**Universidade Nova de Lisboa**

**Instituto de Higiene e Medicina Tropical**

# Reservoirs of zoonotic leishmaniosis: the role played by domestic cats

**André Duarte Belchior Pereira**

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# Reservoirs of zoonotic leishmaniosis: the role played by domestic cats

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*To God*

*To my dear grandfather Duarte*

*To my cat Charles*

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## Resumo

A leishmaniose zoonótica causada por *Leishmania infantum* é um problema grave de saúde pública e veterinária. Contrariamente aos cães, universalmente considerados como principais hospedeiros reservatórios de *L. infantum*, os gatos domésticos (*Felis catus*) foram apontados durante anos como hospedeiros acidentais, cuja relevância para a manutenção e transmissão do parasita seria nula. No entanto, o acumular de evidências contrárias mudou o paradigma ao ponto de estes felídeos serem, atualmente, considerados como possíveis hospedeiros reservatórios de *L. infantum*. Neste contexto, este projeto doutoral teve como objetivo clarificar o papel dos gatos na epidemiologia da leishmaniose zoonótica através da *(i)* avaliação da exposição dos gatos aos vetores de *L. infantum*; *(ii)* determinação da proporção de gatos infetados em focos endémicos; *(iii)* caracterização da infeção por *Leishmania* em gatos através de acompanhamento clínico e parasitológico; *(iv)* realização de tipagem molecular dos parasitas isolados de gatos; e *(v)* avaliação do comportamento *in vitro* e infecciosidade *ex vivo* de estirpes felinas.

Anticorpos contra a saliva de *Phlebotomus perniciosus* foram identificados em 47,7 % (167/350) dos gatos testados, sendo significativamente mais frequente em animais mais velhos e em amostras obtidas durante a época de transmissão (maio a outubro). A presença de anticorpos contra a saliva de *P. perniciosus* foi associada à infeção por *Leishmania* em gatos. O ácido desoxirribonucleico (DNA) de *Leishmania* e/ou anticorpos contra o parasita foram identificados em 7,4 % dos gatos que vivem em focos endémicos. Não foram identificados quaisquer fatores de risco associados à infeção felina. Diagnosticou-se leishmaniose clínica num gato com infeção retroviral (vírus da leucemia felina) concomitante, apresentando sinais clínicos incomuns. A infeção seguiu um padrão crónico e insidioso, sendo refratária à monoterapia com alopurinol. A remissão dos sinais clínicos apenas foi conseguida após tratamento combinado com antimoniato de meglumina. A análise de sequências parciais de citocromo b, glucose-6-fosfato desidrogenase, proteína de choque térmico 70 e espaços intergénicos do DNA ribossomal, revelou que as estirpes isoladas de gatos são geneticamente idênticas a estirpes de *L. infantum* isoladas de cães, humanos e vetores. Além disso, o DNA de *L. major* e de parasitas híbridos *L. major*/*L. donovani* sensu lato foi detetado em dois gatos de diferentes regiões de Portugal Continental. As estirpes felinas apresentaram um perfil de crescimento *in vitro*, reposta adaptativa a condições de stress e suscetibilidade a compostos leishmanicidas e leishmaniostáticos, semelhante à de estirpes de *L. infantum* isoladas de cães e humanos. Os macrófagos de origem felina foram permissivos à infeção *ex vivo* com parasitas isolados de gatos, cães e humanos, que por sua vez apresentaram semelhante capacidade de infeção de macrófagos felinos, caninos e humanos.

Em adição, um algoritmo de diagnóstico para suporte de decisão clínica e um conjunto de orientações para evitar a infeção por *Leishmania* em gatos, são propostos.

Pela interceção dos dados epidemiológicos, moleculares, clínicos e experimentais obtidos no decorrer do presente projeto doutoral, pode-se concluir que *Felis catus* cumpre todos os critérios estabelecidos pela Organização Mundial de Saúde para ser reconhecida como uma espécie hospedeira reservatória de *L. infantum*.

**Palavras-chave:** gato, *Felis catus*, hospedeiro reservatório, *Leishmania infantum*, leishmaniose zoonótica.

# Abstract

Zoonotic leishmaniosis caused by *Leishmania infantum* is a serious veterinary and public health problem. Contrarily to dogs, universally considered the main reservoir hosts of *L. infantum*, domestic cats (*Felis catus*) were regarded for years as accidental hosts whose relevance for parasite maintenance and transmission was nil. However, the accumulation of contrary evidence has shifted the paradigm to the point that these felids are now considered as probable additional reservoir hosts of *L. infantum*. In this context, this doctoral project aimed to clarify the role played by cats in the epidemiology of zoonotic leishmaniosis by: *(i)* assessing the exposure of cats to *L. infantum* vectors; *(ii)* determining the proportion of infected cats in endemic foci; *(iii)* characterising the *Leishmania* infection in cats through clinical and parasitological follow-up; *(iv)* performing molecular typing of the parasites isolated from cats; and *(v)* evaluating the *in vitro* fitness and *ex vivo* infectiousness of feline *Leishmania* strains.

Antibodies to *Phlebotomus perniciosus* saliva were found in 47.7 % of the cats tested (167/350) and were significantly more frequent in older animals and samples collected during the transmission season (May to October). The presence of antibodies to *P. perniciosus* saliva was associated with *Leishmania* infection in cats. *Leishmania* deoxyribonucleic acid (DNA) and/or antibodies to the parasite were identified in 7.4 % of cats living in endemic foci. No risk factors associated with feline infection were found. Clinical leishmaniosis was diagnosed in a cat with concomitant regressive retroviral infection (feline leukaemia virus) showing unusual clinical signs. The infection followed a chronic, and insidious, pattern and was refractory to allopurinol monotherapy. Remission of clinical signs was only achieved after combined treatment with meglumine antimoniate. Analysis of cytochrome b, glucose-6-phosphate dehydrogenase, heat-shock protein 70, and internal transcribed spacers of ribosomal DNA partial sequences revealed that strains isolated from cats are genetically identical to those of well-characterised *L. infantum* strains isolated from dogs, humans, and vectors. Also, the DNA of *L. major* and *L. major*/*L. donovani* sensu lato hybrid parasites was detected in two cats from different regions of mainland Portugal. Feline strains presented a similar *in vitro* growth profile, adaptative response to stress conditions, and susceptibility to antileishmanial drugs to that of *L. infantum* strains isolated from dogs and humans. Macrophages of feline origin were similarly permissive to *ex vivo* infection with parasites isolated from cats, dogs, and humans, which also showed an identical ability to infect feline, canine, and human macrophages.

In addition, a diagnostic algorithm for clinical decision support and a set of prevention guidelines to avoid *Leishmania* infection in cats is herein proposed.

By interception of the epidemiological, molecular, clinical, and experimental data obtained during this doctoral project, it can be concluded that *Felis catus* fulfilled all criteria established by the World Health Organization to be recognised as a reservoir host species.

**Keywords:** cat, *Felis catus*, reservoir host, *Leishmania infantum*, zoonotic leishmaniosis.

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# List of abbreviations

| | |
|---|---|
| **AHCC** | Active hexose correlated compounds |
| **AKI** | Acute kidney injury |
| **ALT** | Alanine aminotransferase |
| **BLAST** | Basic local alignment search tool |
| **BSAVA** | British Small Animal Veterinary Association |
| **CAG** | Crude antigen |
| **CanL** | Canine leishmaniosis |
| ***Ch1*** | Chitinase |
| **CI** | Confidence interval |
| **CL** | Cutaneous leishmaniosis |
| ***COII*** | Cytochrome oxidase II |
| **cRPMI** | Complete RPMI medium |
| **CSV** | Comma-separated value |
| ***cytB*** | Cytochrome b |
| **DAT** | Direct agglutination test |
| **DB** | Dot blot |
| ***df*** | Degrees of Freedom |
| **DMSO** | Dimethyl sulfoxide |
| **DPX** | Dibutyl phthalate polystyrene xylene |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EU** | ELISA units |
| ***F.*** | *Felis* |
| **FBS** | Fetal bovine serum |
| **FCoV** | Feline coronavirus |
| **FeL** | Feline leishmaniosis |
| **FITC** | Fluorescein isothiocyanate |
| **FIV** | Feline immunodeficiency virus |
| **FML** | Fucose-mannose ligand |
| **FNA** | Fine-needle aspiration |

| | |
|---|---|
| **FNB** | Fine-needle biopsy |
| ***g6pdh*** | Glucose-6-phosphate dehydrogenase |
| ***gp63*** | Metalloprotease gp63 |
| **$H_2O_2$** | Hydrogen peroxide |
| **HAART** | Highly active antiretroviral therapy |
| **HIV** | Human immunodeficiency virus |
| **HRM** | High resolution melt |
| **$IC_{50}$** | Half-maximal inhibitory concentration |
| **IFAT** | Immunofluorescence antibody test |
| **IFN-γ** | Interferon-gamma |
| **IgG** | Immunoglobulin G |
| **IHA** | Indirect hemagglutination |
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |
| **IQR,** | Interquartile interval |
| **ITS1** | Internal transcriber spacer 1 |
| **ITS2** | Internal transcriber spacer 2 |
| ***ITS*-rDNA** | Internal transcribed spacers and 5.8 ribosomal DNA |
| **IU** | Internacional unit |
| **kDNA** | Kinetoplast minicircle DNA |
| ***L.*** | *Leishmania* |
| ***Lu.*** | *Lutzomyia* |
| **MCL** | Mucocutaneous leishmaniosis |
| **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **NA** | Not available |
| **NaCl** | Sodium chloride |
| **NCBI** | National Center for Biotechnology Information |
| **nPCR** | Nested PCR |
| **NUTS** | Nomenclature of Units for Territorial Statistics |
| **OIE** | World Organisation for Animal Health |
| **OR** | Odds ratio |
| **OU** | Both eyes |

| | |
|---|---|
| ***p*** | p-value |
| ***P.*** | *Phlebotomus* |
| **PBS** | Phosphate-buffered saline |
| **PBS-Tw** | PBS plus Tween 20 |
| **PCR** | Polymerase chain reaction |
| **PKDL** | Post-kala-azar dermal leishmaniosis |
| **PO** | *Per os* |
| **Prot A** | Protein A |
| **PSG** | Promastigote secretory gel |
| **qPCR** | Quantitative real-time PCR |
| **RFLP** | Restriction fragment length polymorphism |
| **rK39** | Recombinant K39 |
| **RNA** | Ribonucleic acid |
| **ROC** | Receiver operating characteristic curve |
| **ROS** | Reactive oxygen species |
| **RR** | Reference range |
| ***S.*** | *Sergentomyia* |
| **s.l.** | Sensu lato |
| **s.s.** | Sensu stricto |
| **$Sb^{III}$** | Trivalent antimony |
| **$Sb^{V}$** | Pentavalent antimony |
| **SC** | Subcutaneous |
| **SGH** | Salivary gland homogenate |
| **SODe** | Superoxide dismutase excreted |
| **sp.** | Species (singular) |
| **spp.** | Species (plural) |
| ***SSU*-rDNA** | Small subunit ribosomal DNA |
| **Syn.** | Synonymous |
| **Th-0** | T-helper 0 cells |
| **Th-1** | T-helper 1 cells |
| **Th-2** | T-helper 2 cells |
| **TNF-α** | Tumour necrosis factor-alpha |

| | |
|---|---|
| **USA** | United States of America |
| ***V.*** | *Viannia* |
| **VL** | Visceral leishmaniosis |
| **WB** | Western blot |
| **WHO** | World Health Organization |

# CHAPTER 1

## General introduction

**Part of this chapter (section 5) is based on the review article:**

Pereira, A. & Maia, C. (2021) *Leishmania* infection in cats and feline leishmaniosis: an updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Current Research in Parasitology & Vector-Borne Diseases*. 1, 100035. https://doi.org/10.1016/j.crpvbd.2021.100035.

# 1. The pathogen: *Leishmania*

## 1.1. Morphology

*Leishmania* are eukaryotic single-celled protozoan parasites, which are found in two primary morphological forms: the promastigote in the invertebrate host and the amastigote in the vertebrate host (Sunter & Gull, 2017) (Figure 1).

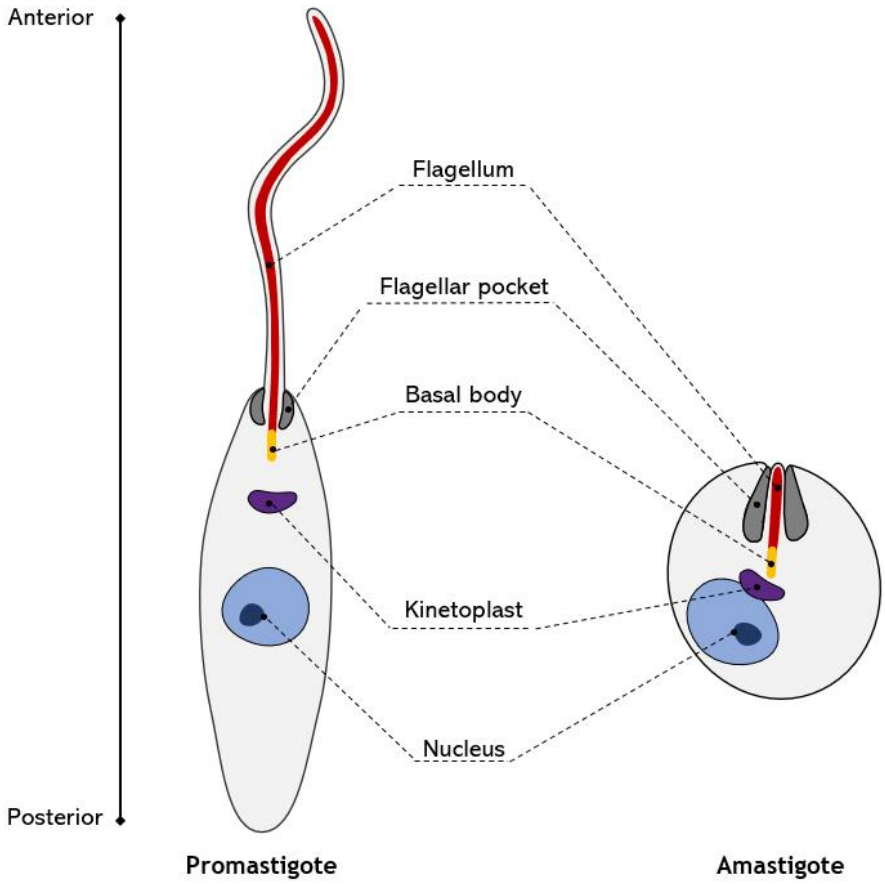


**Figure 1.** Schematic representation of a promastigote and an amastigote of *Leishmania* (author's original).

The promastigote form is characterised by an elongated body of 15 to 20 µm in length and 1.5 to 3.5 µm in width and an anterior prominent free flagellum of variable size. The nucleus is oval and situated centrally. Amastigotes are round or ovoid bodies about 2–6 µm in diameter, with a rudimentary flagellum and a large, typically eccentric, nucleus (Gramiccia & Di Muccio, 2018).

In both forms, a modified mitochondrion, called the kinetoplast, appears anterior to the nucleus as a dense disk-shaped structure that is directly associated with the basal body from which the flagellum emerges through the flagellar pocket. This invagination of the cell membrane is the only site capable of exocytosis and endocytosis (Wheeler, Sunter & Gull, 2016; Sunter & Gull, 2017).

### 1.2. Current taxonomy

According to Akhoundi *et al.* (2016) the genus *Leishmania* Ross, 1903 belongs to the Kingdom Protista Heackel, 1996; Phylum Euglenozoa Cavalier-Smith, 1993; Class Kinetoplastea Honigberg, 1963; Subclass Metakinetoplastina Vickerman, 2004; Order Trypanosomatida Kent, 1880; Family Trypanosomatidae Döflein, 1901; and Subfamily Leishmaniinae Makloves and Lukeš, 2012.

The genus *Leishmania* is divided into the Euleishmania and Paraleishmania sections (Cupolillo *et al.*, 2000). The Euleishmania section includes all species (spp.) classified within the subgenera *Leishmania*, *Mundinia*, *Sauroleishmania* and *Viannia* (Schönian *et al.*, 2018), while the Paraleishmania section comprises the former *Endotrypanum* genus and some *Leishmania* spp. (Akhoundi *et al.*, 2016). Currently, more than 50 species of *Leishmania* are described in the literature (Table 1) (Akhoundi *et al.*, 2017). Nevertheless, some cases of synonymy have been revealed, and taxonomy simplification have been argued for (Schönian, Mauricio & Cupolillo, 2010; Maurício, 2018).

**Table 1.** Revised taxonomy of the genus *Leishmania* (adapted from Akhoundi *et al.*, 2017; Jariyapan *et al.*, 2018; Maurício, 2018).

| Genus | Section | Subgenus | Species complex | Species | Simplified species nomenclature* |
|---|---|---|---|---|---|
| *Leishmania* | Euleishmania | *Leishmania* | *L. donovani* complex | *L. archibaldi*[a] | *L. donovani* |
| | | | | *L. chagasi*[b] | |
| | | | | *L. donovani*[a] | |
| | | | | *L. infantum*[b] | |
| | | | *L. major* complex | *L. arabica* | *L. major* |
| | | | | *L. gerbilli* | |
| | | | | *L. major* | |
| | | | | *L. turanica* | |
| | | | *L. mexicana complex* | *L. amazonensis*[c] | *L. mexicana* |
| | | | | *L. aristidesi* | |
| | | | | *L. forattinii* | |
| | | | | *L. garnhami*[c] | |
| | | | | *L. mexicana*[d] | |
| | | | | *L. pifanoi*[d] | |
| | | | | *L. venezuelensis* | |
| | | | | *L. waltoni* | |
| | | | *L. tropica* complex | *L. aethiopica* | *L. tropica* |
| | | | | *L. killicki*[e] | |
| | | | | *L. tropica*[e] | |
| | | *Mundinia* | | *L. enrietti* | |
| | | | | *L. macropodum* | |
| | | | | *L. martiniquensis* | |
| | | | | *L. orientalis* | |
| | | | | *L.* sp. (Ghana) | |
| | | *Sauroleishmania* | | *L. adleri* | |
| | | | | *L. agamae* | |
| | | | | *L. ceramodactyli* | |
| | | | | *L. chameleonis* | |
| | | | | *L. davidi* | |
| | | | | *L. gulikae* | |
| | | | | *L. gymnodactyli* | |
| | | | | *L. helioscopi* | |
| | | | | *L. hemidactyli* | |

| | | | | |
|---|---|---|---|---|
| | | | *L. henrici* | |
| | | | *L. hoogstraali* | |
| | | | *L. nicollei* | |
| | | | *L. phrynocephali* | |
| | | | *L. platycephala* | |
| | | | *L. senegalensis* | |
| | | | *L. sofieffi* | |
| | | | *L. tarentolae* | |
| | | | *L. zmeevi* | |
| | | | *L. zuckermani* | |
| | | | *L.* sp. I | |
| | | | *L.* sp. II | |
| | *Viannia* | *L. braziliensis* complex | *L. braziliensis* | *L. braziliensis* |
| | | | *L. peruviana* | |
| | | *L. guyanensis* complex | *L. guyanensis* | *L. guyanensis* |
| | | | *L. panamensis* | |
| | | | *L. shawi* | |
| | | | *L. lainsoni* | |
| | | | *L. lindenbergi* | |
| | | | *L. naiffi* | |
| | | | *L. utigensis* | |
| Paraleishmania | | | *Endotrypanum*[**] *monterogeii* | |
| | | | *E. schaudinni* | |
| | | | *L. colombiensis* | |
| | | | *L. deanei* | |
| | | | *L. equatorensis* | |
| | | | *L. herreri* | |
| | | | *L. hertigi* | |

[a-e] Considered synonymous species; [*] purposed by Maurício (2018); [**] suggested as subgenus by Maurício (2018).

### 1.3. Genome

Trypanosomatids have peculiar genomic features compared to other eukaryotes, such as a kinetoplast, genes without introns, a nucleus with polycistronic transcripts and small chromosomes with high gene density (Akhoundi *et al.*, 2017).

*Leishmania* parasites are mostly diploid organisms presenting two genomic pools, the nucleus and the kinetoplast (Díaz & Ponte-Sucre, 2018).

The nuclear genome is organised in 36 chromosomes for the members of the subgenera *Leishmania* except for *L. mexicana* complex, which has 34. The species within the subgenus *Vianna* has 35 chromosomes whereas those within the subgenus *Sauroleishmania* has 38 (Romano *et al.*, 2014; Cantacessi *et al.*, 2015; Akhoundi *et al.*, 2017).

The kinetoplast genome comprises a vast network of catenated circular deoxyribonucleic acid (DNA) molecules, known as kinetoplast DNA (kDNA). The circular DNA present within the kinetoplast is of two types: minicircles and maxicircles. Minicircles usually range from 0.8 to 1.0 kilobase pairs (kb), are heterogeneous in sequence, and are present in several thousand copies, making up to 95 % of kDNA. They encode guide RNA molecules involved in RNA editing. In contrast, maxicircles are present in a few dozen copies of approximately 23 kb and present an analogous structure to mitochondrial DNA of other eukaryotes (Lukeš *et al.*, 2002; Akhoundi *et al.*, 2017).

*Leishmania* reproduction is primarily asexual via clonal propagation (Rogers *et al.*, 2014). Nonetheless, several studies have provided strong evidence for the occurrence of genetic exchanges, sometimes referred to as a meiotic-like sexual cycle, within and between different *Leishmania* spp., during cyclical development in the phlebotomine sand fly vector (Akopyants *et al.*, 2009; Rogers *et al.*, 2014; Romano *et al.*, 2014; Inbar *et al.*, 2019). Experimental hybrid progeny present full genomic complement from both parents, with loss of heterozygosity at some loci and uniparental retention of maxicircle kDNA (Akopyants *et al.*, 2009; Romano *et al.*, 2014).

# 2. The hosts

## 2.1. Invertebrate hosts

*Leishmania* parasites are typically transmitted through the bite of phlebotomine sand flies (Diptera; Psychodidae; Phlebotominae) belonging to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Dvorak, Shaw & Volf, 2018) (see section 4.).

Formal incrimination of a phlebotomine sand fly species as a vector is based on five widely accepted criteria (WHO, 2010):

i. The vector must be anthropophilic.
ii. The vector feeding preferences must include reservoir hosts in zoonotic transmission cycles.
iii. The vector must be infected in nature with the same *Leishmania* spp. as occurs in humans.
iv. The vector must support the complete development of the parasite it transmits after the infecting blood meal has been digested.
v. The vector must be able to transmit the protozoan by bite to a susceptible host.

### 2.1.1. Biology

Briefly, phlebotomine sand flies are small, hairy insects with a body length rarely exceeding 3 mm. They can be found in different parts of the world and types of habitats, from below sea level to over 3,000 m in altitude. Their life cycle is exclusively terrestrial and comprises complete metamorphosis (holometabolous development) through four developmental stages: egg, larva (four instars), pupa and adult (Killick-Kendrick, 1999; Maroli *et al.*, 2013).

The activity of adult phlebotomine sand flies is crepuscular and is affected mainly by temperature and rainfall. Both sexes feed on natural sources of sugars (such as plant sap and nectar) and aphid honeydew. However, females are also hematophagous to successfully produce progeny (Killick-Kendrick, 1999; Maroli *et al.*, 2013).

### 2.1.2. Salivary compounds as biomarkers of vector exposure and *Leishmania* infection

During a blood meal (occurring by telmophagy or pool feeding), the skin of the vertebrate host is damaged by the phlebotomine sand fly's proboscis, and a cocktail of bioactive molecules is inoculated *via* saliva. Some of these salivary compounds called sialogenins have anti-hemostatic, anti-inflammatory and immunomodulatory properties that successfully assist the phlebotomine sand fly female in finishing the blood meal (Lestinova *et al.*, 2017). Moreover, most sialogenins are immunogenic, eliciting cellular and humoral immune responses in mammals, as shown in Figure 2.

