmaniasis. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (accessed September 27, 2022).

Yurčenko V., Kolesnikov A.A., Lukeš J. (2000). Phylogenetic analysis of Trypanosmomatina (Protozoa: Kinetoplastida) based on minicircles conserved regions. Folia Parasitol, 47: 1-5. https://doi.org/10.14411/fp.2000.001.

Zackay A., Nasereddin A., Takele Y., Tadesse D., Hailu W., Hurissa Z., Yifru S., Weldegebreal T., Diro E., Kassahun A., Hailu A., Jaffe C.L. (2013). Polymorphism in the HASPB repeat region of East African *Leishmania donovani* strains. PLoS Negl Trop Dis, 7: e2031. https://doi.org/10.1371/journal.pntd.0002031.

Zanet S., Sposimo P., Trisciuoglio A., Giannini F., Strumia F., Ferroglio E. (2014). Epidemiology of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in absence of domestic reservoir and definitive host. Vet Parasitol, 199: 247-249. http://dx.doi.org/10.1016/j.vetpar.2013.10.023.

Zanoli L., Spoto G. (2013) Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. Biosensors, 3(1): 18–43. https://doi.org/10.3390/bios3010018.

Zemanova E., Jirků M., Mauricio I.L., Horák A., Miles M.A., Lukeš J. (2007). The *Leishmania donovani* complex: Genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. Int J Parasitol, 37: 149-160. https://doi.org/10.1016/j.ijpara.2006.08.008.