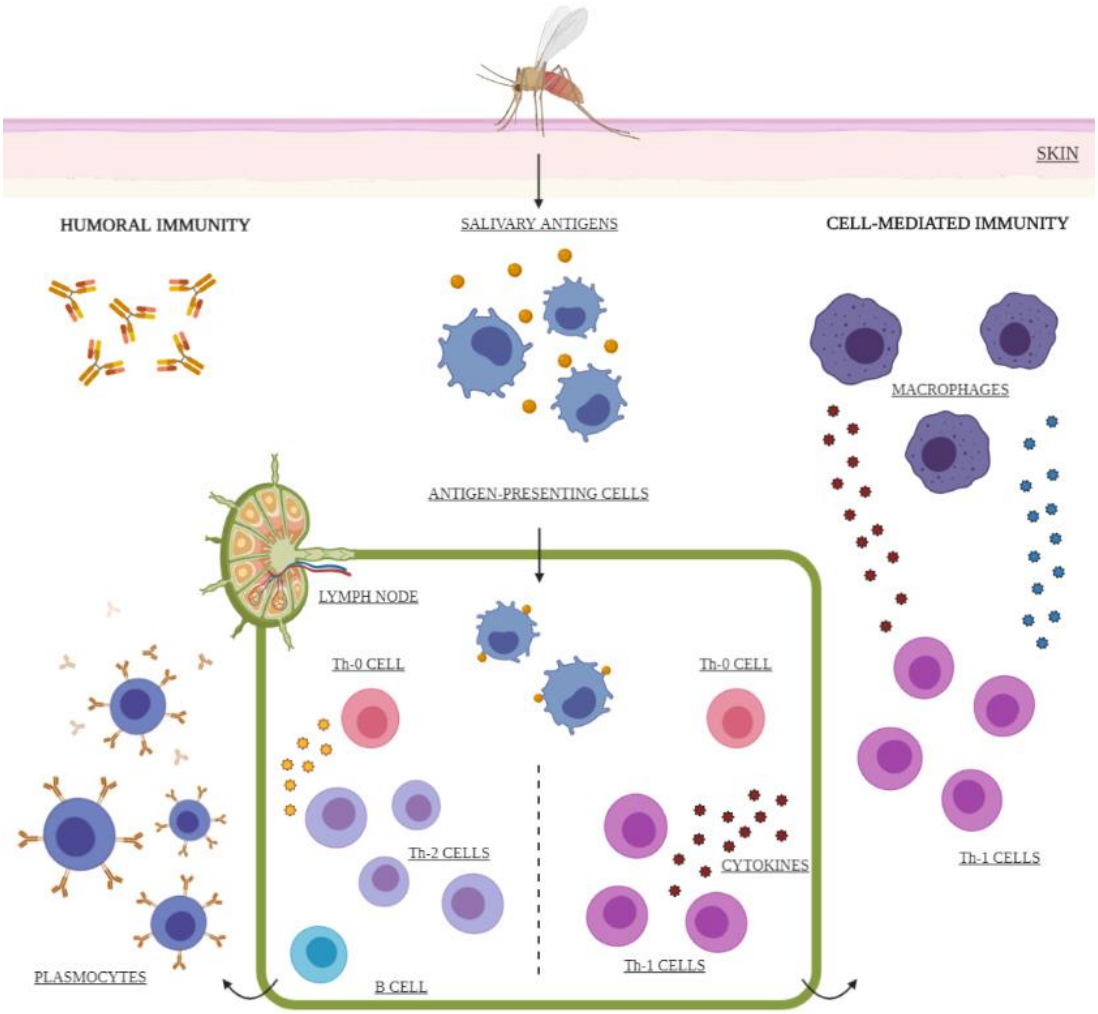


**Figure 2**. Hypothetical model illustrating the immune response in a vertebrate host recurrently exposed to phlebotomine sand fly bites. Eight-pointed stars represent examples of the main cytokines expressed in this model, namely interleukin 4 (yellow), interleukin 12 (red) and interferon-gamma (blue). Abbreviations: Th, T helper cell. (adapted from Lestinova *et al.*, 2017).

The role of phlebotomine sand fly saliva in *Leishmania* transmission was demonstrated for the first time by Titus & Ribeiro (1988). To date, several studies have corroborated that the saliva of phlebotomine sand flies can exacerbate *Leishmania* infection in naïve hosts (reviewed by Lestinova *et al.*, 2017). This phenomenon, called the "enhancing effect", results in severe disease manifestations and an increase in parasite burden (Gomes & Oliveira, 2012).

The use of anti-saliva antibodies as biomarkers of exposure to hematophagous invertebrates and risk of infection appears to be an additional epidemiolocal tool, which may support the design and implementation of new strategies to reduce the incidence of vector-borne diseases in endemic areas (Doucoure *et al.*, 2015).

Exposure to phlebotomine sand flies' bites induce the production of species-specific antibodies [mainly from the immunoglobulin class or isotype G (IgG)] in humans and other animals (reviewed by Andrade & Teixeira, 2012). Also, the levels of anti-saliva IgG have been positively correlated with exposure intensity in rodents/lagomorphs (Martín-Martín, Molina & Jiménez, 2015), dogs (Hostomska *et al.*, 2008) and humans (Clements *et al.*, 2010), and decreases significantly after the last contact with phlebotomine sand flies.

In endemic areas, high levels of anti-saliva IgG specific to *Lu. intermedia*, *P. papatasi* and *P. sergenti* were detected in human patients presenting active cutaneous leishmaniosis (CL), suggesting their use as a risk biomarker of CL (Rohousova *et al.*, 2005; de Moura *et al.*, 2007; Marzouki *et al.*, 2011). In humans living in Latin American countries endemic for visceral leishmaniosis (VL), a positive correlation has been suggested between the presence of anti-*Lutzomyia longipalpis* saliva IgG antibodies and the development of delayed-type hypersensitivity to *Leishmania* antigens (Gomes *et al.*, 2002; Aquino *et al.*, 2010). The use of *P. perniciosus* saliva IgG as a biomarker of *L. infantum* infection in dogs remains controversial. Indeed, positive (Maia *et al.*, 2020), negative (Vlkova *et al.*, 2011) or no correlations (Kostalova *et al.*, 2017) between the levels of anti-*P. perniciosus* saliva IgG and anti-*L. infantum* IgG have been reported in dogs in endemic areas.

## 2.2. Vertebrate hosts

Parasites of the genus *Leishmania* can infect a wide range of vertebrate species, most of which are mammals. Epidemiologically, vertebrates can be divided into primary reservoir hosts, secondary reservoir hosts, or accidental hosts (Maia, Dantas-Torres & Campino, 2018).

A primary reservoir host is responsible for maintaining the parasite indefinitely in nature, in the absence of any other host species, and usually does not show obvious signs of infection. In contrast, a secondary reservoir host may also serve as an infection source for vectors and increases parasite transmissibility but cannot maintain parasite transmission in the absence of the primary reservoir host. Lastly, an accidental host is a host that, despite being susceptible to infection, does not usually transmit the parasite to vectors and therefore plays no essential role in the ecological system in which the parasite is maintained indefinitely in nature (Quinnell & Courtenay, 2009; Maia, Dantas-Torres & Campino, 2018).

### 2.2.1. Incrimination of reservoir hosts

The mere presence of *Leishmania* parasites in a vertebrate host, even in large numbers, is not enough to recognise the latter as a reservoir. In this context, and according to the World Health Organization (WHO) (2010), six criteria should be fulfilled to incriminate a mammal species as a reservoir host of *Leishmania* spp.:

i. A reservoir host is likely to be sufficiently abundant and long-lived to provide a significant food source for phlebotomine sand flies.
ii. Intense host–phlebotomine sand fly contact is necessary.
iii. The proportion of individuals that become infected during their lifetime is usually considerable.
iv. The course of infection in a reservoir host should be long enough, and the infection should be sufficiently non-pathogenic to allow the parasites to survive any non-transmission season.
v. Parasites should be available in the skin or the blood in sufficient numbers to be taken up by a phlebotomine sand fly.
vi. Parasites in reservoir hosts must be the same as those in humans.

## 3. Life cycle

In nature, *Leishmania* parasites have a digenetic life cycle, alternating between a vertebrate host and an insect vector (Figure 3).

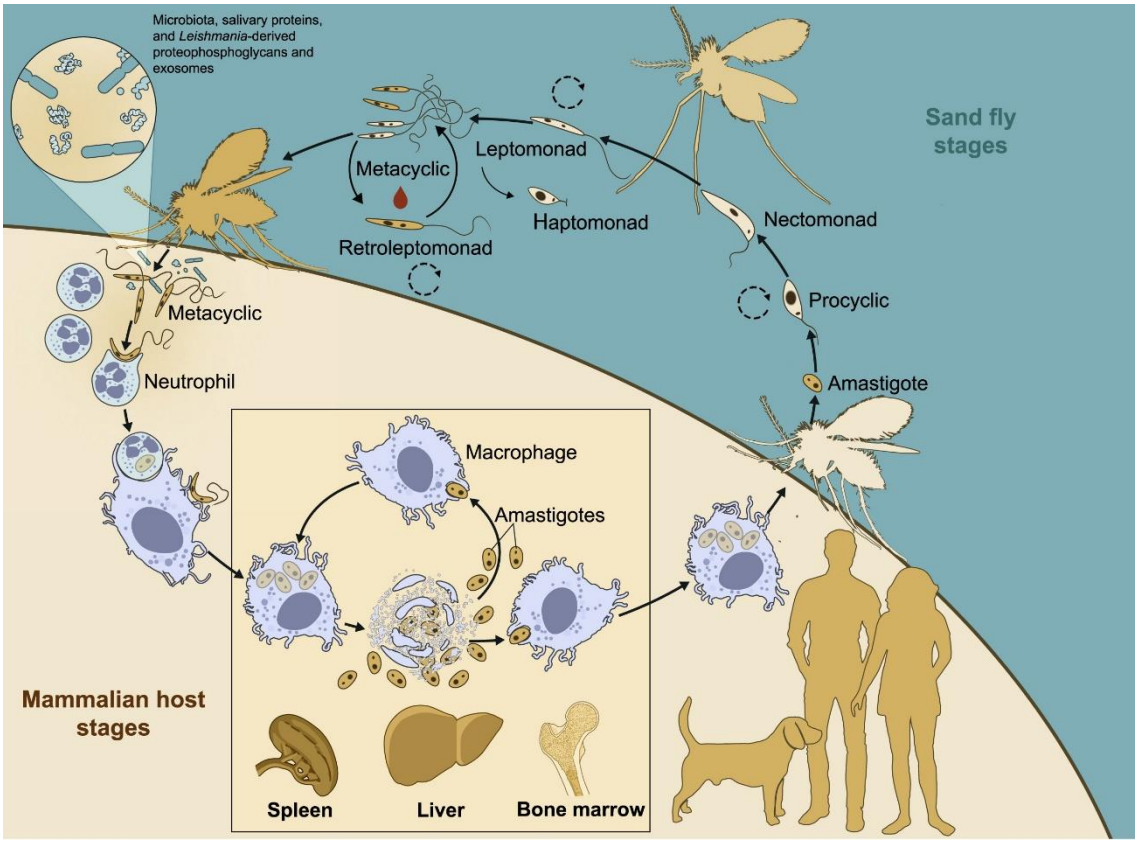


**Figure 3**. Schematic life cycle of *Leishmania* species. Circular arrows represent multiplicative stages. Red drop depicts successive blood meals. (adapted from Serafim, Iniguez & Oliveira, 2020).

The following descriptions are focused on the parasites belonging to the subgenus *Leishmania*, for which the most consistent and detailed information is available. During a blood meal on an infected host, a female phlebotomine sand fly acquires *Leishmania* amastigotes located in phagocytes. The change in conditions moving from the mammalian host to the phlebotomine sand fly midgut, such as the decrease in temperature and increase in pH, triggers the development of the parasites in the vector (Dostálová & Volf, 2012). The amastigotes differentiate into weakly motile forms called procyclic promastigotes, which multiply intensively by binary fission in the blood meal surrounded by the peritrophic matrix (*i.e.*, an acellular mesh of proteins and chitin secreted by the

midgut epithelium which protects the blood meal from digestive proteases and prevents parasites migration anteriorly) (Secundino *et al.*, 2005; Dvorak, Shaw & Volf, 2018). Around 2-3 days later, procyclic promastigotes begin to slow their replication and differentiate into elongated, strongly motile nectomonad promastigotes (Bates, 2007). After the disintegration of the peritrophic matrix by the action of chitinases, nectomonad promastigotes attach by flagella to the microvilli of the midgut epithelium to avoid being expelled during defecation (Ramalho-Ortigão *et al.*, 2005; Sádlová & Volf, 2009; Dostálová & Volf, 2012). Following detachment, nectomonad promastigotes migrate towards the thoracic portion of the midgut and later differentiate into leptomonad promastigotes (shorter forms that resume replication) (Bates, 2007; Dostálová & Volf, 2012), which are responsible for the production and secretion of promastigote secretory gel (PSG) (*i.e.*, a proteophosphoglycan-rich, mucin-like gel that acts as a plug obstructing the midgut and pharynx) (Rogers, Chance & Bates, 2002; Rogers, 2012). Some of the nectomonad/leptomonad promastigotes differentiate into haptomonad promastigotes. The latter attach via hemidesmosomes to the cuticular lining of the stomodeal valve (*i.e.*, the junction between the thoracic midgut and foregut), forming an extensive spherical structure that occludes the valve (Wakid & Bates, 2004; Dvorak, Shaw & Volf, 2018). Haptomonad promastigotes and PSG are responsible for the so-called "blocked fly" effect, forcing infected phlebotomine sand flies to regurgitate parasites (Bates, 2018). Finally, some of the leptomonad promastigotes differentiate into metacyclic promastigotes, which are small, highly motile flagellated infective forms, pre-adapted for survival in the vertebrate host (Bates, 2007). In matured infections, metacyclic promastigotes are typically concentrated in the anterior part of the midgut, on the edge of PSG plug (Dvorak, Shaw & Volf, 2018).

For several years metacyclic promastigotes were considered as the terminal development stage inside the vector. However, Serafim *et al.* (2018) recently demonstrated that a second uninfected blood meal by a *Leishmania*-infected phlebotomine sand fly triggers de-differentiation of metacyclic promastigotes (*i.e.*, those that were not transmitted to the vertebrate host) into retroleptomonad promastigotes. These leptomonad-like forms rapidly multiply and differentiate into metacyclic promastigotes following successive blood meals (reverse metacyclogenesis), enhancing vector infectiousness (Bates, 2018; Serafim *et al.*, 2018).

When the infected phlebotomine sand fly takes another blood meal, the metacyclic promastigotes are directly deposited into the bite wound together with insect saliva and midgut content, including PSG, microbiota, and exosomes (Dvorak, Shaw & Volf, 2018). In the skin, the metacyclics are phagocytised by polymorphonuclear and mononuclear phagocytes, and inside them differentiate (*i.e.*, within the parasitophorous vacuoles) into amastigotes (Serafim, Iniguez & Oliveira, 2020). Once within macrophages (main host cells), the amastigotes multiply actively by binary fission. Following the multiplication phase, the macrophage membrane ruptures, and the parasites are released; free amastigotes in circulation can then infect other phagocytes present in the skin and/or spread through the circulatory system to several internal organs, such as the bone marrow, liver, and spleen (Serafim, Iniguez & Oliveira, 2020). The cycle is completed when amastigotes are taken up by a competent vector.

## 4. Epidemiology of human leishmaniosis

From an epidemiological point of view, leishmaniosis (or "leishmaniasis") can be classified according to the reservoir source of human infection as either an anthroponotic (*i.e.*, when the source is an infected human) or zoonotic disease (*i.e.*, when the source is a non-human vertebrate) (Hubálek, 2003).

All continents, except Antarctica, have endemic areas for human leishmaniosis, in a total of 98 countries and three territories (Alvar *et al.*, 2012). Human leishmaniosis is still one of the world's most neglected diseases, affecting mainly the poorest people in low-income countries, and is associated with malnutrition, poor housing, population displacement, weak immune system, and lack of financial resources (WHO, 2010). Globally, over 1 billion people are potentially at risk of developing leishmaniosis (WHO, 2017b).

At least 20 species of *Leishmania* are known to be pathogenic to humans, and several species of phlebotomine sand flies are implicated in their transmission (*i.e.*, about 100 species are proven or suspected vectors) (Gradoni, 2018) (Table 2 and 3). Human leishmaniosis is primarily zoonotic except for *L. donovani* and *L. tropica*, although non-human reservoir hosts may hypothetically exist for both species across Africa and Asia (reviewed by Maia, Dantas-Torres & Campino, 2018). Leishmaniosis in humans consists

of two primary clinical forms (or syndromes): self-healing or chronic CL and life-threatening VL, also known as kala-azar. Other clinical forms occur more rarely and include localised leishmanial lymphadenopathy, localised mucosal leishmaniosis, mucocutaneous leishmaniosis (MCL), diffuse and disseminated CL, and post-kala-azar dermal leishmaniosis (PKDL) (Gradoni, 2018). Clinical outcomes are determined by the interplay of the infecting species, host genetic factors and immune status (Colmenares *et al.*, 2002).

**Table 2.** Leishmaniosis in the Old World: aetiology, geographical distribution, vectors, reservoir hosts, transmission type and clinical forms (WHO, 2010; Maroli *et al.*, 2013; Ready, 2013; Kwakye-Nuako *et al.*, 2015; Maia, Leelayoova *et al.*, 2017; Burza, Croft & Boelaert, 2018; Jariyapan *et al.*, 2018; Maia, Dantas-Torres & Campino, 2018; Bongiorno *et al.*, 2019; Chanmol *et al.*, 2019).

| ***Leishmania* species** | **Geographical distribution** | **Proven or suspected vector** | **Proven or suspected reservoir** | **Transmission – Clinical form** |
|---|---|---|---|---|
| *L. donovani* | Bangladesh; Bhutan; Chad; China; Cyprus; Djibouti; Ethiopia; India; Iraq; Israel; Kenya; Nepal; Saudi Arabia; Somalia; Sri Lanka; Sudan; Uganda; Ukraine; Yemen | *P. alexandri*; *P. argentipes*; *P. celiae*; *P. chinensis* s.l.; *P. longiductus*; *P. martini*; *P. orientalis*; *P. vansomerenae* | Dogs; Humans; Mongooses; Rodents | Anthroponotic – CL[*]; VL |
| *L. infantum* | Afghanistan; Albania; Algeria; Armenia; Azerbaijan; Bosnia and Herzegovina; Bulgaria; Central African Republic; China; Croatia; Cyprus; Egypt; France; Gambia; Georgia; Greece; Iran; Iraq; Israel; Italy; Jordan; Kazakhstan; Kosovo; Kyrgyzstan; Lebanon; Libya; Macedonia; Malta; Mauritania; Monaco; Montenegro; Morocco; Oman; Pakistan; Palestine; Portugal; Romania; Saudi Arabia; Senegal; Slovenia; Spain; Sudan; Syria; Tunisia; Turkey; Turkmenistan; Ukraine; Uzbekistan; Yemen | *P. alexandri*; *P. ariasi*; *P. balcanicus*; *P. chinensis*; *P. duboscqi*; *P. galileus*; *P. halepensis*; *P. kandelakii*; *P. kandelakii*; *P. langeroni*; *P. longicuspis*; *P. longiductus*; *P. major* s.l.; *P. neglectus*; *P. perfiliewi* s.l.; *P. perniciosus*; *P. sichuanensis*; *P. smirnovi*; *P. syriacus*; *P. tobbi*; *P. transcaucasicus*; *P. turanicus*; *P. wui* | Canids, Cats, Lagomorphs, Rodents | Zoonotic – CL[*]; VL |
| *L. major* | Afghanistan; Algeria; Azerbaijan; Burkina Faso; Cameroon; Chad; Egypt; Ethiopia; Georgia; Gambia; Ghana; Guinea; Guinea-Bissau; India; Iran; Iraq; Israel; Jordan; Kazakhstan; Kenya; Kuwait; Libya; Mali; Mauritania; Mongolia; Morocco; Niger; Nigeria; Oman; Pakistan; Palestine; Saudi Arabia; Senegal; Sudan; Syria; Tunisia; Turkmenistan; Uzbekistan; Yemen | *P. ansarii*; *P. bergeroti*; *P. caucasicus*; *P. duboscqi*; *P. mongolensis*; *P. papatasi*; *P. perniciosus*; *P. salehi*; *S. darlingi*; *S. garnhami* | Rodents | Zoonotic – CL |
| *L. aethiopica* | Ethiopia; Kenya | *P. aculeatus*; *P. longipes*; *P. pedifer*; *P. sergenti* | Hyraxes; Rodents | Zoonotic – CL |
| *L. tropica* | Afghanistan; Algeria; Azerbaijan; Egypt; Ethiopia; Greece; India; Iran; Iraq; Israel; Jordan; Kenya; Libya; Morocco; Namibia; Pakistan; Palestine; Saudi Arabia; Syria; Tunisia; Turkey; Turkmenistan; Uzbekistan<br>Yemen | *P. aculeatus*; *P. arabicus*; *P. chabaudi*; *P. grovei*; *P. guggisbergi*; *P. perniciosus*; *P. rossi*; *P. saevus*; *P. sergenti*; *P. similis* | Dogs; Humans; Hyraxes; Rodents | Anthroponotic – CL |
| *L. martiniquensis* | Myanmar; Thailand | *S. barraudi*; *S. gemmea* | Rodents | Zoonotic – CL; VL |
| *L.* orientalis | Thailand | Biting midges | Unknown | Zoonotic[**] – CL; VL |
| *L.* sp. (Ghana) | Ghana | Unknown | Unknown | Zoonotic[**] – CL |

[*]Rarely; [**]Suspected.

Abbreviations: *L.*, *Leishmania*; *P.*, *Phlebotomus*; *S.*, *Sergentomyia*; s.l., sensu lato; sp., species; syn., synonymous; CL, cutaneous leishmaniosis; VL, visceral leishmaniosis.

**Table 3.** Leishmaniosis in the New World: aetiology, geographical distribution, vectors, reservoir hosts, transmission type and clinical forms (WHO, 2010; Maroli *et al.*, 2013; Ready, 2013; Shaw *et al.*, 2015; Burza, Croft & Boelaert, 2018; Leelayoova *et al.*, 2017; Maia, Dantas-Torres & Campino, 2018).

| ***Leishmania* species** | **Geographical distribution** | **Proven or suspected vector** | **Proven or suspected reservoir** | **Transmission – Clinical form** |
|---|---|---|---|---|
| *L. infantum* | Argentina; Bolivia; Brazil; Colombia; Costa Rica; El Salvador; Guatemala; Honduras; Mexico; Nicaragua; Paraguay; United States of America; Venezuela | *Lu. almerioi*; *Lu. cruzi*; *Lu. evansi*; *Lu. forattinii*; *Lu. longipalpis* s.l.; *Lu. migonei*; *Lu. pseudolongipalpis*; *Lu. sallesi* | Canids; Cats; Marsupials | Zoonotic – CL[*]; VL |
| *L. amazonensis* | Argentina; Bolivia; Brazil; Colombia; Costa Rica; Ecuador; French Guiana; Peru; Suriname; Venezuela | *Lu. flaviscutellata*; *Lu. longipalpis*; *Lu. nuneztovari anglesi*; *Lu. olmeca nociva*; *Lu. olmeca reducta*; *Lu. youngi* | Marsupials; Rodents | Zoonotic – CL |
| *L. mexicana* | Belize; Colombia; Costa Rica; Ecuador; Guatemala; Mexico; United States of America; Venezuela | *Lu. anthophora*; *Lu. ayacuchensis*; *Lu. columbiana*; *Lu. cruciata*; *Lu. diabolica*; *Lu. flaviscutellata*; *Lu. migonei*; *Lu. olmeca bicolor*; *Lu. olmeca olmeca*; *Lu. ovallesi*; *Lu. panamensis*; *Lu. shannoni*; *Lu. ylephiletor* | Dogs; Marsupials; Rodents | Zoonotic – CL |
| *L. venezuelensis* | Venezuela | *Lu. olmeca bicolor* | Cats | Zoonotic – CL |
| *L. waltoni* | Dominican Republic | *Lu. christophei* | Unknoun | Zoonotic[**] – CL |
| *L. martiniquensis* | *French West Indies* | Unknown | Rodents | Zoonotic – CL; VL |
| *L. braziliensis* | Argentina; Belize; Bolivia; Brazil; Colombia; Costa Rica; Ecuador; French Guiana; Guatemala; Honduras; Mexico; Nicaragua; Panama; Paraguay; Peru; Venezuela | *Lu. ayrozai*; *Lu. carrerai*; *Lu. columbiana*; *Lu. complexa*; *Lu. cruciata*; *Lu. edwardsi*; *Lu. fischeri*; *Lu. gomezi*; *Lu. intermedia*; *Lu. llanosmartinsi*; *Lu. migonei* s.l.; *Lu. neivai*; *Lu. nuneztovari anglesi*; *Lu. ovallesi*; *Lu. panamensis*; *Lu. paraensis*; *Lu. pescei*; *Lu. pessoai*; *Lu. pia*; *Lu. shawi*; *Lu. spinicrassa*; *Lu. tejadai*; *Lu. townsendi*; *Lu. trinidadensis*; *Lu. wellcomei*; *Lu. whitmani*; *Lu. ylephiletor*; *Lu. youngi*; *Lu. yucumensis* | Dogs; Equids; Marsupials; Rodents | Zoonotic – CL |
| *L. peruviana* | Peru | *Lu. Ayacuchensis*; *Lu. peruensis* s.l.; *Lu. verrucarum* s.l. | Dogs; Marsupials; Rodents | Zoonotic – CL |
| *L. guyanensis* | Argentina; Bolivia; Brazil; Colombia; Ecuador; French Guiana; Guyana; Peru; Suriname; Venezuela | *Lu. anduzei*; *Lu. ayacuchensis*; *Lu. longiflocosa*; *Lu. migonei*; *Lu. shawi*; *Lu. umbratilis*; *Lu. whitmani* | Edentats; Marsupials; Rodents | Zoonotic – CL |
| *L. panamensis* | Colombia; Costa Rica; Ecuador; Guatemala; Honduras; Nicaragua; Panama | *Lu. cruciata*; *Lu. edentula*; *Lu. gomezi*; *Lu. hartmanni*; *Lu. panamensis*; *Lu. sanguinaria*; *Lu. trapidoi*; *Lu. ylephiletor*; *Lu. yuilli* | Dogs; Edentates; Marsupials; Procyonids; Rodents | Zoonotic – CL |

| | | | | |
|---|---|---|---|---|
| *L. shawi* | Brazil | *Lu. whitmani* | Edentates; Primates; Procyonids | Zoonotic – CL |
| *L. lainsoni* | Bolivia; Brazil; French Guiana; Peru; Suriname | *Lu. nuneztovari anglesi*; *Lu. ubiquitalis* | *Rodents* | Zoonotic – CL |
| *L. naiffi* | Brazil; French Guiana; Panama | *Lu. amazonensis*; *Lu. ayrozai*; *Lu. paraensis*; *Lu. squamiventris*; *Lu. trapidoi* | Armadillos; Edentates | Zoonotic – CL |
| *L. lindenbergi* | Brazi | *Lu. antunesi* | Unknown | Zoonotic** – CL |
| *L. colombiensis* | Colombia; Panama; Venezuela | *Lu. hartmanni*; *Lu. gomezi*; *Lu. panamensis* | Edentates | Zoonotic – CL |

*Rarely; **Suspected.

Abbreviations: *L.*, *Leishmania*; *Lu.*, *Lutzomyia*; s.l., sensu lato; syn., synonymous; CL, cutaneous leishmaniosis; VL, visceral leishmaniosis.

Cutaneous leishmaniosis is the most common form of leishmaniosis and is characterised by the formation of skin lesions that are typically either self-healing or easy to treat, despite being disfiguring (Boelaert & Sundar, 2013). Some 85 % of the estimated 600,000-1,000,000 annual cases occur in Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, Syria, and Tunisia (Figure 4) (WHO, 2020). Forced migration has a dramatic impact on the incidence of CL, with imported cases being relatively common in non-endemic countries (Gradoni, 2018). Several species have been implicated as aetiologic agents of CL, although those mainly responsible are *L. aethiopica*, *L. major* and *L. tropica* in the Old World and *L. amazonensis*, *L. braziliensis*, *L. guyanensis* and *L. mexicana* in the New World. Nonetheless, up to 10 % of CL cases progress to more severe clinical forms, such as MCL (mainly caused by *L. braziliensis* and *L. panamensis*) which is characterised by destructive lesions of the nasal septum, lips, and palate (Burza, Croft & Boelaert, 2018). Over 90 % of MCL cases occur in Bolivia, Brazil, Ethiopia, and Peru (WHO, 2020).

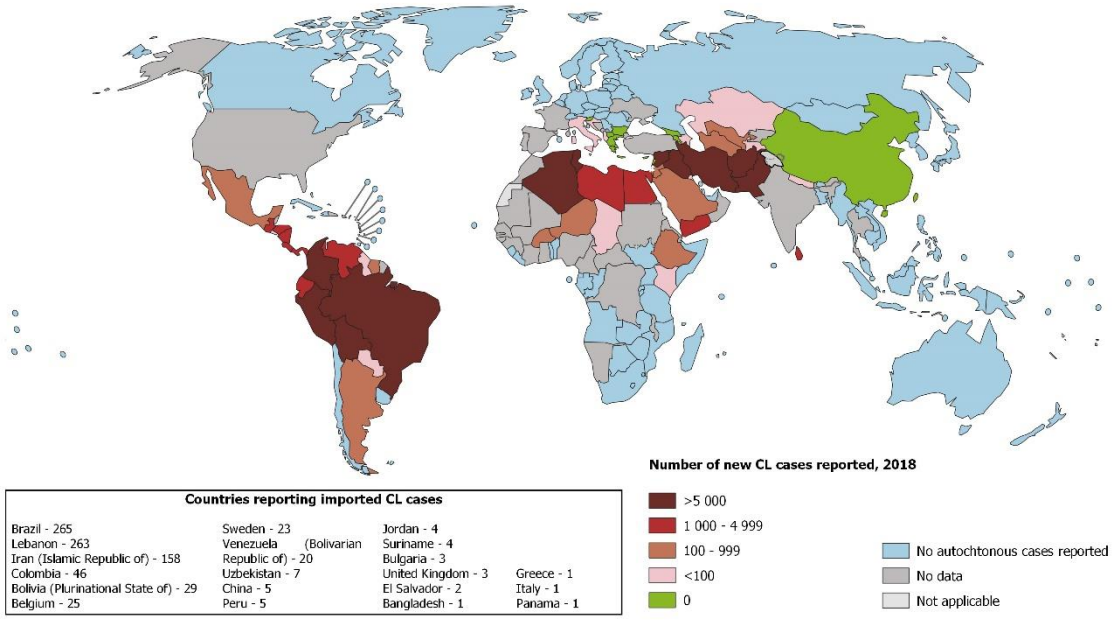


**Figure 4.** Status of endemicity of human cutaneous leishmaniosis (CL) worldwide in 2018 (retrieved from https://www.who.int/leishmaniasis/burden/en/).

Visceral leishmaniosis is a systemic form that is usually fatal if left untreated and is caused by parasites taxonomically classified within the *L. donovani* complex (Boelaert & Sundar, 2013). The World Health Organization estimates VL incidence to be in the

range of 50,000-90,000 cases per year (WHO, 2017b). In 2018, 10 countries (*i.e.*, Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan, and Sudan) reported more than 95 % of the worldwide cases of VL (Figure 5). Nevertheless, VL remains endemic in more than 50 countries, including Portugal (WHO, 2017a; 2020). Immunosuppression represents a significant risk factor for VL development. The epidemiological impact of immunosuppression on VL has been well demonstrated by the effect of the human immunodeficiency virus (HIV) epidemic in the re-emergence of VL in southern Europe in the late 1990s (van Griensven *et al.*, 2014). Thanks to the wide-scale introduction of highly active antiretroviral therapy (HAART), the incidence of VL-HIV cases has been declining in Europe in the past few years (WHO, 2017a).

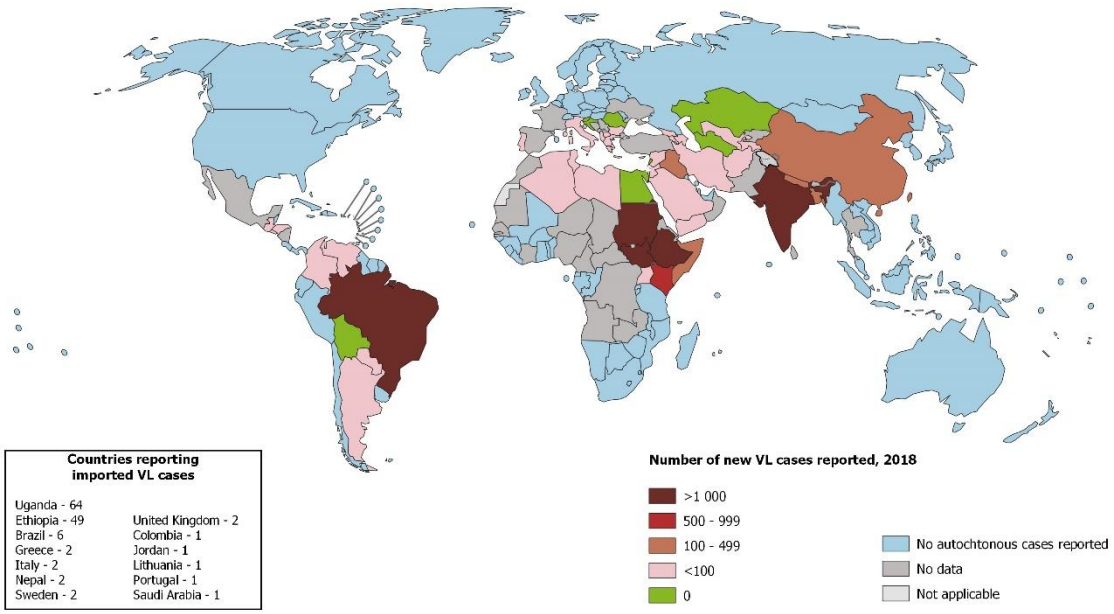


**Figure 5.** Status of endemicity of human visceral leishmaniosis (VL) worldwide in 2018 (retrieved from https://www.who.int/leishmaniasis/burden/en/).

## 5. *Leishmania* infection in cats and feline leishmaniosis

This section is based on the review article:

Pereira, A. & Maia, C. (2021) *Leishmania* infection in cats and feline leishmaniosis: an updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Current Research in Parasitology & Vector-Borne Diseases*. 1, 100035. https://doi.org/10.1016/j.crpvbd.2021.100035.

### 5.1. Abstract

Leishmaniosis is a vector-borne disease caused by protozoans of the genus *Leishmania*, which are transmitted to vertebrates, including cats, through the bites of female phlebotomine sand flies. An increasing number of epidemiological and experimental studies concerning *Leishmania* infection in cats, as well as case reports of clinical leishmaniosis in these felids, have been published in recent years. In the present study, a comprehensive review was made by sourcing the National Library of Medicine resources to provide updated data on epidemiology, immunopathogenesis, diagnosis, treatment, and prevention of feline leishmaniosis. Cats were found infected with *Leishmania* parasites worldwide, and feline leishmaniosis appears as an emergent disease mostly reported in countries surrounding the Mediterranean Sea and in Brazil. Cats with impaired immunocompetence seem to have a higher risk of developing clinical disease. The main clinical and clinicopathological findings are dermatological lesions and hypergammaglobulinemia, respectively. Diagnosis of feline leishmaniosis remains a challenge for veterinarians, in part, due to the lack of diagnosis support systems. For this reason, a diagnostic algorithm for clinical decision support is proposed. No evidence-based treatment protocols are currently available, and these remain empirically based. Control measures are limited and scarce. Thus, a set of prevention guidelines are herein suggested.

**Keywords:** cats, diagnosis algorithm, feline leishmaniosis, *Leishmania*, prevention guidelines, treatment.

## 5.2. Introduction

Leishmaniosis is a disease that affects humans and both domestic and wild animals worldwide and is caused by protozoa of the genus *Leishmania.* The infection typically occurs through the bite of female phlebotomine sand flies of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World (WHO, 2010).

In contrast to dogs, cats had been considered for several years as accidental hosts resistant to leishmaniosis. Nevertheless, this felid now appears as a relevant piece within the ecological system in which *Leishmania* parasites are maintained indefinitely (Asfaram, Fakhar & Teshnizi, 2019). Feline *Leishmania* infection has frequently been reported in endemic areas of South America, Southern Europe and Western Asia, and the number of reported cases of feline leishmaniosis has been increasing in recent years (Pereira *et al.*, 2019b; Baneth *et al.*, 2020; da Costa-Val *et al.*, 2020; Fernandez-Gallego *et al.*, 2020).

The present review aimed to provide updated information concerning the epidemiology of *Leishmania* infection in cats and clinical management of feline leishmaniosis (FeL) with emphasis on immunopathogenesis, diagnosis, treatment, prognosis, and prevention, as well as the development of an algorithm to assist diagnosis and delineate strategic guidelines to prevent feline infection.

## 5.3. Search strategy, eligibility, and review

A comprehensive literature search was performed on 10 March 2021 by sourcing National Library of Medicine resources through PubMed (https://pubmed.ncbi.nlm.nih.gov/) using the following Boolean string: ("leishmania"[MeSH Terms] OR "leishmania"[All Fields] OR "leishmanias"[All Fields] OR "leishmaniae"[All Fields] OR ("leishmaniasis"[MeSH Terms] OR "leishmaniasis"[All Fields] OR "leishmaniosis"[All Fields] OR "leishmaniases"[All Fields])) AND ("cat"[All Fields] OR ("felis"[MeSH Terms] OR "felis"[All Fields]) OR ("felidae"[MeSH Terms] OR "felidae"[All Fields] OR "felid"[All Fields] OR "felids"[All Fields]) OR ("cats"[MeSH Terms] OR "cats"[All Fields] OR "felines"[All Fields] OR "felidae"[MeSH Terms] OR "felidae"[All Fields] OR "feline"[All Fields])). Search results were saved as a comma-separated value (CSV) file, subsequently imported into Microsoft® Excel®. Study eligibility was manually assessed by two independent

investigators in a blinded manner. Only available original research articles concerning *Leishmania* infection in cats were retained, including those published in languages other than English (Figure 6). Except for the epidemiological section (which included data from all *Leishmania* spp. in felids belong to the *Felis* genus), the present review refers exclusively to infection of domestic cats (*Felis catus*) by *L. donovani* (sensu lato). Although this complex is formally comprised of *L. donovani* (sensu stricto), *L. chagasi*, and *L. infantum*, for the remainder of this review, *L. infantum* will be used to refer strictly to feline infection by *L. donovani* (s.l.).

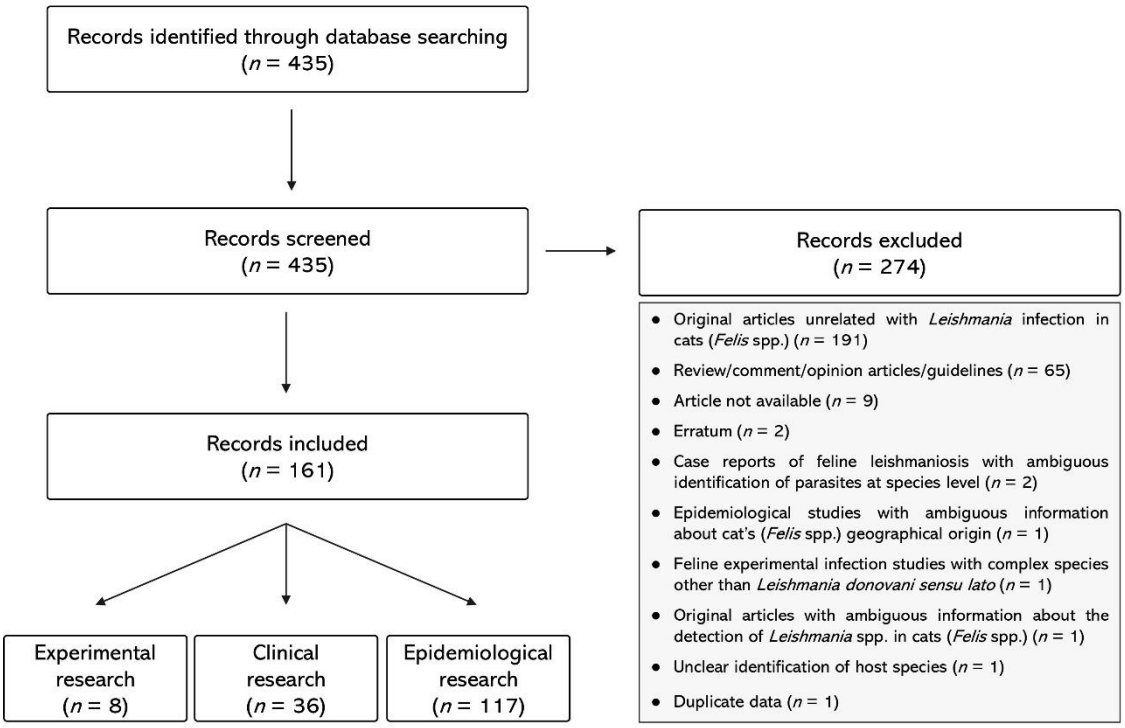


**Figure 6.** Flow diagram of study searching and selection process.

### 5.4. Aetiology, distribution, and risk factors

To date, six species belonging to the subgenus *Leishmania* and one to the subgenus *Viannia* have been identified in domestic cats (*F. catus*) through DNA or isoenzyme-based typing methods (Figure 7):

i. *L.* (*L.*) *amazonensis* in Brazil (de Souza *et al.*, 2005; Carneiro *et al.*, 2020).

ii. *L.* (*L.*) *infantum* in Brazil (Schubach *et al.*, 2004; de Souza *et al.*, 2005; da Silva *et al.*, 2008; Vides *et al.*, 2011; Sobrinho *et al.*, 2012; de Morais *et*

*al.*, 2013; Benassi *et al.*, 2017; Metzdorf *et al.*, 2017; Marcondes *et al.*, 2018; Rocha *et al.*, 2019; Berenguer *et al.*, 2020; da Costa-Val *et al.*, 2020), southern Europe (Ayllón *et al.*, 2008; Maia, Nunes & Campino, 2008; Tabar *et al.*, 2008; Maia *et al.*, 2010; Millán *et al.*, 2011; Ayllón *et al.*, 2012; Chatzis *et al.*, 2014a; Maia *et al.*, 2014; Maia *et al.*, 2015b; Persichetti *et al.*, 2016; Attipa *et al.*, 2017a; Diakou *et al.*, 2017; Otranto *et al.*, 2017; Persichetti *et al.*, 2018; Colella *et al.*, 2019; Ebani *et al.*, 2020; Pereira *et al.*, 2019c, Pereira *et al.*, 2020), western Europe (Ozon *et al.*, 1998; Pratlong *et al.*, 2004; Pocholle *et al.*, 2012; Richter, Schaarschmidt-Kiener & Krudewig, 2014) and western Asia (Hatam *et al.*, 2010; Dincer *et al.*, 2015; Akhtardanesh *et al.*, 2017; Attipa *et al.*, 2017b; Mohebali *et al.*, 2017; Karakuş *et al.*, 2019; Asgari *et al.*, 2020; Baneth *et al.*, 2020).

iii. *L.* (*L.*) *major* in Portugal (Pereira *et al.*, 2020) and Turkey (Paşa *et al.*, 2015).

iv. *L.* (*L.*) *mexicana* in the USA (Craig *et al.*, 1986; Trainor *et al.*, 2010; Minard *et al.*, 2017) and Venezuela (Rivas *et al.*, 2018).

v. *L.* (*L.*) *tropica* in western Asia (Paşa *et al.*, 2015; Can *et al.*, 2016; Akhtardanesh *et al.*, 2017).

vi. *L.* (*L.*) *venezuelensis* in Venezuela (Bonfante-Garrido *et al.*, 1991).

vii. *L.* (*V.*) *braziliensis* in Brazil (Schubach *et al.*, 2004; da Costa-Val *et al.*, 2020) and French Guiana (Rougeron *et al.*, 2011).

Besides, the DNA of *L. infantum* and putative *L. major*/*L. donovani* s.l. hybrid parasites were detected in wild cats (*Felis silvestris*) in Spain (Del Río *et al.*, 2014) and in a domestic cat in mainland Portugal (Pereira *et al.*, 2020), respectively.

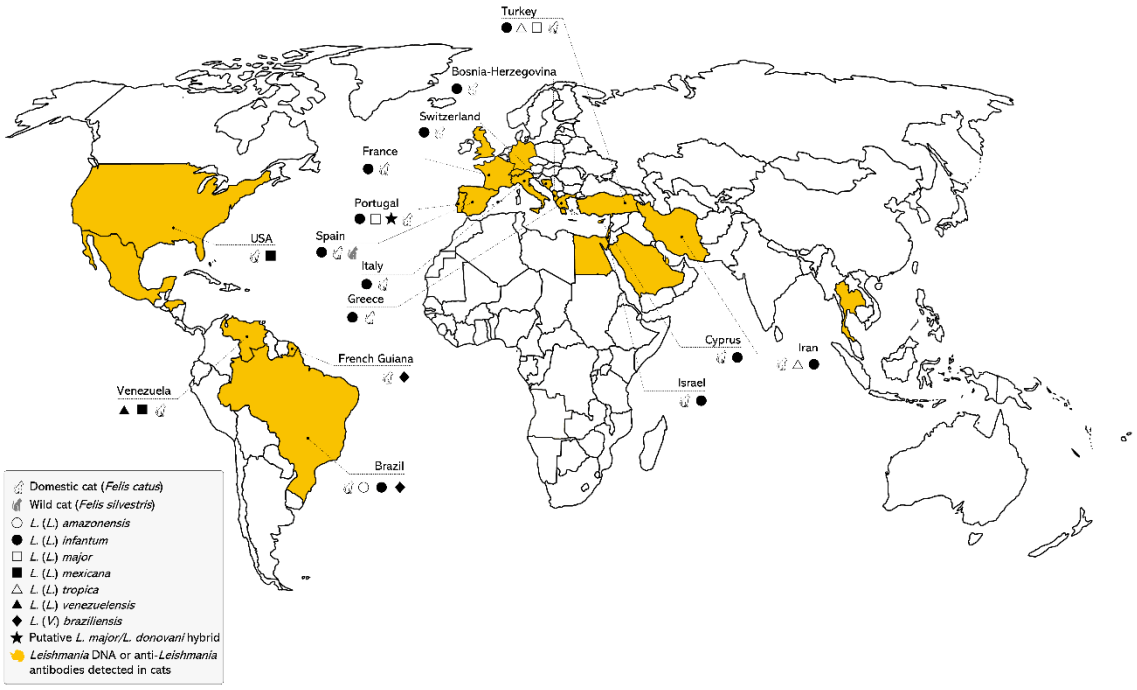


**Figure 7.** Worldwide distribution of *Leishmania* infection in cats (*Felis* sp.) (Author's original).

The proportion of cats infected with or exposed to *Leishmania* has been assessed in several epidemiological studies through parasitological, serological, or molecular methods (Tables 4 and 5). However, reported values vary greatly (from 0 to >70 %) and appear to be influenced by local endemicity, sampling bias and heterogeneity/performance of diagnostic methodologies (mainly cut-off, target gene and sample used for testing).

**Table 4.** Epidemiological studies on the frequency of *Leishmania* infection in cats (*Felis* spp.) in the Old World.

| Country | Study | Sampling year | Species (origin) | No. tested | Method (test, cut-off/target gene) | Sample | % Positive (species)[a] |
|---|---|---|---|---|---|---|---|
| Albania | (Silaghi *et al.*, 2014) | 2008-2010 | *F. catus* (stray) | 146 | Serological (IFAT, 1:64) | Serum | 0.7 (*L. infantum*) |
| | | | | | Molecular (qPCR, kDNA) | Whole blood | 0 |
| Angola | (Lopes *et al.*, 2017) | 2014-2016 | *F. catus* (domestic) | 102 | Serological (DAT, 1:100) | Serum | 0 |
| Bosnia and Herzegovina | (Colella *et al.*, 2019) | 2017 | *F. catus* (domestic) | 5 | Serological (IFAT) | Serum | 0 |
| | | | | | Molecular (qPCR, kDNA) | Whole blood | 20 (*Leishmania* spp.) |
| | | | | 1[b] | Molecular (PCR, kDNA) | Whole blood | 100 (*L. infantum*) |
| | | | | | Molecular (qPCR, ITS2) | Whole blood | 100 (*L. infantum*) |
| Cyprus | (Attipa *et al.*, 2017b) | 2014 | *F. catus* (domestic/shelter) | 164 | Serological (ELISA, 32 EU) | Serum | 4.4 (*L. infantum*) |
| | | | | 174 | Molecular (qPCR, kDNA) | Whole blood | 2.3 (*L. infantum*) |
| Egypt | (Michael *et al.*, 1982) | na | *F. catus* (stray) | 80 | Serological (IHA) | Serum | 3.8 (*Leishmania* spp.) |
| | (Morsy *et al.*, 1988) | na | *F. catus* (stray) | 28 | Serological (IHA) | Serum | 3.6 (*Leishmania* spp.) |
| | (Morsy & Abou el Seoud, 1994) | na | *F. catus* (domestic/stray) | 60 | Serological (IHA, 1:32) | Serum | 10 (*Leishmania* spp.) |
| Germany | (Schäfer *et al.*, 2021) | 2012-2020 | *F. catus* (domestic) | 624 | Serological (IFAT, 1:64) | Serum | 4.0 (*Leishmania* spp.) |
| Greece | (Chatzis *et al.*, 2014a, 2014b) | 2009-2011 | *F. catus* (domestic) | 100 | Parasitological (cytology) | Bone marrow | 0 |
| | | | | | | Lymph node | 0 |
| | | | | | | Skin | 0 |
| | | | | | Serological (ELISA, 0.145) | Serum | 1.0 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:10) | Serum | 10 (*Leishmania* spp.) |
| | | | | | Molecular (PCR, kDNA) | Bone marrow | 16.0 (*L. infantum*) |
| | | | | | | Whole blood | 13.0 (*L. infantum*) |
| | | | | 99 | Molecular (PCR, kDNA) | Skin | 13.1 (*L. infantum*) |
| | | | | 96 | | Conjunctival swab | 3.1 (*L. infantum*) |
| | (Diakou *et al.*, 2017) | 2015 | *F. catus* (stray) | 148 | Serological (IFAT, 1:80) | Serum | 6.1 (*L. infantum*) |
| | | | | | Molecular (nPCR, SSU) | Whole blood | 6.1 (*L. infantum*) |
| | (Diakou, Papadopoulos & Lazarides, 2009) | na | *F. catus* (stray) | 284 | Serological (ELISA) | Serum | 3.9 (*Leishmania* spp.) |
| | (Morelli *et al.*, 2020) | na | *F. catus* | 153 | Serological (IFAT, 1:80) | Serum | 2.0 (*L. infantum*) |
| Iran | (Mohebali *et al.*, 2017) | 2013-2015 | *F. catus* (stray) | 103 | Serological (DAT, 1:320) | Serum | 3.9 (*L. infantum*) |
| | | | | 4[b] | Parasitological (cytology) | Liver | 25.0 (*L. infantum*) |
| | | | | | | Spleen | 25.0 (*L. infantum*) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | 4[b] | Parasitological (culture) | Liver | 0 |
| | | | | | | Spleen | 0 |
| | | | | 1[b] | Molecular (nPCR, ITS2) | Liver | 100 (*L. infantum*) |
| | | | | | | Spleen | 100 (*L. infantum*) |
| | (Akhtardanesh *et al.*, 2020) | 2016 | *F. catus* (stray) | 180 | Molecular (nPCR, kDNA) | Whole blood | 13.9 (*L. infantum*) |
| | (Asgari *et al.*, 2020) | 2016-2018 | *F. catus* (stray) | 174 | Serological (DAT, 1:100) | Serum | 17.2 (*L. infantum*) |
| | | | | | Serological (ELISA) | Serum | 27.6 (*L. infantum*) |
| | | | | | Molecular (nPCR, kDNA) | Buffy coat | 20.7 (*L. infantum*) |
| | (Sarkari *et al.*, 2009) | na | *F. catus* (stray) | 40 | Serological (DAT, 1:20) | Serum | 20 (*L. infantum*) |
| | | | | | Serological (IFAT, 1:10) | Serum | 25.0 (*L. infantum*) |
| | (Hatam *et al.*, 2010) | na | *F. catus* (stray) | 40 | Parasitological (cytology) | Liver | 2.5 (*Leishmania* spp.) |
| | | | | | | Spleen | 2.5 (*Leishmania* spp.) |
| | | | | | Parasitological (culture) | Liver | 7.5 (*Leishmania* spp.) |
| | | | | | | Spleen | 2.5 (*Leishmania* spp.) |
| | | | | | Molecular (PCR, kDNA | Liver | 7.5 (*L. infantum*) |
| | | | | | | Spleen | 5.0 (*L. infantum*) |
| | (Fatollahzadeh *et al.*, 2016) | na | *F. catus* (stray) | 65 | Parasitological (cytology) | Liver | 0 |
| | | | | | | Spleen | 0 |
| | | | | | Parasitological (culture) | Liver | 0 |
| | | | | | | Spleen | 0 |
| | | | | | Serological (DAT, 1:320) | Serum | 23.1 (*L. infantum*) |
| | | | | | Molecular (PCR, kDNA) | Spleen | 0 |
| | (Akhtardanesh *et al.*, 2017) | na | *F. catus* (stray) | 60 | Serological (ELISA) | Serum | 6.7 (*L. infantum*) |
| | | | | | Molecular (nPCR, 7SL RNA) | Whole blood | 16.7 (*L. infantum*) |
| | | | | | | | 1.7 (*L. tropica*) |
| Iraq | (Otranto *et al.*, 2019) | 2008 | *F. catus* (stray) | 207 | Molecular (qPCR, kDNA) | Whole blood | 0 |
| Israel | (Nasereddin, Salant & Abdeen, 2008) | 1999-2000 | *F. catus* (domestic/stray) | 104 | Serological (ELISA) | Serum | 6.7 (*L. infantum*) |
| | (Baneth *et al.*, 2020) | 2018 | *F. catus* (shelter) | 67 | Serological (ELISA, 0.4) | Serum | 75.0 (*L. infantum*) |
| | | | | | Molecular (qPCR, kDNA) | Whole blood | 16.0 (*L. infantum*) |
| | | | | | Molecular ($_{HRM}$PCR, ITS1) | Whole blood | 0 |
| Italy | (Vita *et al.*, 2005) | 2002-2004 | *F. catus* (domestic/stray) | 203 | Serological (IFAT, 1:40) | Serum | 16.3 (*L. infantum*) |
| | | | | 11[b] | Molecular (PCR) | Lymph node | 100 (*L. infantum*) |
| | | | | | | Whole blood | 45.5 (*L. infantum*) |
| | (Spada *et al.*, 2013) | 2008-2010 | *F. catus* (stray) | 233 | Serological (IFAT, 1:40) | Serum | 25.3 (*L. infantum)* |
| | | | | | Molecular (qPCR, kDNA) | Whole blood | 0 |
| | (Morganti *et al.*, 2019) | 2010-2016 | *F. catus* (shelter/stray) | 286 | Serological (IFAT, 1:40) | Serum | 9.1 (*L. infantum*) |
| | | | | | Molecular (nPCR, SSU) | Buffy coat | 0 |
| | | | | | | Conjunctival swab | 15.7 (*L. infantum*) |
| | (Dedola *et al.*, 2018) | 2011-2013 | *F. catus* (domestic) | 90 | Serological (IFAT, 1:40) | Serum | 10 (*L. infantum*) |
| | | | | | Molecular (nPCR, ITS) | Whole blood | 5.5 (*L. infantum*) |
| | (Veronesi *et al.*, 2016) | 2011-2014 | *F. silvestris* (wild) | 21 | Molecular (qPCR, *COII*) | Spleen | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| (Persichetti *et al.*, 2016) | 2012-2013 | *F. catus* (domestic) | 42 | Serological (IFAT, 1:80) | Serum | 2.4 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Whole blood | 7.1 (*L. infantum*) |
| (Persichetti *et al.*, 2018) | 2012-2013 | *F. catus* (domestic) | 197 | Parasitological (cytology) | Whole blood | 0 |
| | | | | Serological (IFAT, 1:80) | Serum | 9.6 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Conjunctival swab | 1.5 (*L. infantum*) |
| | | | 181 | Molecular (qPCR, kDNA) | Lymph node | 1.7 (*L. infantum*) |
| | | | 143 | Molecular (qPCR, kDNA) | Urine | 2.1 (*L. infantum*) |
| | | | 197 | Molecular (qPCR, kDNA) | Oral Swab | 1.5 (*L. infantum*) |
| | | | | | Whole blood | 2.0 (*L. infantum*) |
| (Spada *et al.*, 2016) | 2014 | *F. catus* (stray) | 90 | Serological (IFAT, 1:40) | Serum | 30 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Conjunctival swab | 0 |
| | | | | | Lymph node | 1.1 (*L. infantum*) |
| | | | | | Whole blood | 1.1 (*L. infantum*) |
| (Brianti *et al.*, 2017) | 2015 | *F. catus* (domestic) | 159 | Serological (IFAT, 1:80) | Serum | 9.4 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Conjunctival swab | 3.8 (*L. infantum*) |
| | | | | | Whole blood | 7.5 (*L. infantum*) |
| (Otranto *et al.*, 2017) | 2015-2016 | *F. catus* (domestic) | 330 | Serological (IFAT, 1:40) | Serum | 25.7 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Conjunctival swab | 1.8 (*L. infantum*) |
| | | | | | Whole blood | 2.1 (*L. infantum*) |
| (Abbate *et al.*, 2019) | 2015-2017 | *F. silvestris* (wild) | 11 | Molecular (qPCR, kDNA) | Lymph node/skin/spleen | 0 |
| (Priolo *et al.*, 2019) | 2016-2017 | *F. catus* (domestic/stray) | 66 | Serological (ELISA) | Serum | 17.0 (*L. infantum*) |
| | | | | Serological (IFAT, 1:80) | Serum | 14.0 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Whole blood | 4.0 (*L. infantum*) |
| (Spada *et al.*, 2020) | 2016-2018 | *F. catus* (stray) | 102 | Serology (IFAT, 1:80) | Serum | 4.9 (*L. infantum*) |
| | | | 117 | Molecular (qPCR, kDNA) | Conjunctival swab | 0 |
| | | | 115 | Molecular (qPCR, kDNA) | Lymph node | 4.3 (*L. infantum*) |
| | | | 109 | Molecular (qPCR, kDNA) | Whole blood | 0 |
| (Urbani *et al.*, 2020) | 2017 | *F. catus* (domestic) | 152 | Serological (IFAT, 1:80) | Serum | 11.8 (*L. infantum*) |
| | | | 150 | Molecular (qPCR, kDNA) | Conjunctival swab | 0 |
| | | | | | Hair | 0.7 (*L. infantum*) |
| | | | 146 | Molecular (qPCR, kDNA) | Whole blood | 0 |
| (Iatta *et al.*, 2019) | 2017-2018 | *F. catus* (domestic) | 2659 | Serological (IFAT, 1:80) | Serum | 3.3 (*L. infantum*) |
| | | | | Molecular (qPCR, kDNA) | Whole blood | 0.8 (*L. infantum*) |
| (Ebani *et al.*, 2020) | 2018-2019 | *F. catus* (stray) | 85 | Serological (IFAT) | Serum | 2.4 (*Leishmania* spp.) |
| | | | | Molecular (PCR, SSU) | Blood[c] | 5.9 (*Leishmania* spp.) |
| (Persichetti *et al.*, 2017) | 2013 | na | 76 | Serological (ELISA, 40 EU) | Serum | 2.6 (*L. infantum*) |
| | | | | Serological (IFAT, 1:80) | Serum | 17.1 (*L. infantum*) |
| | | | | Serological (WB) | Serum | 18.4 (*L. infantum*) |
| | | | 21[b] | Serological (ELISA, 40 EU) | Serum | 100 (*L. infantum*) |
| | | | | Serological (IFAT, 1:80) | Serum | 95.2 (*L. infantum*) |
| | | | | Serological (WB) | Serum | 100 (*L. infantum*) |
| (Poli *et al.*, 2002) | na | F. catus (domestic) | 110 | Serological (IFAT, 1:80) | Serum | 0.9 (*Leishmania* spp.) |
| (Morelli *et al.*, 2019) | na | *F. catus* (domestic) | 167 | Serological (IFAT, 1:80) | Serum | 3.0 (*L. infantum*) |
| (Morelli *et al.*, 2020) | na | *F. catus* | 116 | Serological (IFAT, 1:80) | Serum | 4.3 (*L. infantum*) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Portugal | (Duarte *et al.*, 2010) | 2003-2005 | *F. catus* (stray) | 180 | Serology (IFAT, 1:40) | Serum | 0.6 (*L. infantum*) |
| | (Maia, Nunes & Campino, 2008) | 2004 | *F. catus* (stray) | 20 | Serological (IFAT, 1:64) | Serum | 0 |
| | | | | 23 | Molecular (PCR, ITS1) | Blood on filter paper | 30.4 (*Leishmania* spp.) |
| | | | | | Molecular (PCR, kDNA) | Blood on filter paper | 30.4 (*Leishmania* spp.) |
| | | | | 4[b] | Molecular (PCR-RFLP, ITS1) | Blood on filter paper | 100 (*L. infantum*) |
| | (Cardoso *et al.*, 2010) | 2004-2008 | *F. catus* (domestic) | 316 | Serological (DAT, 1:100) | Serum | 1.9 (*L. infantum*) |
| | | | | | Serological (ELISA) | Serum | 2.8 (*L. infantum*) |
| | (Maia *et al.*, 2010) | 2007-2008 | *F. catus* (domestic/stray) | 76 | Serological (IFAT, 1:64) | Serum | 1.3 (*Leishmania* spp.) |
| | | | | 138 | Molecular (PCR, kDNA) | Whole blood | 20.3 (*L. infantum*) |
| | (Maia *et al.*, 2015a) | 2011-2014 | *F. catus* (domestic/stray) | 271 | Serological (DAT, 1:100) | Serum | 3.7 (*L. infantum*) |
| | (Maia *et al.*, 2014) | 2012-2013 | *F. catus* (domestic/stray) | 649 | Molecular (nPCR, SSU) | Whole blood | 9.9 (*Leishmania* spp.) |
| | (Pereira *et al.*, 2019a, 2019b, 2020) | 2017-2018 | *F. catus* (domestic/ shelter/stray) | 373 | Serological (IFAT, 1:64) | Serum | 1.6 (*Leishmania* spp.) |
| | | | | 465 | Molecular (nPCR, SSU) | Buffy coat | 5.4 (*Leishmania* spp.) |
| | | | | 25[b] | Molecular (nPCR, *cytB*) | Buffy coat | 12.0 (*L. donovani* s.l.)<br>4.0 (*L. major*)<br>4.0 (*L. major*/*L. donovani* s.l.)[f] |
| | | | | | Molecular (PCR, *g6pdh*) | Buffy coat | 4.0 (L. donovani s.l.) |
| | | | | | Molecular (nPCR, *hsp70*) | Buffy coat | 12.0 (*L. donovani* s.l.)<br>4.0 (*L. major*/*L. donovani* s.l.)[f] |
| | | | | | Molecular (nPCR, ITS) | Buffy coat | 12.0 (*L. donovani* s.l.)<br>4.0 (*L. major*) |
| | (Neves *et al.*, 2020) | 2018-2019 | *F. catus* (domestic) | 141 | Serological (DAT, 1:100) | Serum | 0 |
| | (Vilhena *et al.*, 2013) | na | *F. catus* (domestic) | 320 | Molecular (qPCR, kDNA) | Whole blood | 0.3 (*L. infantum*) |
| Portugal/Spain | (Mesa-Sanchez *et al.*, 2020) | 2015-2020 | *F. catus* (domestic)[g] | 173 | Molecular (nPCR, SSU) | Whole blood | 0 |
| Qatar | (Lima *et al.*, 2019) | 2016-2018 | *F. catus* (domestic/stray) | 79 | Molecular (qPCR, kDNA) | Whole blood/on dried spot | 1.3 (*Leishmania* spp.) |
| Saudi Arabia | (Morsy, Aldakhil & el-Bahrawy, 1999) | na | *F. margarita* (wild) | 10 | Parasitological (cytology) | Liver | 20 (*Leishmania* spp.) |
| | | | | | | Spleen | 40 (*Leishmania* spp.) |
| | | | | | Serological (IHA, 1:64) | Serum | 40 (*Leishmania* spp.) |
| Spain | (Del Río *et al.*, 2014) | 2001-2006 | *Felis silvestris* (wild) | 4 | Molecular (qPCR, kDNA) | Liver and/or spleen | 25.0 (*L. infantum*) |
| | | | | 1[b] | Molecular (PCR, ITS2) | Liver and/or spleen | 100 (*L. infantum*) |
| | (Martín-Sánchez *et al.*, 2007) | 2003-2004 | *F. catus* (domestic) | 183 | Serological (IFAT, 1:40) | Serum | 28.3 (*Leishmania* spp.) |
| | | | | | Molecular (PCR-ELISA, kDNA) | Whole blood | 25.7 (*L. infantum*) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | 7[b] | Parasitological (culture) | Leucoconcentrate | 0 |
| | | | | | Parasitological (cytology) | Leucoconcentrate | 42.9 (*Leishmania* spp.) |
| | (Ayllón *et al.*, 2008) | 2005-2006 | *F. catus* (domestic) | 233 | Serological (IFAT, 1:100) | Serum | 1.3 (*L. infantum*) |
| | | | | | Molecular (PCR, kDNA) | Whole blood | 0.4 (*L. infantum*) |
| | (Ayllón *et al.*, 2012) | 2005-2008 | *F. catus* (domestic/stray) | 680 | Serological (IFAT, 1:50) | Serum | 3.7 (*L. infantum*) |
| | | | | | Molecular (PCR, kDNA) | Whole blood | 0.6 (*L. infantum*) |
| | (Tabar *et al.*, 2008) | 2006 | *F. catus* (domestic) | 100 | Molecular (qPCR, kDNA) | Whole blood | 3.0 (*L. infantum*) |
| | (Sherry *et al.*, 2011) | 2008 | *F. catus* (shelter) | 105 | Serological (ELISA) | Serum | 13.2 (*L. infantum*) |
| | | | | 104 | Molecular (qPCR, kDNA) | Whole blood | 8.7 (*L. infantum*) |
| | (Millán *et al.*, 2011) | 2008-2009 | *F. catus* (stray) | 83 | Serological (WB) | Serum | 15.7 (*L. infantum*) |
| | | | | 73 | Molecular (PCR, kDNA) | Blood and/or spleen | 25.6 (*L. infantum*) |
| | | | | 14[b] | Molecular (PCR-RFLP, kDNA) | Blood and/or spleen | 100 (*L. infantum*) |
| | (Miró *et al.*, 2014) | 2012-2013 | *F. catus* (stray) | 346 | Serological (IFAT, 1:100) | Serum | 3.2 (*L. infantum*) |
| | | | | 57[d] | Molecular (nested-PCR, ITS1) | Whole blood | 0 |
| | | | | | Molecular (nested-PCR, SSU) | Whole blood | 0 |
| | (Risueño *et al.*, 2018) | 2013-2015 | *F. silvestris* (wild) | 2 | Molecular (qPCR, kDNA) | Skin | 50 (*L. infantum*) |
| | | | | | | Other organs[e] | 0 |
| | (Marenzoni *et al.*, 2018) | 2014-2015 | *F. catus* (domestic) | 31[g] | Molecular (PCR, kDNA) | Whole blood | 0 |
| | (Montoya *et al.*, 2018a) | 2014-2017 | *F. catus* (stray) | 249 | Serological (IFAT, 1:100) | Serum | 4.8 (*L. infantum*) |
| | | | | | Molecular (PCR, ITS) | Skin/whole blood | 0 |
| | (Priolo *et al.*, 2019) | 2016-2017 | *F. catus* (domestic/stray) | 113 | Serological (ELISA) | Serum | 7.0 (*L. infantum*) |
| | | | | | Serological (IFAT, 1:80) | Serum | 19.0 (*L. infantum*) |
| | | | | | Molecular (qPCR, kDNA) | Whole blood | 5.0 (*L. infantum*) |
| | (Villanueva-Saz *et al.*, 2021) | 2020 | *F. catus* (stray) | 114 | Serological (ELISA, 13 EU) | Serum | 16.7 (*L. infantum*) |
| | (Solano-Gallego *et al.*, 2007) | na | *F. catus* (domestic/stray) | 445 | Serological (ELISA-IgG, 53 EU) | Serum | 5.3 (*L. infantum*) |
| | | | | | Serological (ELISA-Prot A, 44 EU) | Serum | 6.3 (*L. infantum*) |
| | (Alcover *et al.*, 2020) | na | *F. catus* (wild) | 1 | Molecular (qPCR, kDNA) | Liver | 100 (*Leishmania* spp.) |
| | | | | | | Skin | 100 (*Leishmania* spp.) |
| | | | | | | Spleen | 100 (*Leishmania* spp.) |
| | (Miró *et al.*, 2011) | na | *F. catus* (breeding) | 20 | Serological (IFAT, 1:100) | Serum | 15.0 (*L. infantum*) |
| | (Moreno *et al.*, 2014) | na | *F. catus* (stray) | 43 | Serological (IFAT, 1:50) | Serum | 4.3 (*L. infantum*) |
| | (Montoya *et al.*, 2018b) | na | *F. catus* (stray) | | Serological (IFAT, 1:100) | Serum | 0 |
| Thailand | (Sukmee *et al.*, 2008) | 2006 | *F. catus* | 15 | Serological (DAT; 1:100) | Serum | 60 (*Leishmania* spp.) |
| | | | | 9[b] | Molecular (PCR, ITS1) | Whole blood | 0 |
| | | | | | Molecular (PCR, kDNA) | Whole blood | 0 |
| | (Junsiri *et al.*, 2017) | 2013 | *F. catus* (domestic) | 250 | Serological (ELISA, 0.2) | Serum | 5.6 (*L. infantum*) |
| | | | | | Molecular (PCR, kDNA) | Whole blood | 0 |
| | (Kongkaew *et al.*, 2007) | na | na | 5 | Serological (DAT, 1:100) | Serum | 20 (*Leishmania* spp.) |
| | | | | 1[b] | Molecular (PCR) | Whole blood | 0 |
| Turkey | (Dincer *et al.*, 2015) | 2013 | *F. catus* (domestic/shelter) | 22 | Molecular (nPCR, kDNA) | Whole blood | 4.5 (*L. infantum*) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (Karakuş *et al.*, 2019) | 2014 | *F. catus* (stray) | 5 | Molecular (nPCR, SSU) | Conjunctival swab | 0 |
| | | 2015 | | 8 | Molecular (qPCR, ITS1) | Conjunctival swab | 12.5 (*L. infantum*) |
| | | 2016 | | 6 | Molecular (qPCR, ITS1) | Conjunctival swab | 0 |
| | (Dincer *et al.*, 2016) | 2015 | *F. catus* (domestic/shelter) | 50 | Molecular (nPCR, kDNA) | na | 0 |
| | (Dinçer *et al.*, 20121) | na | *F. catus* (domestic) | 1 | Serological (IFAT) | Serum | 0 |
| | (Paşa *et al.*, 2015) | na | *F. catus* (domestic) | 147 | Molecular (qPCR, ITS1) | Whole blood | 2.7 (*L. major*) |
| | | | | | | | 8.8 (*L. tropica*) |
| | | | | | Molecular (qPCR, *hsp70*) | Whole blood | 2.0 (*L. major*) |
| | | | | | | | 2.7 (*L. tropica*) |
| | | | | | | | 2.7 (*Leishmania* spp.) |
| | (Can *et al.*, 2016) | na | *F. catus* (shelter) | 1101 | Serological (ELISA) | Serum | 10.8 |
| | | | | | Serological (IFAT, 1:40) | Serum | 15.2 |
| | | | | | Molecular (qPCR, ITS1) | Whole blood | 0.1 (*L. tropica*) |
| | | | | | Molecular (nPCR, kDNA) | Whole blood | 0.1 (*L. infantum*) |
| | | | | | | | 0.5 (*L. tropica*) |
| UK | (Persichetti *et al.*, 2017) | 2013 | *F. catus* | 64 | Serological (ELISA, 40 EU) | Serum | 1.6 (*L. infantum*) |
| | | | | | Serological (IFAT, 1:80) | Serum | 0 |
| | | | | | Serological (WB) | Serum | 3.1 (*L. infantum*) |
| Uzbekistan | (Kovalenko *et al.*, 2011) | na | *F. catus* | 1 | Serological (ELISA) | Serum | 0 |

[a] Species defined according to the original study; [b] previously identified as positive by another test; [c] DNA extracted from the sediment obtained after centrifugation of the blood samples; [d] Seropositive for *L. infantum* and/or for feline retrovirus (feline leukemia virus and/or feline immunodeficiency virus); [e] not specified; [f] putative hybrid; [g] cats eligible for blood donation.

Abbreviations: *COII*, cytochrome oxidase II; *cytB*, cytochrome b; DAT, direct agglutination test; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; *F.*, *Felis*; *g6pdh*, glucose-6-phosphate dehydrogenase; $_{HRM}$PCR, high resolution melt PCR; *hsp70*, heat-shock protein 70; IFAT, immunofluorescence antibody test; IgG, Immunoglobulin G; IHA, indirect hemagglutination; ITS, internal transcriber spacers; ITS1, internal transcriber spacer 1; ITS2, internal transcriber spacer 2; kDNA, kinetoplast minicircle DNA; *L.*, *Leishmania*; na, not available; nPCR, nested-PCR; PCR, one-step PCR (polymerase chain reaction); Prot A, Protein A; qPCR, real-time PCR; RFLP, restriction fragment length polymorphism; s.l., sensu lato; SSU, small subunit ribosomal DNA; WB, western blot.

**Table 5.** Epidemiological studies on the frequency of *Leishmania* infection in cats (*Felis* spp.) in the New World.

| Country | Study | Sampling year | Species (origin) | No. tested | Method (test, cut-off/target gene) | Sample | % Positive (species)[a] |
|---|---|---|---|---|---|---|---|
| Brazil | (de Matos *et al.*, 2018) | 2004-2014 | *F. catus* | 679 | Serological (ELISA) | Serum | 43.4 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:40) | Serum | 15.8 (*Leishmania* spp.) |
| | (Figueiredo *et al.*, 2009) | 2005 | *F. catus* (domestic) | 43 | Serological (ELISA) | Serum | 2.4 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:40) | Serum | 0.0 |
| | (Coelho *et al.*, 2011a) | 2007-2009 | *F. catus* | 70 | Serological (ELISA) | Serum | 4.2 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:40) | Serum | 0.0 (*Leishmania* spp.) |
| | (Vides *et al.*, 2011) | 2008-2009 | *F. catus* | 55 | Parasitological (cytology) | Bone marrow | 12.7 (*Leishmania* spp.) |
| | | | | | | Liver | 3.6 (*Leishmania* spp.) |
| | | | | | | Lymph node | 5.5 (*Leishmania* spp.) |
| | | | | | | Spleen | 7.3 (*Leishmania* spp.) |
| | | | | | Parasitological (IHC) | Skin | 16.4 (*Leishmania* spp.) |
| | | | | | Serological (ELISA, 0.277) | Serum | 25.4 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:40) | Serum | 10.9 (*Leishmania* spp. |
| | | | | 3 | Molecular (qPCR, *gp63*) | Whole blood | 100.0 (*L. chagasi*) |
| | (Cardia *et al.*, 2013) | 2010 | *F. catus* (shelter/stray) | 386 | Serological (IFAT, 1:40) | Serum | 0.5 (*Leishmania* spp.) |
| | (Silva *et al.*, 2014) | 2010 | *F. catus* (domestic/shelter) | 153 | Serological (ELISA) | Serum | 3.9 (*L. infantum*) |
| | (de Sousa Oliveira *et al.*, 2015) | 2012 | *F. catus* | 52 | Molecular (PCR, kDNA) | Conjunctival swab | 13.5 (*Leishmania* spp.) |
| | (de Sousa *et al.*, 2014) | 2013 | *F. catus* (domestic/stray) | 151 | Serological (IFAT, 1:40) | Serum | 6.6 (*L. infantum*) |
| | (Metzdorf *et al.*, 2017) | 2013-2014 | *F. catus* (domestic/shelter) | 100 | Parasitological (cytology) | Bone marrow | 4.0 (*Leishmania* spp.) |
| | | | | | | Lymph node | 4.0 (*Leishmania* spp.) |
| | | | | | | Whole blood | 4.0 (*Leishmania* spp.) |
| | | | | | Molecular (PCR-RFLP, kDNA) | Bone marrow | 6.0 (*L. infantum*) |
| | | | | | | Lymph node | 3.0 (*L. infantum*) |
| | | | | | | Whole blood | 4.0 (*L. infantum*) |
| | (Leonel *et al.*, 2020) | 2014 | *F. catus* (shelter) | 94 | Serological (ELISA) | Serum | 31.9 (*Leishmania* spp.) |
| | | | | | Serological (IFAT, 1:40) | Serum | 29.8 (*Leishmania* spp.) |
| | | | | | Molecular (PCR, kDNA) | Conjunctival swab | 0.0 |
| | | | | | | Whole blood | 0.0 |
| | (Marcondes *et al.*, 2018) | 2014-2015 | *F. catus* (domestic/shelter) | 50[b] | Parasitological (cytology) | Bone marrow | 14.0 (*Leishmania* spp.) |
| | | | | | Molecular (qPCR, kDNA) | Bone marrow | 86.0 (*L. infantum*) |
| | | | | | | Whole blood | 72.0 (*L. infantum*) |
| | (Rocha *et al.*, 2019) | 2016-2017 | *F. catus* (domestic) | 105 | Serological (IFAT, 1:40) | Serum | 30.5 (*L. infantum*) |
| | | | | | Molecular (PCR, CH1) | Whole blood | 2.9 (*L. infantum*) |
| | | | | | Molecular (PCR, ITS1) | Whole blood | 5.7 (*L. infantum*) |
| | (Pedrassani *et al.*, 2019) | 2017 | *F. catus* (domestic) | 30 | Serological (IFAT, 1:80) | Serum | 6.6 (*L. infantum*) |
| | | | | | Molecular (PCR, kDNA) | Whole blood | 0.0 |
| | (Berenguer *et al.*, 2020) | 2017 | *F. catus* (domestic) | 128 | Molecular (PCR, kDNA) | Conjunctival swab | 0.0 |
| | | | | | | Whole blood | 0.8 (*L. infantum*) |
| | | | | 3[c] | Parasitological (cytology) | Lymph node | 33.3 (*Leishmania* ssp.) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Molecular (PCR, kDNA) | Lymph node | 33.3 (*L. infantum*) |
| (Bezerra *et al.*, 2019) | 2017-2018 | *F. catus* (domestic) | 91 | Serological (IFAT, 1:40) | Serum | 15.4 (*Leishmania* spp.) |
| | | | | Molecular (PCR, kDNA) | Whole blood | 0.0 |
| (da Silva *et al.*, 2008) | na | *F. catus* (domestic) | 8 | Serological (IFAT, 1:40) | Serum | 25.0 (*Leishmania* spp.) |
| | | | 3 | Molecular (multiplex PCR, kDNA) | Whole blood | 66.7 (*Leishmania* spp.) |
| | | | 2[b] | Molecular (DB) | Whole blood | 100 (*L. chagasi*) |
| (Bresciani *et al.*, 2010) | na | *F. catus* (domestic) | 283 | Parasitological (cytology) | Lymph node | 0.7 (*Leishmania* spp.) |
| | | | | Serological (IFAT, 1:40) | Serum | 0.0 |
| (da Silveira Neto *et al.*, 2011) | na | *F. catus* (shelter) | 130 | Serological (CAG-ELISA, 0.449) | Serum | 23.0 (*Leishmania* spp.) |
| | | | | Serological (FML-ELISA, 0.215) | Serum | 13.3 (*Leishmania* spp.) |
| | | | | Serological (rK39-ELISA, 0.347) | Serum | 15.9 (*Leishmania* spp.) |
| (Coelho *et al.*, 2011b) | na | *F. catus* (domestic) | 52 | Parasitological (cytology) | Bone marrow | 0.0 |
| | | | | | Lymph node | 3.8 (*Leishmania* spp.) |
| | | | | | Spleen | 0.0 |
| | | | | Molecular (PCR, kDNA) | Bone marrow | 0.0 |
| | | | | | Lymph node | 3.8 (*L. chagasi*) |
| | | | | | Spleen | 1.9 (*L. chagasi*) |
| (Sobrinho *et al.*, 2012) | na | *F. catus* (shelter/stray) | 302 | Parasitological (Cytology) | Bone marrow | 7.0 (*Leishmania* spp.) |
| | | | | | Lymph node | 7.9 (*Leishmania* spp.) |
| | | | | Serological (ELISA, 0.301) | Serum | 13.0 (*Leishmania* spp.) |
| | | | | Serological (IFAT, 1:40) | Serum | 4.6 (*Leishmania* spp.) |
| | | | 5[b] | Molecular (qPCR, *gp63*) | Whole blood | 100 (*L. infantum*) |
| (de Morais *et al.*, 2013) | na | *F. catus* (domestic) | 5 | Molecular (qPCR, kDNA) | Whole blood | 80.0 (*L. infantum*) |
| | | | | Molecular (PCR, kDNA) | Whole blood | 80.0 (*L. infantum*) |
| (Braga *et al.*, 2014) | na | *F. catus* (domestic) | 50 | Serological (IFAT, 1:40) | Serum | 4.0 (*Leishmania* spp.) |
| (Braga, Langoni & Lucheis, 2014) | na | *F. catus* | 100 | Parasitological (culture) | Whole blood | 2.0 (*Leishmania* spp.) |
| | | | | Serological (IFAT, 1:40) | Serum | 15.0 (*Leishmania* spp.) |
| | | | | Molecular, PCR, kDNA) | Whole blood | 0.0 |
| (Oliveira *et al.*, 2015) | na | *F. catus* (domestic) | 443 | Serological (DAT, 1:40) | Serum | 5.6 (*Leishmania* spp.) |
| | | | | Serological (IFAT, 1:40) | Serum | 4.1 (*Leishmania* spp.) |
| (Benassi *et al.*, 2017) | na | *F. catus* (domestic/stray) | 108 | Molecular (PCR, kDNA) | Conjunctival swab | 1.9 (*Leishmania* spp.) |
| | | | | | Whole blood | 0.0 |
| | | | | Molecular (qPCR, kDNA) | Conjunctival swab | 1.9 (*Leishmania* spp.) |
| | | | | | Whole blood | 0.0 |
| | | | 2[b] | Molecular (PCR, ITS1) | Conjunctival swab | 50.0 (*L. infantum*) |
| (Coura *et al.*, 2018) | na | *F. catus* (shelter) | 100 | Parasitological (cytology) | Bone marrow | 0.0 |
| | | | | Parasitological (culture) | Bone marrow | 0.0 |
| | | | | Serological (IFAT, 1:40) | Serum | 54.0 (*Leishmania* spp.) |
| | | | 54[b] | Molecular (PCR, kDNA) | Bone marrow/skin | 0.0 |
| (da Costa-Val *et al.*, 2020) | na | *F. catus* (domestic) | 64 | Serological (ELISA, 0.955) | Serum | 29.8 (*Leishmania* spp.) |
| | | | 64 | Molecular (PCR, kDNA) | Conjunctival swab | 6.3 (*Leishmania* spp.) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | 64 | Molecular (PCR, kDNA) | Oral swab | 4.7 (*Leishmania* spp.) |
| | | | | 8[b] | Molecular (PCR-RFLP, ITS1) | Conjunctival swab | 12.5 (*L. infantum*) |
| | | | | | | Oral swab | 37.5 (*L. infantum*) |
| | | | | | | | 12.5 (*L. braziliensis*) |
| Honduras | (Mccown & Grzeszak, 2010) | na | *F. catus* (stray) | 12 | Serological (IFAT, 1:32) | Serum | 25.0 (*L. donovani*) |
| Mexico | (Longoni *et al.*, 2012) | 2008-2009 | *F. catus* (stray) | 95 | Serological (ELISA-H) | Serum | 5.3 (*L. baziliensis*) |
| | | | | | | | 13.7 (*L. infantum*) |
| | | | | | | | 1.1 (*L. mexicana*) |
| | | | | | Serological (ELISA-SODe) | Serum | 11.6 (*L. baziliensis*) |
| | | | | | | | 22.1 (*L. infantum*) |
| | | | | | | | 10.5 (*L. mexicana*) |
| | | | | | Serological (WB) | Serum | 10.5 (*L. baziliensis*) |
| | | | | | | | 20.0 (*L. infantum*) |
| | | | | | | | 10.5 (*L. mexicana*) |
| Venezuela | (Viettri *et al.*, 2018) | na | na | 5 | Molecular (nested-PCR, ITS1) | Blood on filter paper | 20.0 (*Leishmania* spp.) |
| | | | | | Molecular (nPCR, *SSU*-rDNA) | Blood on filter paper | 20.0 (*Leishmania* spp.) |
| | (Rivas *et al.*, 2018) | | *F. catus* (domestic/stray) | 6 | Parasitological (cytology) | Skin lesions | 66.7 (*Leishmania* spp.) |
| | | | | 5 | Parastiological (histology) | Skin lesions | 80.0 (*Leishmania* spp.) |
| | | | | 5 | Parasitological (IHC) | Skin lesions | 100 (*Leishmania* spp.) |
| | | | | 30 | Serological (ELISA, 15.3 EU) | Serum | 6.7 (*L. braziliensis*) |
| | | | | | Serological (ELISA, 15.3 EU) | Serum | 6.7 (*L. infantum*) |
| | | | | | Serological (WB) | Serum | 33.3 (*L. braziliensis*) |
| | | | | | Serological (WB) | Serum | 33.3 (*L. infantum*) |
| | | | | 31 | Molecular (qPCR, kDNA) | Whole blood | 9.7 (*Leishmania* spp.) |
| | | | | 5 | Molecular (qPCR, kDNA) | Skin lesions | 100 (*Leishmania* spp.) |
| | | | | | Molecular (qPCR, ITS1) | Skin lesions | 40.0 (*L. mexicana*) |
| | | | | 2[b] | Molecular (PCR-RFLP, ITS1) | Skin lesions | 50.0 (*L. mexicana*) |
| | (Paniz Mondolfi *et al.*, 2019) | na | na | 12 | Molecular (nPCR, *cytB*) | Skin lesions | 83.3 (*L. mexicana*) |
| | | | | | | | 16.7 (*Leishmania* spp.) |

[a] Species defined according to the original study; [b] previously identified as positive by another test, [c] cats with lymphadenomegaly.

Abbreviations: CAG, crude antigen; CH1, chitinase; *cytB*, cytochrome b; DAT, direct agglutination test; DB, dot blot; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; *F.*, *Felis*; FML, fucose–mannose ligand; *gp63*, metalloprotease gp63; H, total parasite extract; IFAT, immunofluorescence antibody test; IHC, immunohistochemistry; ITS1, internal transcriber spacer 1; kDNA, kinetoplast minicircle DNA; *L.*, *Leishmania*; na, not available; nPCR, nested-PCR; PCR, one-step PCR (polymerase chain reaction); qPCR, real time-PCR; RFLP, restriction fragment length polymorphism; rK39, recombinant K39; SODe - superoxide dismutase excreted; SSU, small subunit ribosomal DNA; WB, western blot.

Specific antibodies or *Leishmania* DNA have been mostly detected in domestic cats living in endemic areas of South America, the Mediterranean Region and western Asia. Some studies also suggest that wild cats from Spain (Del Río *et al.*, 2014; Risueño *et al.*, 2018) and sand cats (*Felis margarita*) from Saudi Arabia (Morsy, Aldakhil & el-Bahrawy, 1999) are frequently exposed to *Leishmania* infection.

In non-endemic countries, as seen in dogs, feline *Leishmania* infection has been particularly associated with cats travelling to or rehomed from southern Europe and Brazil (Rüfenacht *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Maia & Cardoso, 2015; Schäfer *et al.*, 2021). Also, antibodies to *Leishmania* were detected in three domestic cats living in the United Kingdom, but in all cases, the travel and clinical history were unknown (Persichetti *et al.*, 2017).

Although blood transfusion is regarded as a probable non-vector-borne transmission pathway of *Leishmania* in cats, no feline infection cases by this parasite (screened by PCR) were identified among eligible blood donors (Marenzoni *et al.*, 2018; Mesa-Sanchez *et al.*, 2020).

Several factors have been highlighted as possibly associated with *Leishmania* infection in cats based on univariate analysis, including old age (Akhtardanesh *et al.*, 2017; Junsiri *et al.*, 2017; Morganti *et al.*, 2019; Asgari *et al.*, 2020), male sex (Cardoso *et al.*, 2010; Sobrinho *et al.*, 2012; Montoya *et al.*, 2018a; Asgari *et al.*, 2020; Latrofa *et al.*, 2020), non-neutered status (Otranto *et al.*, 2017; Latrofa *et al.*, 2020), presence of clinical or clinicopathological abnormalities (such as crusting skin lesions, leukopaenia, increase in alanine aminotransferase [ALT] levels, lymphadenomegaly, lymphocytosis and neutrophilia) (Ayllón *et al.*, 2008; Sherry *et al.*, 2011; Sobrinho *et al.*, 2012; Spada *et al.*, 2013; Akhtardanesh *et al.*, 2017; Otranto *et al.*, 2017; Latrofa *et al.*, 2020), concomitant infections (such as feline coronavirus [FCoV], feline immunodeficiency virus [FIV], feline leukaemia virus [FeLV] and *Toxoplasma gondii*) (Sherry *et al.*, 2011; Sobrinho *et al.*, 2012; Spada *et al.*, 2013, 2016; Montoya *et al.*, 2018a), geographical area/local environment (such as altitude and rural areas) (Nasereddin, Salant & Abdeen, 2008; Cardoso *et al.*, 2010; Asgari *et al.*, 2020), lifestyle (such as access to the outdoors) (Rocha *et al.*, 2019) and cohabitation with dogs (Rocha *et al.*, 2019; Morelli *et al.*, 2020). Epidemiological studies using logistic regression models (a powerful analytic research

tool that avoids confounding effects) have evidenced that being adult (Iatta *et al.*, 2019; Akhtardanesh *et al.*, 2020), males (Iatta *et al.*, 2019; Akhtardanesh *et al.*, 2020), non-neutered (Iatta *et al.*, 2019), or with concomitant infections by FeLV (Martín-Sánchez *et al.*, 2007; Sherry *et al.*, 2011; Spada *et al.*, 2013; Akhtardanesh *et al.*, 2020), FIV (Iatta *et al.*, 2019; Akhtardanesh *et al.*, 2020), "*Candidatus* Mycoplasma turicensis" or *Hepatozoon* spp. (Attipa *et al.*, 2017b) have an increased risk for *Leishmania* infection.

### 5.5. Immunopathogenesis

In dogs, several studies have provided evidence demonstrating that the course of *L. infantum* infection is directly linked to the immune response. Development of progressive disease in susceptible dogs is typically characterised by high antibody levels and an impaired ability to mount a strong and effective cell-mediated response characterised by the expression of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukin (IL)-2 (reviewed by Maia and Campino, 2018). However, very limited data are available on the pathogenesis of leishmaniosis in cats. Experimental studies involving intravenous/intraperitoneal inoculation of axenic promastigotes suggest that cats are hypothetically less susceptible to developing disease by *L. infantum* when compared to dogs, despite also presenting a long-lasting parasitaemia (Kirkpatrick, Farrell & Goldschmidt, 1984; Akhtardanesh *et al.*, 2018). Recently, Priolo et al. (2019) demonstrated that cats naturally exposed to *L. infantum* infection produce IFN-γ following *ex vivo* blood stimulation with parasite antigens, as reported in dogs (Solano-Gallego *et al.*, 2016). This finding suggests that *Leishmania* parasites can elicit a protective cell-mediated immune response in cats. The only study assessing the role of the complement system in feline *L. infantum* infection showed that, contrary to humans and dogs, cat's proteins are consumed by parasites in the lectin pathway, which hypothetically may justify their low predisposition to develop clinical disease (Tirado *et al.*, 2021).

### 5.6. Clinical presentation and clinicopathological findings

Feline leishmaniosis caused by *L. infantum* is mostly reported in adult (median age: 7 years; range: 2-21 years) domestic-short hair cats living in or travelling to endemic countries of southern Europe and Brazil. The disease has a chronic course and may be manifested by a plethora of clinical signs and/or clinicopathological abnormalities, which are summarised in Tables 6 and 7, respectively. About one-third of cats with leishmaniosis showed concomitant infections/diseases including FIV (Hervás *et al.*, 2001; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Grevot *et al.*, 2005; Pocholle *et al.*, 2012; Pimenta *et al.*, 2015; Fernandez-Gallego *et al.*, 2020), FeLV (Poli *et al.*, 2002; Grevot *et al.*, 2005; Pereira *et al.*, 2019c), FCoV (Pennisi *et al.*, 2004; Savani *et al.*, 2004), *Toxoplasma gondii* (Pennisi *et al.*, 2004), *Bartonella henselae* (Pennisi *et al.*, 2004), *diabetes mellitus* (Leiva *et al.*, 2005), *pemphigus foliaceus* (Rüfenacht *et al.*, 2005), neoplasia (Grevot *et al.*, 2005; Pocholle *et al.*, 2012; Maia *et al.*, 2015b) and/or were under immunosuppressive therapies at the time of diagnosis (Fernandez-Gallego *et al.*, 2020).

Dermatological disorders were found in about 75 % of reported clinical cases. Although uncommon, they may occur in the apparent absence of other obvious signs of disease (Fernandez-Gallego *et al.*, 2020). Nodular dermatitis seems to be the main cutaneous lesion associated with FeL and is typically found on the eyelids (Hervás *et al.*, 2001; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020). Erosive/ulcerative dermatitis is another clinical finding suggestive of FeL and has been identified on the head (Hervás et al., 2001; Grevot et al., 2005; Coelho et al., 2010; Pocholle et al., 2012; Maia et al., 2015b; Basso et al., 2016; Brianti et al., 2019; Headley et al., 2019; Fernandez-Gallego et al., 2020), extremities (Rüfenacht et al., 2005; Coelho et al., 2010; Basso et al., 2016; Fernandez-Gallego et al., 2020; Silva et al., 2020), trunk (Pocholle *et al.*, 2012; Fernandez-Gallego *et al.*, 2020), and over bony prominences (Hervás *et al.*, 1999). Although less frequent, some cats with clinical leishmaniosis showed onychogryphosis (da Silva *et al.*, 2010; Headley *et al.*, 2019), a rather specific sign of canine leishmaniosis (CanL) (Maia & Campino, 2018). Generalised or focal lymphadenopathy appears as a common finding in FeL (Hervás *et al.*, 1999, 2001; Poli *et al.*, 2002; Savani *et al.*, 2004; Pennisi *et al.*, 2004; Maroli *et al.*, 2007; da Silva *et al.*,

2010; Brianti *et al.*, 2019; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) as well as non-specific signs including lethargy/depression (Poli *et al.*, 2002; Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Pocholle *et al.*, 2012; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Fernandez-Gallego *et al.*, 2020), anorexia/inappetence (Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Fernandez-Gallego *et al.*, 2020), and weigh loss (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Savani *et al.*, 2004; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020).

Approximately one-fourth of cats with clinical leishmaniosis showed uveitis (Hervás *et al.*, 2001; Pennisi *et al.*, 2004; Verneuil, 2013; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020), stomatitis (Hervás *et al.*, 2001; Leiva *et al.*, 2005; Maroli *et al.*, 2007; Verneuil, 2013; Maia *et al.*, 2015b; Migliazzo *et al.*, 2015; Fernandez-Gallego *et al.*, 2020) and/or cardiorespiratory signs such as dyspnoea/tachypnoea, pallor, abnormal respiratory sounds, nasal discharge and sneezing (Hervás *et al.*, 2001; Pennisi *et al.*, 2004; Marcos *et al.*, 2009; da Silva *et al.*, 2010; Richter *et al.*, 2014; Maia *et al.*, 2015b; Migliazzo *et al.*, 2015; Basso *et al.*, 2016; Leal *et al.*, 2018; Headley *et al.*, 2019; Altuzarra *et al.*, 2020; Silva *et al.*, 2020). Musculoskeletal (*i.e.*, muscle atrophy; da Silva et al., 2010), neurological (*i.e.*, ataxia; Fernandez-Gallego et al., 2020), and urogenital (*i.e.*, vaginal bleeding; Maia et al., 2015) signs were also occasionally described, but in some cases, they appear to be secondary to concomitant diseases (Maia *et al.*, 2015b; Fernandez-Gallego *et al.*, 2020). Other clinical manifestations rarely found and which may represent a further diagnostic challenge to veterinarians include: depigmentation (Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012), cutaneous bloody cyst (Pennisi *et al.*, 2004), pruritus (Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012), footpad hyperkeratosis (Fernandez-Gallego *et al.*, 2020), hepatomegaly (Pennisi *et al.*, 2004; Leiva *et al.*, 2005), splenomegaly (Poli *et al.*, 2002; Leal *et al.*, 2018) , bruising (Maia *et al.*, 2015), mastitis (Pereira *et al.*, 2019c), chorioretinitis (Pennisi *et al.*, 2004; Fernandez-Gallego *et al.*, 2020), corneal opacification (Hervás *et al.*, 2001; Pimenta *et al.*, 2015), glaucoma (Leiva *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014), blepharitis (Brianti *et al.*, 2019), chemosis (Fernandez-Gallego *et al.*, 2020), ocular masses (Hervás *et al.*, 2001), glossitis (Fernandez-Gallego *et al.*, 2020), jaundice (Hervás *et al.*, 1999;

Fernandez-Gallego *et al.*, 2020), abdominal distension (Leiva *et al.*, 2005), and vomiting/diarrhoea (Hervás *et al.*, 1999; Fernandez-Gallego *et al.*, 2020).

**Table 6.** Frequency of clinical signs in domestic cats (*Felis catus*) with clinical leishmaniosis caused by *Leishmania infantum*.

| Historical or physical signs | Frequency (%)[a] | Reference |
|---|---|---|
| Dermatological | | |
| Nodules | 38 | (Poli *et al.*, 2002; Savani *et al.*, 2004; Rüfenacht *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Brianti *et al.*, 2019; Headley *et al.*, 2019; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) |
| Erosive/ulcerative skin disease | 37 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Hervás *et al.*, 2001; Pennisi *et al.*, 2004; Grevot *et al.*, 2005; Rüfenacht *et al.*, 2005; Coelho *et al.*, 2010; Pocholle *et al.*, 2012; Maia *et al.*, 2015b; Basso *et al.*, 2016; Brianti *et al.*, 2019; Headley *et al.*, 2019; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) |
| Scaling/crusting | 21 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Coelho *et al.*, 2010; da Silva *et al.*, 2010; Headley *et al.*, 2019; Fernandez-Gallego *et al.*, 2020) |
| Alopecia | 12 | (Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Fernandez-Gallego *et al.*, 2020) |
| Onychogryphosis | 6 | (da Silva *et al.*, 2010; Headley *et al.*, 2019) |
| Bloody cyst | 4 | (Pennisi *et al.*, 2004) |
| Depigmentation | 4 | (Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012) |
| Pruritus | 4 | (Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012) |
| Pustule/papule | 4 | (Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012) |
| Footpad hyperkeratosis | 2 | (Fernandez-Gallego *et al.*, 2020) |
| General/miscellaneous | | |
| Lymphadenomegaly | 27 | (Hervás *et al.*, 1999, 2001; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Savani *et al.*, 2004; Maroli *et al.*, 2007; da Silva *et al.*, 2010; Brianti *et al.*, 2019; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) |
| Lethargy/depression | 25 | (Poli *et al.*, 2002; Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Pocholle *et al.*, 2012; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Fernandez-Gallego *et al.*, 2020) |
| Anorexia/inappetence | 21 | (Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020) |
| Weight loss | 21 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Savani *et al.*, 2004; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) |
| Hyperthermia | 12 | (Leiva *et al.*, 2005; Basso et al., 2016; Headley *et al.*, 2019; Fernandez-Gallego *et al.*, 2020) |
| Hepatomegaly | 4 | (Pennisi *et al.*, 2004; Leiva *et al.*, 2005) |
| Splenomegaly | 4 | (Poli *et al.*, 2002; Leal *et al.*, 2018) |
| Bruising | 2 | (Maia *et al.*, 2015b) |
| Mastitis | 2 | (Pereira *et al.*, 2019c) |
| Ocular | | |
| Uveitis | 27 | (Hervás *et al.*, 2001; Pennisi *et al.*, 2004; Verneuil, 2013; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020) |
| Corneal oedema | 10 | (Hervás *et al.*, 2001; Pimenta *et al.*, 2015; Fernandez-Gallego *et al.*, 2020) |
| Conjunctivitis | 8 | (Migliazzo *et al.*, 2015; Brianti *et al.*, 2019; Fernandez-Gallego *et al.*, 2020) |
| Chorioretinitis | 4 | (Pennisi *et al.*, 2004; Fernandez-Gallego *et al.*, 2020) |
| Corneal opacification | 4 | (Hervás *et al.*, 2001; Pimenta *et al.*, 2015) |
| Glaucoma | 4 | (Leiva *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014) |
| Keratitis | 4 | (Richter, Schaarschmidt-Kiener & Krudewig, 2014; Fernandez-Gallego *et al.*, 2020) |
| Blepharitis | 2 | (Brianti *et al.*, 2019) |
| Chemosis | 2 | (Fernandez-Gallego *et al.*, 2020) |
| Masse | 2 | (Hervás *et al.*, 2001) |
| Gastrointestinal/abdominal | | |

| | | |
|---|---|---|
| Stomatitis | 21 | (Hervás *et al.*, 2001; Leiva *et al.*, 2005; Maroli *et al.*, 2007; Verneuil, 2013; Migliazzo *et al.*, 2015; Maia *et al.*, 2015b; Fernandez-Gallego *et al.*, 2020) |
| Glossitis | 4 | (Fernandez-Gallego *et al.*, 2020) |
| Jaundice | 4 | (Hervás *et al.*, 1999; Fernandez-Gallego *et al.*, 2020) |
| Vomiting | 4 | (Hervás *et al.*, 1999; Fernandez-Gallego *et al.*, 2020) |
| Abdominal distension | 2 | (Leiva *et al.*, 2005) |
| Diarrhoea | 2 | (Fernandez-Gallego *et al.*, 2020) |
| Cardiorespiratory | | |
| Dispnoea/tachypnoea | 12 | (da Silva *et al.*, 2010; Basso et al., 2016; Leal *et al.*, 2018; Headley *et al.*, 2019; Silva *et al.*, 2020) |
| Pallor | 10 | (Hervás *et al.*, 2001; Pennisi *et al.*, 2004; Marcos *et al.*, 2009; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Maia *et al.*, 2015b) |
| Abnormal respiratory sounds | 4 | (Leal *et al.*, 2018; Altuzarra *et al.*, 2020) |
| Nasal discharge | 4 | (Migliazzo *et al.*, 2015; Altuzarra *et al.*, 2020) |
| Sneezing | 2 | (Leal *et al.*, 2018) |
| Musculoskeletal | | |
| Muscle atrophy | 2 | (da Silva *et al.*, 2010) |
| Neurological | | |
| Ataxia | 2 | (Fernandez-Gallego *et al.*, 2020) |
| Urogenital | | |
| Vaginal bleeding | 2 | (Maia *et al.*, 2015b) |

[a] $n = 52$.

Most consistent laboratory abnormalities found in FeL cases include anaemia (generally of the normochromic, normocytic type) (Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Marcos *et al.*, 2009; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020) and hyperproteinaemia with hypergammaglobulinaemia (Ozon *et al.*, 1998; Herv *et al.*, 1999; Pennisi *et al.*, 2004; Poli *et al.*, 2002; Leiva *et al.*, 2005; Marcos *et al.*, 2009; Richter *et al.*, 2014; Basso *et al.*, 2016; Leal *et al.*, 2018; Brianti *et al.*, 2019; Pereira *et al.*, 2019c; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al.*, 2020). The latter was detected in more than 80 % of sick cats and should be investigated as a possible biomarker of FeL. Leukocytosis (Ozon *et al.*, 1998; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020) and leukopaenia (Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014) are inconsistent findings, whereas thrombocytopenia (Pennisi *et al*., 2004; Marcos et al., 2009; Richter *et al.*, 2014; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Pereira *et al.*, 2019c) and azotaemia (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Marcos *et al.*, 2009; Leal *et al.*, 2018; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020) have been frequently reported. About a quarter of the sick cats presented proteinuria (Marcos *et al*., 2009; Leal *et al.*, 2018; Fernandez-Gallego *et al.*, 2020), suggesting a possible association between FeL and kidney disease as described in dogs. Recently, Chatzis et al. (2020) observed that cats infected with *Leishmania* parasites had higher concentrations of inorganic phosphorus than non-infected cats, reinforcing this assumption. Mild increases of liver enzyme activities are also described (Fernandez-Gallego *et al.*, 2020), but less frequently than in cases of CanL (Maia & Campino, 2018).

**Table 7**. Frequency of clinicopathological abnormalities in domestic cats (*Felis catus*) with leishmaniosis caused by *Leishmania infantum*.

| Parameter | Frequency (%)[a] | Reference |
|---|---|---|
| Hemogram | | |
| Anaemia | 31 | (Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Marcos *et al.*, 2009; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020) |
| Neutrophilia | 19 | (Poli *et al.*, 2002; Leiva *et al.*, 2005; da Silva *et al.*, 2010; Verneuil, 2013; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020) |
| Thrombocytopenia | 17 | (Pennisi *et al.*, 2004; Marcos *et al.*, 2009; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Basso et al., 2016; Pereira *et al.*, 2019c) |
| Leukocytosis | 10 | (Ozon *et al.*, 1998; da Silva *et al.*, 2010; Fernandez-Gallego *et al.*, 2020) |
| Leukopaenia | 10 | (Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014) |
| Eosinophilia | 7 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Altuzarra *et al.*, 2020) |
| Neutropaenia | 5 | (Marcos *et al.*, 2009; Fernandez-Gallego *et al.*, 2020) |
| Lymphopaenia | 2 | (Rüfenacht *et al.*, 2005) |
| Monocytosis | 2 | (Leiva *et al.*, 2005) |
| Blood chemistry | | |
| Hyperproteinaemia | 36 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Pimenta *et al.*, 2015; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Brianti *et al.*, 2019; Fernandez-Gallego *et al.*, 2020) |
| Hyperglobulinaemia | 31 | (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Brianti *et al.*, 2019; Altuzarra *et al.*, 2020) |
| Azotaemia | 21 | (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Marcos *et al.*, 2009; da Silva *et al.*, 2010; Leal *et al.*, 2018; Fernandez-Gallego *et al.*, 2020) |
| Hypoalbuminaemia | 10 | (Hervás *et al.*, 1999; Rüfenacht *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Fernandez-Gallego *et al.*, 2020) |
| Hyperglycaemia | 8 | (Leiva *et al.*, 2005; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Fernandez-Gallego *et al.*, 2020) |
| Bilirubinaemia | 5 | (Fernandez-Gallego *et al.*, 2020) |
| Hyperphosphataemia | 3 | (Fernandez-Gallego *et al.*, 2020) |
| Hypophosphataemia | 3 | (Fernandez-Gallego *et al.*, 2020) |
| Increased alanine aminotransferase | 3 | (Fernandez-Gallego *et al.*, 2020) |
| Increased aspartate transaminase | 3 | |
| Increased creatinine kinase | 3 | (Fernandez-Gallego *et al.*, 2020) |
| Protein electrophoresis | | |
| Hypergammaglobulinaemia | 84 | (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Marcos *et al.*, 2009; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Basso et al., 2016; Leal *et al.*, 2018; Brianti *et al.*, 2019; Pereira *et al.*, 2019c; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al.*, 2020) |
| Increased $\alpha_2$ globulins | 13 | (Basso et al., 2016; Fernandez-Gallego *et al.*, 2020) |
| Hyperbetaglobulinaemia | 3 | (Hervás *et al.*, 1999) |
| Urinalysis | | |
| Proteinuria | 25 | (Marcos *et al.*, 2009; Leal *et al.*, 2018; Fernandez-Gallego *et al.*, 2020) |
| Bilirubinuria | 4 | (Marcos *et al.*, 2009) |
| Glycosuria | 4 | (Leiva *et al.*, 2005) |

[a] Hemogram, $n = 42$; Blood chemistry, $n = 39$; Serum protein electrophoresis, $n = 32$; Urianalysis, $n = 24$.

### 5.7. Diagnosis

Clinical presentation combined with epidemiological context may lead to suspicion of FeL, but for a definitive diagnosis, *Leishmania*-specific laboratory tests are required (Table 8). These include direct tests (cytology, histology, immunohistochemistry, culture, and PCR), demonstrating the presence of the parasite or its components, and indirect tests (serology) assessing the host's response to infection.

Cytology is strongly advised in cats presenting erosive/ulcerative skin disease, nodular lesions and/or lymphadenomegaly (Herv et al., 1999; Poli et al., 2002; Savani *et al.*, 2004; Coelho *et al.*, 2010; Richter *et al.*, 2014; Maia *et al.*, 2015b; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Brianti *et al.*, 2019; Headley *et al.*, 2019; Pereira *et al.*, 2019c; Silva *et al.*, 2020). Material for diagnosis can be obtained by fine-needle biopsy (with or without aspiration), scraping or imprinting. The presence of *Leishmania* parasites has been demonstrated in cytological examinations of feline nodular lesions (Poli et al., 2002; Savani *et al.*, 2004; Richter *et al.*, 2014; Basso *et al.*, 2016; Pimenta *et al.*, 2015; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Brianti *et al.*, 2019; Pereira *et al.*, 2019c; Fernandez-Gallego *et al.*, 2020; Silva et al., 2020), erosive/ulcerative lesions (Maia *et al.*, 2015b; Headley *et al.*, 2019; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), whole-blood (Marcos *et al.*, 2009; Metzdorf *et al.*, 2017), buffy coat/leucoconcentrate (Martín-Sánchez *et al.*, 2007; Marcos *et al.*, 2009), lymph nodes (Hervás *et al.*, 1999; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Bresciani *et al.*, 2010; Coelho *et al.*, 2010, 2011b; Vides *et al.*, 2011; Sobrinho *et al.*, 2012; Metzdorf *et al.*, 2017; Berenguer *et al.*, 2020; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), bone marrow (Pennisi *et al.*, 2004; Marcos *et al.*, 2009; Vides *et al.*, 2011; Sobrinho *et al.*, 2012; Metzdorf *et al.*, 2017; Marcondes *et al.*, 2018; Fernandez-Gallego *et al.*, 2020), liver (Vides *et al.*, 2011; Mohebali *et al.*, 2017; Fernandez-Gallego *et al.*, 2020), spleen (Vides *et al.*, 2011; Mohebali *et al.*, 2017; Fernandez-Gallego *et al.*, 2020), nasal exudate (Migliazzo *et al.*, 2015), corneal impression (Pimenta *et al.*, 2015), and inflammatory breast fluid (Pereira *et al.*, 2019c). Cytologic preparations consistent with FeL typically have a cell composition characteristic of pyogranulomatous, granulomatous or lymphoplasmacytic inflammation (Poli *et al.*, 2002; Headley *et al.*, 2019; Pereira *et al.*, 2019c). Similar patterns are reported in histological studies on feline paraffin-embedded specimens (Poli *et al.*, 2002; Navarro *et al.*, 2010; Migliazzo *et al.*, 2015; Di Mattia *et al.*,

2018; Leal *et al.*, 2018; Altuzarra *et al.*, 2020). Nevertheless, compared with cytology, histology has the main advantage of providing more detailed diagnostic information on the tissue architecture, which allow to determine if parasites are indeed associated with lesions (Paltrinieri *et al.*, 2016). Immunohistochemistry may be further performed to confirm the presence of *Leishmania* organisms in biological samples obtained from cats (Poli *et al.*, 2002; Navarro *et al.*, 2010; Migliazzo *et al.*, 2015). Based on histological and immunohistochemical examinations, it has been observed that this parasite may invade several feline organs/tissues such as skin (Ozon *et al.*, 1998; Poli *et al.*, 2002; Grevot *et al.*, 2005; Rüfenacht *et al.*, 2005; Attipa *et al.*, 2017a; Rivas *et al.*, 2018; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), nasal and oral mucosa (Pennisi *et al.*, 2004; Migliazzo *et al.*, 2015; Leal *et al.*, 2018), eyes (Hervás *et al.*, 2001; Fernandez-Gallego *et al.*, 2020), nasopharynx (Leal *et al.*, 2018), stomach (Hervás *et al.*, 1999), liver (Hervás *et al.*, 1999; Silva *et al.*, 2020), kidneys (Ozon *et al.*, 1998), spleen (Hervás *et al.*, 1999; Grevot *et al.*, 2005; Marcos *et al.*, 2009; Maia *et al.*, 2015b; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), bone marrow (Ozon *et al.*, 1998; Pimenta *et al.*, 2015; Silva *et al.*, 2020), and lymph nodes (Hervás *et al.*, 1999), and may also be associated with neoplasia (Grevot *et al.*, 2005; Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012; Maia *et al.*, 2015b; Altuzarra *et al.*, 2020).

Parasite culture is an accurate test allowing conclusive diagnosis of active infection. However, this test is not suitable for rapid diagnosis and is restricted to specialised laboratories. Parasite culture is a starting point for parasite identification and characterisation by isoenzyme electrophoresis (Pratlong *et al.*, 2004). Viable parasites have been isolated from whole blood (Pocholle *et al.*, 2012), nodular lesions (Poli et al., 2002; Basso et al., 2016), liver (Maia *et al.*, 2015b; Silva *et al.*, 2020), spleen (Maia *et al.*, 2015b; Silva *et al.*, 2020), lymph nodes (Pennisi *et al.*, 2004; Maroli *et al.*, 2007; Maia *et al.*, 2015b; Basso *et al.*, 2016; Silva *et al.*, 2020), and bone marrow (Silva *et al.*, 2020) of cats with leishmaniosis.

Polymerase chain reaction (PCR)-based tests has been allowed the identification of *Leishmania* DNA in several feline samples, including whole blood (Marcos *et al.*, 2009; Pocholle *et al.*, 2012; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Attipa *et al.*, 2017a; Brianti *et al.*, 2019; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), buffy coat (Pereira *et al.*, 2019c), conjunctival and oral swabs (Migliazzo *et al.*, 2015; Brianti *et al.*, 2019; da

Costa-Val *et al.*, 2020), hair (Urbani *et al.*, 2020), skin (Rüfenacht *et al.*, 2005; da Silva *et al.*, 2010; Richter et al., 2014; Maia *et al.*, 2015b; Basso *et al.*, 2016; Fernandez-Gallego *et al.*, 2020; Silva *et al.*, 2020), nasal tissue (Leal *et al.*, 2018), liver (Maia *et al.*, 2015b; Silva *et al.*, 2020), spleen (Savani *et al.*, 2004; Coelho *et al.*, 2010; da Silva *et al.*, 2010; Maia *et al.*, 2015b; Pimenta *et al.*, 2015; Silva *et al.*, 2020; Fernandez-Gallego *et al.*, 2020), kidneys (da Silva *et al.*, 2010), lymph nodes (Poli *et al.*, 2002; Pennisi *et al.*, 2004; Coelho *et al.*, 2010; ; da Silva *et al.*, 2010; Maia *et al.*, 2015b; Migliazzo *et al.*, 2015; Pimenta *et al.*, 2015; Silva *et al.*, 2020), bone marrow (da Silva *et al.*, 2010; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Pimenta *et al.*, 2015; Silva *et al.*, 2020; Fernandez-Gallego *et al.*, 2020), and inflammatory breast fluid (Pereira *et al.*, 2019c). Conventional PCR, nested PCR, and real-time PCR (qPCR) targeting kinetoplast minicircle DNA (kDNA) or the small subunit ribosomal DNA (*SSU*-rDNA) multicopy genes have been widely used in routine veterinary practise for FeL diagnosis (Pimenta *et al.*, 2015; Pereira *et al.*, 2019c; Brianti *et al.*, 2019) as well as in epidemiological studies concerning *Leishmania* infection in cats (Vilhena *et al.*, 2013; Maia *et al.*, 2014; Pereira *et al.*, 2020). Nevertheless, two-step PCR when used to amplify stretches of multicopy genes has increased the sensitivity of detection and should be preferred for sample testing under suboptimal conditions (*i.e.*, where the parasite load tends to be low) such as when whole blood is used (Pereira *et al.*, 2020). On the other hand, quantitative qPCR may further provide information about the amount of parasite DNA present in the sample (Galluzzi *et al.*, 2018). This aspect is particularly relevant for monitoring the efficacy of anti-*Leishmania* treatments (Pocholle *et al.*, 2012; Basso *et al.*, 2016). However, it is important to highlight that a PCR positive result may only reflect a transient infection and, for this reason, should be carefully interpreted in a clinical context. PCR products may be followed subsequently analysed by restriction enzyme digestion (*i.e.*, restriction fragment length polymorphism) and/or DNA sequencing for parasite species identification (Metzdorf *et al.*, 2017; Pereira *et al.*, 2020).

The most common serological tests used to detect anti-*Leishmania* antibodies in cats are based on enzyme-linked immunosorbent assay (ELISA) and immunofluorescent antibody test (IFAT). The latter is considered as the reference test for the serodiagnosis of canine and human leishmaniosis (WHO, 2010; OIE, 2018). Persichetti *et al.* (2017) established 1:80 serum dilution as IFAT cut-off for FeL serodiagnosis, and demonstrated

that this test helps to detect subclinical or early *Leishmania* infections in cats. More recently, Iatta *et al.* (2020) validated IFAT as an accurate test to assess the exposure of cats to *L. infantum*, reporting positive and negative predictive values of 80.7 % and 89.9 %, respectively. Compared to IFAT, ELISA (cut-off 40 ELISA units) presents a better performance for the serodiagnosis of clinical FeL (Persichetti *et al.*, 2017). Western blot analysis is mainly intended for research and is rarely available in routine practice. However, this test seems to offer the best diagnostic performance (considering an 18 kDa band as a marker for positivity) to detect antibodies against *L. infantum* in cats (Persichetti *et al.*, 2017). Direct agglutination test has also occasionally been used in both clinical and epidemiological contexts for serological diagnosis of FeL (Pimenta *et al.*, 2015; Asgari *et al.*, 2020). Some authors have considered a cut-off value of 1:100 to distinguish infected from uninfected cats (Kongkaew *et al.*, 2007; Cardoso *et al.*, 2010; Maia *et al.*, 2015a; Lopes *et al.*, 2017; Asgari *et al.*, 2020; Neves *et al.*, 2020). Indirect hemagglutination was exclusively performed in epidemiological studies in domestic cats in Egypt (Michael *et al.*, 1982; Morsy *et al.*, 1988; Morsy & Abou el Seoud, 1994).

Cats with clinical leishmaniosis tend to present high antibody levels (Richter *et al.*, 2014; Maia *et al.*, 2015b; Pimenta *et al.*, 2015; Basso *et al.*, 2016), and specific treatment frequently leads to the reduction of anti-*Leishmania* antibodies (Pennisi *et al.*, 2004; Richter *et al.*, 2014; Basso *et al.*, 2016; Pereira *et al.*, 2019c). In some cases, an increase of antibody titres was associated with clinical relapse. Nevertheless, it is essential to emphasise that a positive serological result formally only reflects exposure to pathogens and should be interpreted in a clinical context (Paltrinieri *et al.*, 2016).

In conclusion, the diagnosis of FeL can be a real challenge for veterinarians and is seldom considered during the differential diagnosis. Therefore, the algorithm illustrated in Figure 8 is proposed for clinically healthy cats used as blood donors or for breeding purposes, and for cats with suspected leishmaniosis.

**Table 8.** Common laboratory tests performed for diagnostic of *Leishmania* infection in domestic cats (*Felis catus*).

| Type/test | Aim | Confirmation of clinical disease | Confirmation of subclinical disease | Preferential sample | Advantages | Disadvantages | Observations |
|---|---|---|---|---|---|---|---|
| Parasitological | | | | | | | |
| Cytology | Detection of parasites | +++ | + | - Bone-marrow (FNB)<br>- Lymph node (FNB)<br>- Nodular lesions (FNB)<br>- Erosive/ulcerative skin lesions (Scraping) | - Does not require specific laboratory equipment<br>- Low cost<br>- Rapid<br>- High specificity | -Requires experienced observers<br>- Strictly qualitative<br>- Not suitable for identification at species level | - Amastigotes can be found in both intracellular and extracellular areas |
| Histopathology | Detection of parasites | +++ | + | - Skin/ocular lesions<br>- Bone marrow<br>- Lymph-nodes<br>- Spleen | - Preserves structure and maintains tissue pathology<br>- High specificity<br>- Good sensitivity using IHC | - Invasive<br>- Requires experienced observers<br>- Requires specific laboratory equipment<br>- More laborious and time-consuming<br>- IHC is not widely available<br>- Only qualitative<br>- Not suitable for identification at the species level | |
| Parasite culture | Isolation of viable parasites | ++ | + | - Biopsy lesions<br>- Bone marrow<br>- Lymph node | - Provide parasites for further analysis<br>- Confirms active infection<br>- High specificity | - Labour-intensive<br>- Restricted to specialised reference laboratories<br>- Up to more than 30 days to provide a result<br>- Only qualitative<br>- Not suitable for identification at species level | - Aseptic sampling should be ensured<br>- Biopsy sample must be homogenised in saline or culture medium under sterile conditions |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Molecular | | | | | | | |
| PCR | Detection of parasite DNA | +++ | +++ | - Biopsy lesions<br>- Bone marrow<br>- Lymph node | - Allows identification at species level<br>- High sensitivity and specificity | - Transient infection cannot be excluded<br>- Requires specific laboratory equipment<br>- Requires vigilance against false-positive results<br>- Only qualitative<br>- Expensive | - Protocols targeting multicopy genes are preferable for diagnosis<br>- Nested-PCR has more sensitivity than conventional PCR |
| qPCR | Detection of parasite DNA | +++ | +++ | - Biopsy lesions<br>- Bone marrow<br>- Lymph node | - Allows identification at species level<br>- High sensitivity and specificity<br>- Quantification of parasite load<br>- Reduced cross-contamination probability<br>- Valuable for treatment follow-up<br>- Qualitative/quantitative | - Transient infection cannot be excluded<br>- Standardised methods to parasite load quantification may not be offered by some laboratories.<br>- Expensive | - Protocols targeting multicopy genes are preferable for diagnosis |
| Serological | | | | | | | |
| ELISA | Detection of specific antibodies | +++ | ++ | - Serum<br>- Plasma | - Valuable for treatment follow-up<br>- Relatively low cost<br>- Qualitative/quantitative | - Possible cross-reactivity<br>- Difficult to assess results at threshold of positivity<br>- Not suitable for unambiguous identification at species level | - Established cut-off (40 EU) |
| IFAT | Detection of specific antibodies | ++ | +++ | - Serum<br>- Plasma | - Valuable for treatment follow-up<br>- Relatively low cost<br>- Qualitative/quantitative | -Requires experienced observer<br>- Subjective interpretation<br>- Possible cross-reactivity<br>- Not suitable for unambiguous | - Reference method for the serodiagnosis of human and canine leishmanioses<br>- Established cut-off (1:80) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | identification at species level | |
| Western Blot | Detection of specific antibodies | +++ | +++ | - Serum<br>- Plasma | - High sensitivity and specificity | - Labour-intensive<br>- Expensive<br>- Not available in routine practice | - Marker for positivity: 18 kDA band |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; FNB, fine-needle biopsy; IFAT, immunofluorescence antibody test; IHC, immunohistochemistry, KDa, kilodaltons; PCR, conventional/nested polymerase chain reaction; qPCR, real time polymerase chain reaction; WB, western blot. +++, recommended test; ++ suitable test; +, limited test.

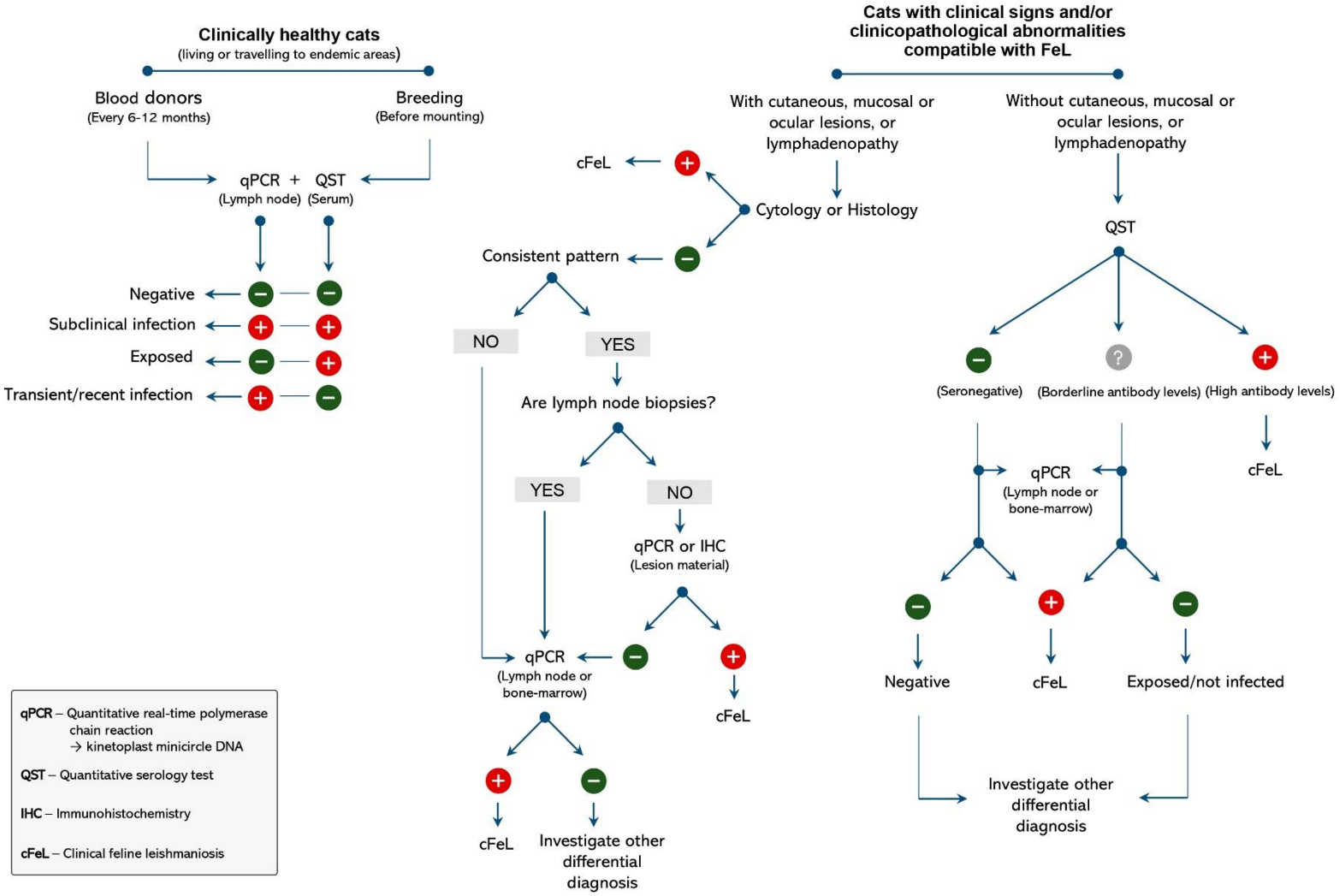


**Figure 8**. Proposed diagnostic algorithm for clinically healthy cats used as blood donors or for breeding, and cats with suspected leishmaniosis.

### 5.8. Treatment and prognosis

Treatment should be considered only after confirmation of disease (see section 6.). Although several treatment regimens have been empirically used for FeL (Table 9), no controlled studies about their efficacy and safety have yet been performed. Long-term administration of allopurinol as monotherapy is the most common regimen prescribed for FeL (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Pocholle *et al.*, 2012; Richter *et al.*, 2014; Maia *et al.*, 2015b; Migliazzo *et al.*, 2015; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Brianti *et al.*, 2019; Pereira *et al.*, 2019c; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al.*, 2020). This drug is generally well-tolerated, but possible cases of cutaneous adverse reactions (Leal *et al.*, 2018; Brianti *et al.*, 2019), coprostasis (Maia *et al.*, 2015b), and elevated liver enzymes (Rüfenacht *et al.*, 2005) have been sporadically reported. Favourable results (*i.e.*, clinical cure or improvement of clinical status) with allopurinol as monotherapy have been commonly obtained (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Rüfenacht *et al.*, 2005; Pocholle *et al.*, 2012; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Migliazzo *et al.*, 2015; Pimenta *et al.*, 2015; Attipa *et al.*, 2017a; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al.*, 2020). Nevertheless, relapse after discontinuation or low-dose administration (Pennisi *et al.*, 2004; Leiva *et al.*, 2005; Brianti *et al.*, 2019; Pereira *et al.*, 2019c) and no or poor response to allopurinol therapy have been occasionally reported, even in cats with no apparent history of concomitant infections or immunosuppressive therapies (Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Basso *et al.*, 2016; Fernandez-Gallego *et al.*, 2020). Therefore, the combination of meglumine antimoniate and allopurinol has been proposed for FeL treatment, appearing to be more effective (Basso *et al.*, 2016; Pereira *et al.*, 2019c), but acute kidney injury has already been reported (Leal *et al.*, 2018). Although controversial, this drug is suspected of inducing nephrotoxicity in dogs (reviewed by Roura *et al.*, 2021). Thus, its use in cats with altered renal function should be carefully considered. Meglumine antimoniate plus ketoconazole was used in a cat with cutaneous and systemic signs of FeL, resulting in apparent clinical cure (Hervás *et al.*, 1999). Miltefosine was recently used as an alternative to meglumine antimoniate in an azotemic cat, resulting in rapid clinical improvement (Leal *et al.*, 2018). In this case, transient vomiting episodes were reported in the first week of treatment but were managed using antiemetics (*i.e.*, maropitant). Nevertheless, Fernandez-Gallego *et al.* (2020)

recently reported a case of FeL with concomitant FIV infection not responsive to miltefosine plus allopurinol (combination therapy). Pennisi *et al.* (2004) reported treatment failure in a seropositive cat for FIV, *T. gondii* and *B. henselae* suffering from leishmaniosis. In this case, three distinct regimens were used (*i.e.*, metronidazole plus spiramycin, fluconazole and itraconazole) (Pennisi *et al.*, 2004). In another cat with leishmaniosis associated with an invasive squamous cell carcinoma, domperidone was used after unsuccessful allopurinol monotherapy, but clinical signs remained after one month of treatment (Maia *et al.*, 2015b). The dietary supplement active hexose correlated compounds (AHCC) was recently suggested as a possible alternative maintenance therapy to allopurinol (Leal *et al.*, 2018). Surgical removal of lesions was also reported as an additional therapeutic approach (Hervás *et al.*, 2001; Rüfenacht *et al.*, 2005; Basso *et al.*, 2016).

Like in dogs, *Leishmania* parasites may persist in treated cats (Pocholle *et al.*, 2012; Pimenta *et al.*, 2015; Attipa *et al.*, 2017a), suggesting that treatment may lead to clinical cure but may not eliminate the infection.

Overall, FeL has a good prognosis even in cases with underlying viral infections (*i.e.*, FIV or FeLV) (Hervás *et al.*, 1999; Pennisi *et al.*, 2004; Rüfenacht *et al.*, 2005; Richter *et al.*, 2014; Migliazzo *et al.*, 2015; Pimenta *et al.*, 2015; Basso *et al.*, 2016; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al.*, 2020). On the other hand, panleukopaenia, acute kidney injury and lack of treatment seem to be critical factors associated with poor prognosis (Ozon *et al.*, 1998; Hervás *et al.*, 1999; Poli *et al.*, 2002; Pennisi *et al.*, 2004; Pimenta *et al.*, 2015; Fernandez-Gallego *et al.*, 2020).

**Table 9.** Treatment regimens used for feline leishmaniosis.

| Type | Drug (regimen and dose) | Outcome | Adverse reactions[a] | Issues to consider | Reference |
|---|---|---|---|---|---|
| Monotherapy | | | | | |
| | Allopurinol (10-30 mg/kg or 100 mg/cat PO q12-24h; for long-term) | Variable (no response to clinical cure) | Increased liver enzymes; coprostasis[b]; toxidermia | Secondary xanthine urolithiasis has been reported in dogs | (Pennisi *et al*., 2004; Rüfenacht *et al.*, 2005; Marcos *et al.*, 2009; Pocholle *et al.*, 2012; Richter, Schaarschmidt-Kiener & Krudewig, 2014; Maia *et al*., 2015b; Migliazzo *et al*., 2015; Pimenta *et al.*, 2015; Basso *et al*., 2016; Attipa *et al.*, 2017a; Leal *et al.*, 2018; Pereira *et al.*, 2019c; Brianti *et al.*, 2019; Altuzarra *et al.*, 2020; Fernandez-Gallego *et al*., 2020) |
| | Domperidone (0.5 mg/kg PO q24h for 1 month) | No improvement | Not reported | Immunomodulatory drug used on prevention and treatment of CanL | (Maia *et al.*, 2015b) |
| | Fluconazole (5 mg/kg PO q24h for 2 months) | No response | Not reported | May be hepatotoxic | (Pennisi *et al*., 2004) |
| | Itraconazole (50 mg/cat PO q24h for 2 months) | No response | Not reported | Hepatotoxic drug; may lead to suppression of adrenal function | (Pennisi *et al*., 2004) |
| | Meglumine antimoniate (50 mg/kg SC q24h for 25 days) | Not applicable | AKI - suspected | Treatment stopped due to AKI development; painful to administer; may be nephrotoxic (controversial) | (Leal et al., 2018) |
| | Meglumine antimoniate (300 mg/cat SC q24h for 4 months) | Resolution of clinical signs | See previous line | See previous line | (Fernandez-Gallego *et al*., 2020) |
| Combination therapy | | | | | |
| | Meglumine antimoniate (50 mg/kg SC q24h for 30 days) plus allopurinol (10 mg/kg PO q12-24h for long-term) | Variable (partial resolution of clinical signs to clinical cure) | See meglumine antimoniate and allopurinol monotherapy | Proposed for FeL refractory cases | (Pimenta *et al.*, 2015; Basso *et al*., 2016; Pereira *et al*., 2019c; Fernandez-Gallego *et al.*, 2020) |
| | Meglumine antimoniate (5 mg/kg SC q24h) plus ketoconazole (10 mg/kg q24h); 3 cycles of 4 weeks, 10 days apart | Resolution of lesions | Not reported; see meglumine antimoniate monotherapy | According to BSAVA (2020) ketoconazole is not recommended for cats | (Hervás *et al.*, 1999) |
| | Metronidazole (25 mg/kg PO q24h for 35 days) plus spiramycin (150,000 IU/kg PO q24h for 35 days) | No response | Not reported | | (Pennisi *et al*., 2004) |

| | | | | | |
|---|---|---|---|---|---|
| | Miltefosine (2 mg/kg PO q24h for 28 days) plus AHCC (½ tablet once daily for long-term) | Resolution of clinical signs | Transient vomiting associated with miltefosine administration | Miltefosine licenced formulations for CanL contain propylene glycol which can hypothetically induce Heinz body haemolytic anaemia in cats (Pennisi & Persichetti, 2018) | (Leal *et al*., 2018) |
| | Miltefosine (2 mg/kg PO q24h for 28 days) plus allopurinol (10 mg/kg PO q12 for long-term) | No response | See previous line | See previous line | (Fernandez-Gallego *et al*., 2020) |

[a] Reported during treatment of cats with clinical leishmaniosis; [b] associated with high doses (50 mg/kg q24h).

Abbreviations: AHCC, active hexose correlated compounds; AKI, acute kidney injury; CanL, canine leishmanosis; FeL, feline leishmaniosis; IU, internacional unit; PO, *per os*; SC, subcutaneous.

### 5.9. Prophylaxis and control

No vaccines or drugs preventing leishmaniosis are currently available for use in cats, and most repellents avoiding infection in dogs are toxic to these felids. In endemic areas, cats are frequently exposed to phlebotomine sand fly bites, and this is associated with an increased risk of *Leishmania* infection (Pereira *et al.*, 2019b). Chemoprophylaxis may be achieved by using a matrix collar containing 10 % imidacloprid and 4.5 % flumethrin. This formulation showed to be safe and effective in reducing infection risk by *L. infantum* in cats (Brianti *et al.*, 2017). Nevertheless, keeping cats indoors from dusk to dawn during the period of vector activity (April to November in Mediterranean areas; see Alten *et al.*, 2016), as well as using physical barriers such as nets (*i.e.*, mesh size – 1,240 holes/in$^2$) on windows and doors (Faiman, Cuño & Warburg, 2009) may eschew exposure to phlebotomine sand fly bites, thereby minimising the risk of *Leishmania* infection. Spraying with residual insecticides on walls and roofs of human houses and animal shelters has been proposed as an additional measure for preventing CanL (Maroli *et al.*, 2010). However, their use in environments with cats should be carefully considered since most of these products contain compounds (*i.e.*, pyrethrins or pyrethroids) that can induce feline toxicosis. Isoxazolines, namely afoxolaner and fluralaner, have been regarded as a new promising class of drugs for controlling CanL and human leishmaniosis in endemic areas (Miglianico *et al.*, 2018; Bongiorno *et al.*, 2020; Queiroga *et al.*, 2020). A spot-on formulation of fluralaner (112.5-500 mg) is licensed for ectoparasite (*i.e.*, ticks, fleas, and mites) control in cats. This systemic insecticide induced long-term mortality of *Lutzomyia longipalpis* and *Phlebotomus perniciosus* (vectors of *L. infantum* in the New and Old Worlds, respectively) after feeding on treated dogs (Bongiorno *et al.*, 2020; Queiroga *et al.*, 2020). Similar results are expected to be observed in cats. Although studies are undoubtedly needed, this drug may also hypothetically represent an affordable indirect method for reducing *Leishmania* infection in cats in endemic areas. The detection and treatment of cats with leishmaniosis is also likely a beneficial control measure, as they may serve as a source of infection to phlebotomine sand fly vectors (Maroli *et al.*, 2007; da Silva *et al.*, 2010; Mendonça *et al.*, 2020). In the absence of evidence indicating otherwise, *Leishmania*-infected cats should not be used for breeding or as blood donors due to the potential risk of transmission through blood transfusion and

venereal/congenital infection, as reported in dogs (Owens *et al.*, 2001; Naucke & Lorentz, 2012).

In summary, and according to the current knowledge, the following prophylactic measures are proposed to prevent and control feline infection:

i. In endemic areas, keeping cats indoors from dusk to dawn during the phlebotomine sand fly season should be encouraged.

ii. Use of physical barriers on houses and animal shelters located in endemic areas with high vector density.

iii. Use of a matrix collar containing 10 % imidacloprid and 4.5 % flumethrin as well topical solutions containing 112.5-500 mg of fluralaner in cats living in or travelling to (cover the time of travel) endemic areas during the known transmission season.

iv. After the return from endemic areas, cats should be clinically evaluated and tested.

v. Cats eligible for breeding and blood transfusion should be periodically tested.

vi. Infected cats should not be used for breeding or as blood donors.

vii. Cats with leishmaniosis should be treated and periodically monitored.

### 5.10. Public health considerations

Zoonotic visceral leishmaniosis (ZVL) caused by *L. infantum* is a life-threatening human disease endemic in the Mediterranean Basin, the Middle East, western Asia, and Brazil (WHO, 2010). Domestic dogs are considered the primary source of human infection, which typically occurs *via* the bites of female phlebotomine sand flies (WHO, 2010). Nevertheless, during the last years, cats have been deserved attention due to their potential enrolment in ZVL epidemiology, appearing now as possible primary or secondary reservoir hosts (Asfaram, Fakhar & Teshnizi, 2019). This hypothesis arises by the following reasons (Maroli *et al.*, 2007; da Silva *et al.*, 2010; GfK, 2016; Pereira *et al.*, 2019b, 2019c; Carneiro *et al.*, 2020; Fernandez-Gallego *et al.*, 2020; Mendonça *et al.*, 2020; Pereira *et al.*, 2020):

i. Cats are frequently exposed to the bites of competent vectors.

ii. Cats are naturally susceptible to *L. infantum* infection.

iii. Feline infection often runs a subclinical course.

iv. Parasites are frequently found in the skin and blood of infected cats.

v. Naturally infected cats are infectious to competent vectors.

vi. Naturally infected cats may be the source of infection to other mammals through competent vectors.

vii. Strains of feline origin seem to be indistinguishable from those isolated from dogs, humans, and competent vectors.

viii. Cats are among the most popular animals owned as a pet.

ix. Cats are often present in domestic/peridomestic areas where transmission cycles occur.

### 10. Conclusion

In the last few years, several studies concerning *Leishmania* infection in cats have been conducted. Feline leishmaniosis has also gained importance appearing nowadays as an emergent disease. Nevertheless, its immunopathogenesis is poorly known. This protozoonosis is manifested by a broad spectrum of clinical signs and clinicopathological abnormalities, which, associated with the lack of standardised protocols, makes its diagnosis even more challenging for veterinarians. In this review, a diagnostic algorithm for FeL is proposed for clinical decision support. Treatment options currently available are empirical and suboptimal. The main form to prevent disease is to avoid infection. However, in contrast to dogs, very limited options are currently available to keep infective sand flies away from cats. Thus, a set of prevention guidelines were herein suggested.

## 6. Objectives and experimental design

The general objective of this PhD research project was to evaluate the putative role played by domestic cats (*Felis catus*) in the epidemiology of zoonotic leishmaniosis caused by *L. infantum*. For this purpose, the following specific objectives were set:

i. Assess the exposure of cats to proven phlebotomine sand fly vectors of *L. infantum*.

ii. Determine the proportion of *Leishmania* infection in cats in endemic *foci*.

iii. Follow-up naturally infected cats (through clinical and parasitological examination).

iv. Genetic characterisation of *Leishmania* strains isolated from cats.

v. Assess the phenotypic behaviour of *L. infantum* strains isolated from cats under optimal, stress, and drug conditions.

vi. Evaluate the infectivity of feline strains of *L. infantum* for feline, canine, and human host cells (macrophages).

The experimental design underlying the objectives outlined above is illustrated in Figure 9.

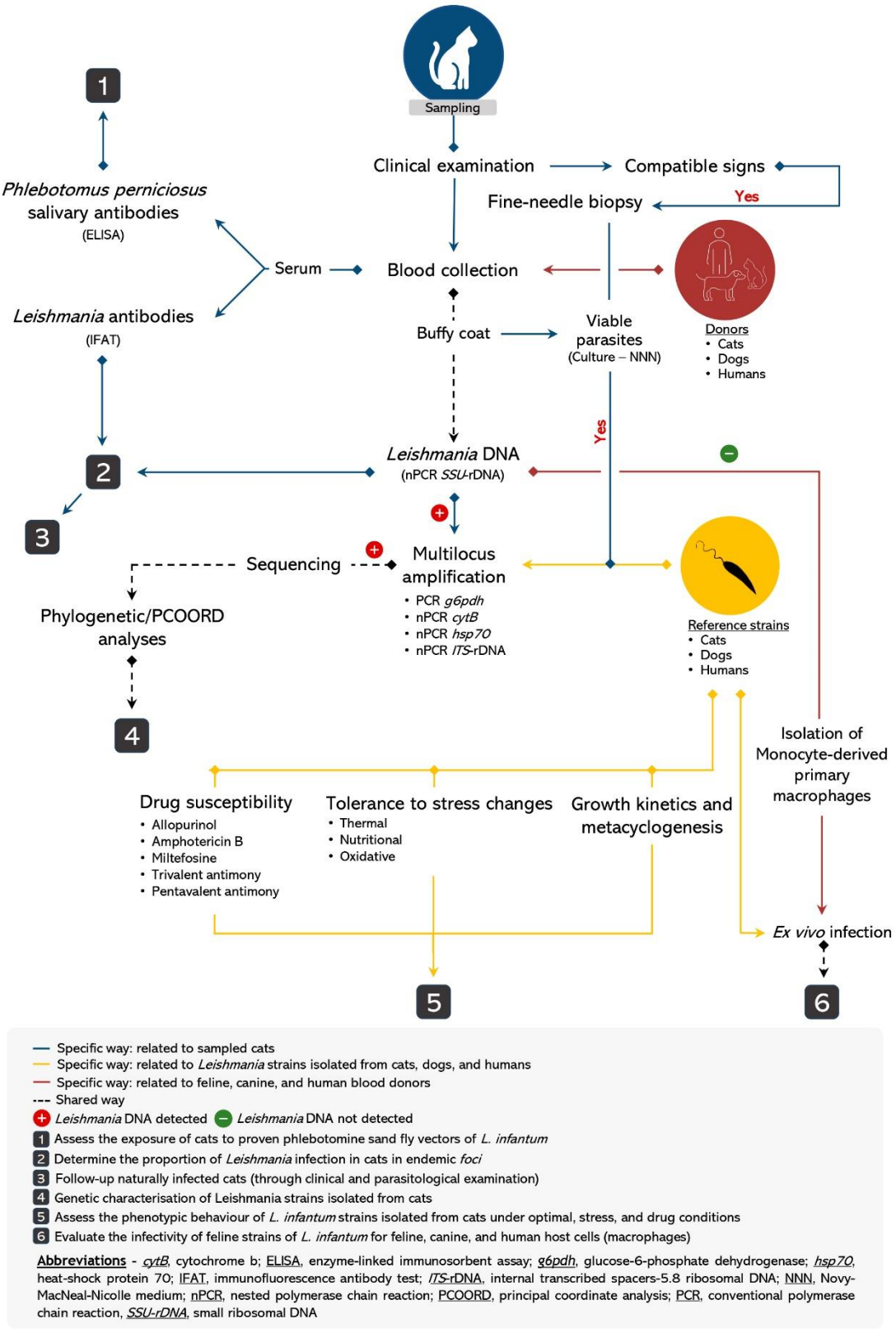


**Figure 9.** Flowchart of the experimental design.

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# CHAPTER 2

## Estimation of *Leishmania* infection prevalence in cats and exposure to phlebotomine sand flies

**This chapter is based on the research article:**

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# 1. Abstract

## 1.1. Background

Zoonotic leishmaniosis, caused by the protozoan *Leishmania infantum*, is a public and animal health problem in Asia, Central and South America, the Middle East and the Mediterranean Basin. Several phlebotomine sand fly species from the subgenus *Larrousius* are vectors of *L. infantum*. Data from dogs living in endemic areas of leishmaniosis support the use of antibody responses to phlebotomine sand fly saliva as an epidemiological biomarker for monitoring vector exposure. The aim of this study was to analyse the exposure of cats to phlebotomine sand flies using detection of IgG antibodies to *Phlebotomus perniciosus* saliva. The association between phlebotomine sand fly exposure and the presence of *Leishmania* infection was also investigated.

## 1.2. Results

IgG antibodies to *P. perniciosus* saliva were detected in 167 (47.7 %) out of 350 cats; higher antibody levels were present in sera collected during the period of phlebotomine sand fly seasonal activity (OR = 19.44, 95 % CI: 9.84-38.41). Cats of age 12-35 months had higher antibody levels than younger ones (OR = 3.56, 95 % CI: 1.39-9.16), this difference was significant also with old cats (for 36-95 months old OR = 9.43, 95 % CI: 3.62-24.48, for older than 95 months, OR = 9.68, 95 % CI: 3.92-23.91). *Leishmania* spp. DNA was detected in the blood of 24 (6.9 %) cats, while antibodies to *L. infantum* were detected in three (0.9 %). Only one cat was positive to *Leishmania* by both techniques. Cats presenting IgG antibodies to *P. perniciosus* had a significantly higher risk to be positive for *Leishmania* infection.

## 1.3. Conclusions

To our knowledge, this is the first study demonstrating anti-*P. perniciosus* antibodies in cats. The evaluation of the contact of this animal species with the vector is important for the development of prophylactic measures directed to cats, with the aim of reducing the prevalence of infection in an endemic area. Therefore, studies evaluating whether the use of imidacloprid/flumethrin collars reduces the frequency of *P. perniciosus* bites in cats are needed. It is also important to evaluate if there is a correlation between the number of phlebotomine sand fly bites and IgG antibody levels.

**Keywords:** antibodies, cat, *Leishmania infantum*, *Phlebotomus perniciosus*, saliva, Portugal.

## 2. Background

Zoonotic leishmaniosis, caused by the protozoan *Leishmania infantum*, is a serious public and animal health problem in several countries of Asia, Central and South America, the Middle East, and the Mediterranean Basin. Domestic dogs are the major hosts of the parasite and the main domestic reservoir hosts for human infection. Nevertheless, the number of feline leishmaniosis reports and subclinical *L. infantum* infections in cats living in endemic areas has increased in recent years [1]. In fact, there is an increasing trend to consider cats as a potential primary or secondary reservoir host of *L. infantum*, rather than being an accidental host [2]. This assumption is based on several premises, namely natural susceptibility to infection, suitability to serve as a blood source for phlebotomine sand flies, infectivity to the vector, and close contact with humans where the peridomestic and domestic transmission cycles of the parasite occur [3].

*Leishmania* parasites are transmitted by phlebotomine sand flies (Diptera: Psychodidae). During the blood meal, immunogenic components present in phlebotomine sand fly saliva are injected into the vertebrate host leading to the development of anti-saliva antibodies [4]. Data from dogs living in endemic areas of leishmaniosis caused by *L. infantum* suggest the use of antibody responses to salivary antigens as an epidemiological biomarker for monitoring vector exposure [5-11]. The levels of specific IgG antibodies against phlebotomine sand fly saliva positively correlate with the number of blood-fed sand flies [6-12] and decays after the end of phlebotomine sand fly seasonal activity [7, 10].

In the Old World, *L. infantum* is transmitted by several phlebotomine sand fly species belonging to the subgenus *Larrossius*, of which *Phlebotomus perniciosus* is the principal vector in the west part of Mediterranean, including Portugal [13]. Portugal is endemic for canine leishmaniosis [14] and hypoendemic for human visceral leishmaniosis [15]. Feline leishmaniosis [16] and *L. infantum* infection in cats have been documented

in Portugal [17-20]. The phlebotomine sand fly season usually lasts from May until late October [13, 21, 22].

The aim of this work was to analyse the exposure of cats to phlebotomine sand flies through the detection of antibodies to *P. perniciosus* saliva, and to assess associated risk factors. The possible association between phlebotomine sand fly exposure and the presence of *Leishmania* infection was also investigated.

## 3. Methods

### 3.1. Animals and samples

From April to December 2017, a total of 350 cats with access to the outdoors from veterinary medical centres, animal shelters and from colonies (captured under the scope of trap-neuter-return programs) from Portugal, were studied. Cats were from three continental Portuguese NUTS II (Nomenclature of Units for Territorial Statistics): Centre (Coimbra and Guarda regions; $n = 61$), Lisbon (Lisbon and Setúbal regions; $n = 266$) and the Algarve region ($n = 23$).

Peripheral blood (1–2 ml) was obtained by cephalic or jugular venipuncture from each animal and collected into EDTA and serum-separating tubes. Serum and buffy coat were obtained by centrifugation and stored at -20 °C until use in serological analyses and DNA extraction, respectively.

Whenever available, data on sex, breed, fur length, age, reproductive status, lifestyle, use of insecticides/acaricides, co-habitation with other animals, presence of concomitant diseases and of clinical signs compatible with leishmaniosis (*i.e.*, anorexia, muscular atrophy, dermatological manifestations, exercise intolerance, fever, dyspnea, epistaxis, spleen/hepatomegaly, gingivostomatitis, gastrointestinal alterations, lameness, lymphadenopathy, lethargy, ocular manifestations, pale mucous membranes polyuria/polydipsia or weight loss) were recorded for each cat.

### 3.2. *Phlebotomus perniciosus* salivary glands and detection of anti-*P. perniciosus* saliva antibodies

Salivary gland homogenate (SGH) was obtained by dissecting salivary glands from 4–6 days-old *P. perniciosus* females reared under standard conditions [23]. Groups

of 20 salivary glands were pooled in 20 mM Tris buffer with 150 mM NaCl and then kept lyophilized at 4 °C until used.

Anti-*P. perniciosus* IgG was measured in all sera samples by indirect enzyme-linked immunosorbent assay (ELISA). The ELISA was performed in accordance with previous studies [7] with minor modifications. Briefly, flat-bottom microtiter plates (Nunc; VWR, Radnor, Pennsylvania, U.S.A.) were coated with *P. perniciosus* SGH (0.2 salivary gland per well) in 20 mM carbonate-bicarbonate buffer (pH 9, 100 µl/well) and incubated overnight at 4 °C. The plates were washed with PBS + 0.05 % Tween 20 (PBS-Tw) and blocked with 6 % (w/v) low fat dry milk diluted in PBS-Tw at 37 °C for 60 min. Feline sera diluted 1/50 in 2 % (w/v) low fat dry milk/PBS-Tw was added to the wells (100 µl/well) after washing twice with PBS-Tw. After 90 min incubation at 37 °C, the plates were washed with PBS-Tw and incubated at 37 °C for 45 min with secondary antibodies [AAI26P; Bio-Rad (AbD Serotec), Hercules, California, U.S.A.] (100 µl/well) diluted 1:5000 in PBS-Tw. Following another washing cycle, the ELISA was developed using orthophenylendiamine (P23938; Sigma-Aldrich, St. Louis, Missouri, U.S.A.) (0.5 mg/ml) in a phosphate citrate buffer (pH 5.5) with 0.001 % hydrogen peroxide (30 %; Merck, Darmstadt, Germany). The reaction was stopped after 5 min with 10 % sulphuric acid and absorbance (OP value) was measured at 492 nm using a NanoQuant (Infinite M200 Pro; Tecan, Zürich, Switzerland). Each serum was tested in duplicate. Wells without serum (but coated with SGH) were used as blanks while sera from cats living in non-endemic countries, namely Germany and Switzerland, served as negative controls. The cut-off value was calculated by the addition of three standard deviations to the mean optical density of the control sera.

### 3.3. Detection of anti-Leishmania IgG

Anti-*Leishmania* antibodies were determined in sera by the immunofluorescence antibody test (IFAT) as previously described [18]. Briefly, a *L. infantum* MON-1 (MCAN/PT/05/IMT-373) suspension of $10^7$ promastigotes was used as antigen and the anti-cat IgG (whole molecule)-FITC (F4262; Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was used in a dilution of l:20. A serum sample from a seropositive cat (IFAT titre 1:1204) was used as positive control [16] while the serum sample of a cat from a non-endemic

country of leishmaniosis was used as negative control. The IFAT cut-off value was established at a serum dilution of 1:64 (the same as used in the laboratory for dogs) [24].

### 3.4. DNA extraction and PCR amplification

DNA was extracted from buffy coat using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Detection of *Leishmania* DNA was done using a nested PCR protocol with primers targeting the small subunit ribosomal RNA (*SSU* rRNA) gene [25]. A positive control containing *L. infantum* MON-1 (MHOM/PT/88/IMT318) DNA and a negative control without DNA template were included in each amplification. The DNA amplicons were resolved by conventional electrophoresis on 1.5 % agarose gels stained with Green Safe Premium (Nzytech, Lisbon, Portugal), using a 100 bp DNA ladder as a molecular weight marker, then visualized under UV illumination.

### 3.5. Statistical analysis

An exploratory and descriptive data analysis was conducted for the main variables of the dataset. Cats were considered infected with *Leishmania* if they tested positive for at least one of the techniques (*i.e.*, PCR or IFAT). For the quantitative variable "age in months", the normality and the homogeneity of variance were evaluated using Kolmogorov-Smirnov/Shapiro-Wilk tests and the Levenne test, respectively. When these prerequisites were not valid, the non-parametric Mann-Whitney test was used. To explore the associations between qualitative variables and to compare proportions a Chi-square test, the alternative Fisher's exact test or the Freeman-Halton test was performed. Confidence intervals (95 % CI) for proportions were obtained by the Wilson method. This initial approach was followed by multivariate analysis that was developed to evaluate, in an integrated way, possible factors associated with the presence of antibodies against *P. perniciosus* saliva and with the presence of *Leishmania* DNA and/or antibodies to the parasite (outcome variables). First, crude odds ratios (OR crude) and 95 % CIs were obtained by a simple logistic regression model to screen the effect of each explanatory variable on the outcome variables. In a second step, explanatory variables with a *P*-value $\leq 0.2$ in the univariate analysis were selected and included in the multiple logistic regression model. Finally, a backward stepwise elimination procedure was implemented, using a *P*-value $\leq 0.05$ as the criterion for variables to remain in the model. The Hosmer

& Lemeshow goodness-of-fit test, residual analysis and determination of the area under the receiver operating characteristic curve (ROC) were performed. All statistical analyses were conducted using IBM® SPSS® Statistics version 25.0 and OpenEpi version 3.01 software.

## 4. Results

Antibodies to *Phlebotomus perniciosus* saliva (cut-off ≥ 0.173) were detected in 167 (47.7 %) sera (Table 1). One hundred and seven (73.8 %) and 72 (35.8 %) blood samples of domestic and stray cats, respectively, were collected during phlebotomine sand fly activity. There were significant differences between the ELISA result and the seven variables studied: (i) age group ($\chi^2$ = 38.335, *df* = 3, *P* < 0.001); (ii) fur length ($\chi^2$ = 6.229, *df* = 1, *P* = 0.043); (iii) lifestyle ($\chi^2$ = 31.806, *df* = 1, *P* < 0.001); (iv) region ($\chi^2$ = 14.246, *df* = 2, *P* < 0.001); (v) reproductive status ($\chi^2$ = 47.881, *df* = 1, *P* < 0.001); (vi) the use of acaricides/insecticides ($\chi^2$ = 20.516, *df* = 1, *P* < 0.001); and (vii) phlebotomine period activity ($\chi^2$ = 102.048, *df* = 1, *P* < 0.001). According to the multivariate logistic regression models, factors with a predicting effect on the presence of antibodies to *P. perniciosus* (Table 2) were age and phlebotomine activity period (Figure 1). First, cats of 12–35 months had 3.56 higher odds (95 % CI: 1.39–9.16; $\chi^2_{Wald}$ = 6.953, *df* = 1, *P* = 0.008) of presenting antibodies to *P. perniciosus* saliva than younger ones. This difference remained significant with higher magnitude when comparing young cats with those 36–95 months-old (OR = 9.43, 95 % CI: 3.62–24.48; $\chi^2_{Wald}$ = 21.224, *df* = 1, *P* < 0.001) and those older than 95 months (OR = 9.68, 95 % CI: 3.92–23.91; $\chi^2_{Wald}$ = 24.222, *df* = 1, *P* < 0.001). Secondly, sera collected during the period of phlebotomine sand fly seasonal activity exhibited nearly 19 times higher odds of having IgG antibody levels than those collected outside phlebotomine sand fly season (95 % CI: 9.84–38.41; $\chi^2_{Wald}$ = 72.947, *df* = 1, *P* < 0.001).

*Leishmania* infection was detected in 26 cats (7.7 %): *Leishmania* spp. DNA was detected using a set of general primers that target *SSU* rRNA in the blood samples of 24 (6.9 %) cats, while antibodies to *L. infantum* were detected in three (0.9 %) sera.

Only one cat was positive to *Leishmania* by both techniques. No significant differences were detected in positivity to *L. infantum* among all the variables/categories studied (Table 1).

**Table 1.** Prevalence of *Leishmania* (molecular and/or serological) and antibodies to *Phlebotomus perniciosus* saliva in cats from three regions of mainland Portugal.

| Variable/Categories | | Antibodies to *P. perniciosus* saliva | | | Antibodies to *Leishmania* and/or parasite DNA | | |
|---|---|---|---|---|---|---|---|
| | Tested cats | Positive cats | 95 % CI | *P*-value | Positive cats | 95 % CI | *P*-value |
| Sex, *n* (%) | 349 | | | 0.111 ($\chi^2$ = 2.535, *df* = 1) | | | 0.468 ($\chi^2$ = 0.526, *df* = 1) |
| Female | 191 (54.7) | 84 (44.0) | 37.1–51.1 | | 16 (8.4) | 5.2–13.2 | |
| Male | 158 (45.3) | 83 (52.5) | 44.8–60.2 | | 10 (6.3) | 3.5–11.3 | |
| Age, median (IQR) | 36 (12–96) | 72 (24–121) | | <0.001 (*Z* = -6.379) | 28 (8–96) | | 0.301 (*Z* = -1.034) |
| Age group, *n* (%) | 310 | | | <0.001 ($\chi^2$ = 38.335, *df* = 3) | | | 1.866 ($\chi^2$ = 1.866, *df* = 3) |
| 2–11 months | 70 (22.6) | 16 (22.9)[a,b] | 14.6–34.0 | | 7 (10.0) | 4.9–19.2 | |
| 12–35 months | 67 (21.6) | 25 (37.3)[c] | 26.7–49.3 | | 6 (9.0) | 4.2–18.2 | |
| 36–95 months | 85 (27.4) | 39 (45.9)[a,d] | 35.7–56.4 | | 4 (4.7) | 1.8–11.5 | |
| More than 95 months | 88 (28.4) | 62 (70.5)[b,c,d] | 60.2–79.0 | | 6 (6.8) | 3.2–14.1 | |
| Reproductive status, *n* (%) | 334 | | | <0.001 ($\chi^2$ = 47.881, *df* = 1) | | | 0.693 ($\chi^2$ = 0.156, *df* = 1) |
| Entire | 216 (64.7) | 72 (33.3) | 27.4–39.9 | | 14 (6.5) | 3.9–10.6 | |
| Neutered | 118 (35.3) | 86 (72.9) | 64.2–80.1 | | 9 (7.6) | 4.1–13.9 | |
| Breed, *n* (%) | 347 | | | 0.811 ($\chi^2$ = 0,057 *df* = 1) | | | 0.635[f] |
| Defined | 18 (5.2) | 9 (50.0) | 29.0–71.0 | | 2 (11.1) | 3.1–32.8 | |
| Mongrel | 329 (94.8) | 155 (47.1) | 41.8–52.5 | | 24 (7.3) | 5.0–10.6 | |
| Fur lenght, *n* (%) | 349 | | | 0.013 ($\chi^2$ = 6.229, *df* = 1) | | | 0.191[f] |
| Short | 310 (88.8) | 141 (45.5) | 40.0–51.0 | | 21 (6.8) | 4.5–10.1 | |
| Medium or long | 39 (11.2) | 26 (66.7) | 51.0–79.4 | | 5 (12.8) | 5.6–26.7 | |
| Lifestyle, *n* (%) | 346 | | | <0.001 ($\chi^2$ = 31.806, *df* = 1) | | | 0.522 ($\chi^2$ = 0.411, *df* = 1) |
| Domestic | 145 (41.9) | 95 (65.5) | 57.5–72.8 | | 12 (8.3) | 4.8–13.9 | |
| Shelter/stray | 201 (58.1) | 70 (34.8) | 28.6–41.6 | | 13 (6.5) | 3.8–10.8 | |
| Region, *n* (%) | 350 | | | 0.001 ($\chi^2$ = 14.246, *df* = 2) | | | 0.467[f] |
| Centre | 61 (17.4) | 41 (67.2)[e] | 54.7–77.7 | | 5 (8.2) | 3.6–17.8 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lisbon metropolitan area | 266 (76.0) | 112 (42.1)[e] | 36.3–48.1 | | 18 (6.8) | 5.6–26.7 | |
| Algarve | 23 (6.6) | 14 (60.9) | 40.8–77.8 | | 3 (13.0) | 4.5–32.1 | |
| Other animals, *n* (%) | 343 | | | 0.149 ($\chi^2$ = 2.082, *df* = 1) | | | 0.197[f] |
| No | 39 (11.4) | 23 (59.0) | 43.4–72.9 | | 5 (12.8) | 5.6–26.7 | |
| Yes | 304 (88.6) | 142 (46.7) | 41.2–52.3 | | 21 (6.9) | 4.6–10.3 | |
| Ectoparasiticides, *n* (%) | 332 | | | <0.001 ($\chi^2$ = 20.516, *df* = 1) | | | 0.147 ($\chi^2$ = 2.101, *df* = 1) |
| No | 257 (77.4) | 102 (39.7) | 33.9–45.8 | | 15 (5.8) | 3.6–9.4 | |
| Yes | 75 (22.6) | 52 (69.3) | 58.2–78.6 | | 8 (10.7) | 5.5–19.7 | |
| Clinical signs, *n* (%) | 350 | | | 0.137 ($\chi^2$ = 2.212, *df* = 1 | | | 0.899 ($\chi^2$ = 0.899, *df* = 1) |
| No | 252 (72.0) | 114 (45.2) | 39.2–51.4 | | 19 (7.5) | 4.9–11.5 | |
| Yes | 98 (28.0) | 53 (54.1) | 44.3–63.6 | | 7 (7.1) | 3.5–14.0 | |
| Concomitant diseases, *n* (%) | 181 | | | 0.185 ($\chi^2$ = 1.760, *df* = 1) | | | 0.384 ($\chi^2$ = 0.759, *df* = 1) |
| No | 99 (54.7) | 62 (62.6) | 52.8–71.5 | | 11 (11.1) | 6.3–18.8 | |
| Yes | 82 (45.3) | 59 (72.0) | 61.4–80.5 | | 6 (7.3) | 3.4–15.1 | |
| Phlebotomine activity period, *n* (%) | 350 | | | <0.001 ($\chi^2$ = 102.048, *df* = 1) | | | 0.156 ($\chi^2$ = 2.016, *df* = 1) |
| No | 168 (48.0) | 33 (19.6) | 14.3–26.3 | | 9 (5.4) | 2.8–9.9 | |
| Yes | 182 (52.0) | 134 (73.6) | 66.8–79.5 | 0.398 ($\chi^2$ = 5.148, *df* = 5) | 17 (9.3) | 5.9–14.5 | 0.653 ($\chi^2$ = 3.308, *df* = 5) |
| May | 25 (7.1) | 20 (80.0) | 60.9–91.1 | | 3 (12.0) | 2.5–31.2 | |
| June | 20 (5.7) | 14 (70.0) | 48.1–85.5 | | 1 (5.0) | 0.9–23.6 | |
| July | 22 (6.3) | 20 (90.9) | 72.2–97.5 | | 2 (9.1) | 2.5–27.8 | |
| August | 18 (5.1) | 13 (72.2) | 49.1–87.5 | | 0 (0.0) | 0.0–17.6 | |
| September | 24 (6.9) | 17 (70.8) | 50.8–85.1 | | 2 (8.3) | 2.3–25.9 | |
| October | 73 (20.9) | 50 (68.5) | 57.1–78.0 | | 9 (12.3) | 6.6–21.8 | |
| Total, *n* (%) | 350 | 167 (47.7) | 42.5–52.9 | | 26 (7.4) | 5.1–10.7 | |

[a] $P = 0.003$ ($\chi^2 = 8.832$, $df = 1$); [b] $P < 0.001$ ($\chi^2 = 35.110$, $df = 1$); [c] $P < 0.001$ ($\chi^2 = 16.860$, $df = 1$); [d] $P = 0.001$ ($\chi^2 = 10.680$, $df = 1$); [e] $P < 0.001$ ($\chi^2 = 12.530$, $df = 1$); [f] Fisher's exact test or Freeman-Halton test.

Abbreviations: CI, confidence interval; IQR, interquartile interval (quartile 1 - quartile 3).

**Table 2.** Presence of antibodies against *Phlebotomus perniciosus* saliva: odds-ratios, 95 % confidence intervals and significances, obtained by simple (OR crude) and multiple (OR adjusted) logistic regression models.

| Variable/categories | OR crude | 95 % CI | *P*-value | OR adjusted | 95 % CI | *P*-value |
|---|---|---|---|---|---|---|
| Sex[b] | | | | | | |
| Female[a] | | | | | | |
| Male | 1.41 | 0.92–2.15 | 0.112 ($\chi^2_{Wald}$ = 2.528, *df* = 1) | | | |
| Age group | | | | | | |
| 2–11 months[a] | | | <0.001 ($\chi^2_{Wald}$ = 35.190, *df* = 3) | | | <0.001 ($\chi^2_{Wald}$ = 29.553, *df* = 3) |
| 12–35 months | 2.01 | 0.95–4.24 | 0.067 ($\chi^2_{Wald}$ = 3.360, *df* = 1) | 3.56 | 1.39–9.16 | 0.008 ($\chi^2_{Wald}$ = 6.953, *df* = 1) |
| 36–95 months | 2.86 | 1.42–5.78 | 0.003 ($\chi^2_{Wald}$ = 8.608, *df* = 1) | 9.43 | 3.62–24.48 | <0.001 ($\chi^2_{Wald}$ = 21.224, *df* = 1) |
| More than 95 months | 8.05 | 3.91–16.56 | <0.001 ($\chi^2_{Wald}$ = 32.070, *df* = 1) | 9.68 | 3.92–23.91 | <0.001 ($\chi^2_{Wald}$ = 24.222, *df* = 1) |
| Reproductive status[b] | | | | | | |
| Entire[a] | | | | | | |
| Neutered | 5.38 | 3.28–8.82 | <0.001 ($\chi^2_{Wald}$ = 44.393, *df* = 1) | | | |
| Breed[b] | | | | | | |
| Defined[a] | | | | | | |
| Mongrel | 0.89 | 0.35–2.30 | 0.811 ($\chi^2_{Wald}$ = 0.057, *df* = 1) | | | |
| Fur lenght[b] | | | | | | |
| Short[a] | | | | | | |
| Medium or long | 2.40 | 1.19–4.84 | 0.015 ($\chi^2_{Wald}$ = 5.953, *df* = 1) | | | |
| Lifestyle[b] | | | | | | |
| Domestic[a] | | | | | | |
| Shelter/stray | 0.28 | 0.180–0.44 | <0.001 ($\chi^2_{Wald}$ = 30.684, *df* = 1) | | | |
| Region[b] | | | | | | |
| Centre[a] | | | 0.001 ($\chi^2_{Wald}$ = 13.693, *df* = 2) | | | |
| Lisbon metropolitan area | 0.36 | 0.20–0.64 | 0.001 ($\chi^2_{Wald}$ = 11.957, *df* = 1) | | | |
| Algarve | 0.76 | 0.28–2.05 | 0.586 ($\chi^2_{Wald}$ = 0.297, *df* = 1) | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Other animals[b] | | | | | | |
| No[a] | | | | | | |
| Yes | 0.61 | 0.31–1.20 | 0.152 ($\chi^2_{Wald}$ = 2.053, *df* = 1) | | | |
| Ectoparasiticides[b] | | | | | | |
| No[a] | | | | | | |
| Yes | 3.44 | 1.98–5.96 | <0.001 ($\chi^2_{Wald}$ = 19.290, *df* = 1) | | | |
| Clinical signs[b] | | | | | | |
| No[a] | | | | | | |
| Yes | 1.43 | 0.89–2.28 | 0.138 ($\chi^2_{Wald}$ = 2.203, *df* = 1) | | | |
| Concomitant diseases[b] | | | | | | |
| No[a] | | | | | | |
| Yes | 1.53 | 0.82–2.88 | 0.186 ($\chi^2_{Wald}$ = 1.751, *df* = 1) | | | |
| Phlebotomine activity period[b] | | | | | | |
| No[a] | | | | | | |
| Yes | 11.42 | 6.90–18.90 | <0.001 ($\chi^2_{Wald}$ = 89.858, *df* = 1) | 19.44 | 9.84–38.41 | <0.001 ($\chi^2_{Wald}$ = 72.947, *df* = 1) |

[a]Reference category; [b]Variable that did not have a statistically significant association in the fitted model.

Abbreviations: OR, odds ratio; CI, confidence interval.

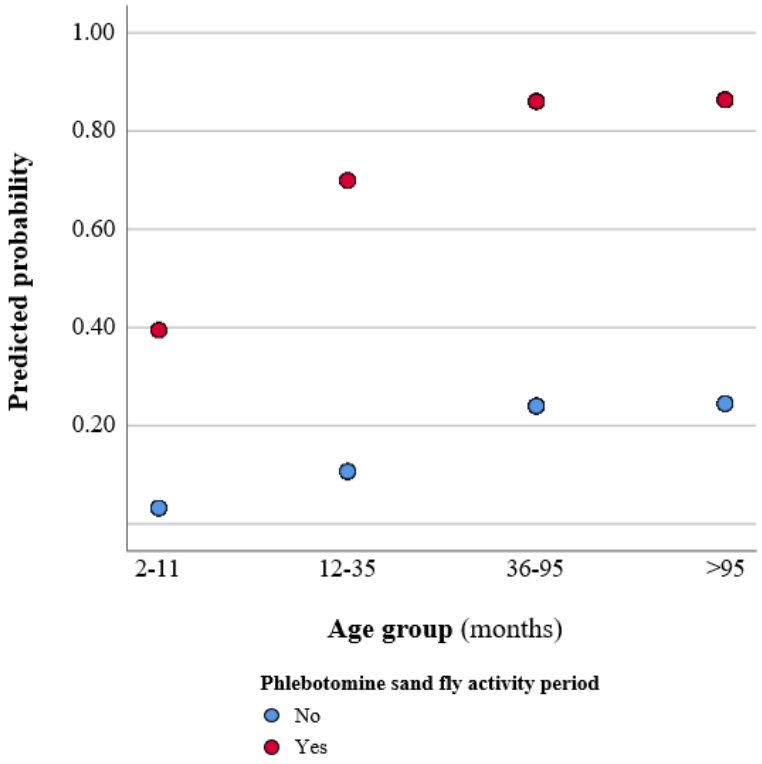


**Figure 1.** Predicted probability of the presence of antibodies against *Phlebotomus perniciosus* saliva related with cat age and phlebotomine sand fly activity period.

*Leishmania* DNA or specific antibodies to the parasite were detected in 18 cats seropositive to phlebotomine sand fly saliva. Of these 18 cats, all but one had a blood sample taken during phlebotomine sand fly activity. Cats presenting IgG antibodies to *P. perniciosus* had significantly higher risk ($\chi^2_{Wald} = 4.893$, $df = 1$, $P = 0.027$; OR = 2.64, 95 % CI: 1.12–6.25) of being infected with *Leishmania* (Table 3).

**Table 3.** Association between the presence of antibodies to *Phlebotomus perniciosus* saliva and a serological and/or molecular positive result for *Leishmania*.

| **Variable/categories** | **Antibodies to *Leishmania* and/or parasite DNA** | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | **Chi-Square test** | | **Simple logistic regression model** | | |
| | **Tested cats, *n* (%)** | **Positive cats, *n* (%)** | **95% CI** | ***P*-value** | **OR** | **95 % CI** | ***P*-value** |
| *Phlebotomus perniciosus* saliva | 350 | | | 0.022 ($\chi^2$ = 5.212, *df* = 1) | | | |
| Seronegative[a] | 183 (52.3) | 8 (4.4) | 2.2–8.4 | | | | |
| Seropositive | 167 (47.7) | 18 (10.8) | 6.9–16.4 | | 2.64 | 1.12–6.25 | 0.027 ($\chi^2_{Wald}$ = 4.893, *df* = 1) |

[a]Reference category.

Abbreviations: CI, confidence interval; OR, odds ratio.

## 5. Discussion

To our knowledge, this study describes for the first time feline antibody response against *P. perniciosus* saliva in cats naturally exposed to phlebotomine sand flies. The detection of antibodies to *P. perniciosus* in 47.7 % of sera shows that cats are frequently bitten by this species of sand fly, which is the most abundant *Phlebotomus* species in the three studied Portuguese regions [13, 26]. The presence of IgGs in 73.6 % of sera tested during phlebotomine activity period corroborates the results obtained in dogs from the Lisbon Metropolitan Area, where antibodies to *P. perniciosus* SGH were detected in 181 (75.1 %) out of 241 animals at the beginning of phlebotomine sand fly activity (*i.e.*, May) and in 209 (86.7 %) out of 241 at the end of phlebotomine sand fly season (*i.e.*, October) [27].

Previous studies have demonstrated that canine antibodies to phlebotomine sand fly-saliva correlate with biting intensity, fluctuate within phlebotomine sand fly season and decline significantly after the end of the biting season [6, 7, 12], emphasizing their usefulness as biomarkers for evaluating the exposure to phlebotomine sand flies and efficacy of vector control campaigns [4, 28].

In the present study, two non-casual associations were observed in the univariate analysis, namely the presence of a higher percentage of *P. perniciosus* antibodies in domestic and treated cats with ectoparasiticides than in stray and untreated cats, respectively. The reasons for these non-casual associations can be explained with the fact that most (73.8 %; 107/145) of the blood samples of the domestic cats with access to the outdoors were taken during the exposure period to phlebotomine sand fly bites, while only 35.8 % (72/201) of stray cats were sampled during phlebotomine sand fly season activity. On the other hand, the fact that cats treated with ectoparasiticides did not show a lower prevalence of positivity to *P. perniciosus* saliva than untreated cats was not entirely surprising because the only repellents effective against phlebotomine sand flies, the pyrethroids, are toxic to cats, with the exception of flumethrin. However, the application of imidacloprid/flumethrin collars in cats is still quite low in Portugal [29]. Nevertheless, and despite the lack of repellent effect of the most common ectoparasiticides applied to cats, they can potentially prevent parasite transmission from treated animals to other vertebrate hosts.

However, based on multivariate analysis, the presence of *P. perniciosus* antibodies in the peripheral blood of cats was neither associated with lifestyle nor with the use of ectoparasiticides, suggesting that these two variables are confounders. These results reinforce the importance of multivariate analysis in addressing confounding in epidemiological studies [30]. Based on this analysis, an association between IgG positivity and phlebotomine sand fly seasonal activity was observed in the present study, being significantly higher between May and October, than during winter months, when phlebotomine sand flies are inactive, suggesting that feline antibodies to saliva are relatively short-living. Unfortunately, no data are available for cats regarding the kinetics of specific antibodies to phlebotomine sand fly saliva or their correlation with the number of phlebotomine sand fly bites; therefore, it is not possible to precisely correlate feline antibodies against sand fly SGH and the seasonal abundance of *P. perniciosus.*

In cats, the antibody levels to *P. perniciosus* saliva were significantly increased with age group, suggesting accumulative exposure of older animals to sand fly bites. A similar positive correlation has repeatedly been demonstrated in dogs [8] which is probably related to the re-exposure of vertebrate hosts to phlebotomine sand flies following antigenic priming in the previous seasons. Interestingly, cats presenting antibodies to saliva were significantly more at risk of being positive to *Leishmania* infection. Whether saliva antigens could be used as biomarkers for *Leishmania* infection remains controversial, since both positive [7, 10, 11] and negative [6] associations between anti-*P. perniciosus* SGH antibodies and active *L. infantum* infection have been observed in dogs from endemic areas of leishmaniosis [4].

Regarding *Leishmania* infection, antibodies to the parasite or its DNA were detected in 26 cats (7.7 %). The positivity of detection of *Leishmania* DNA (6.9 %) was higher than the 0.3 % previously obtained in the north and centre of Portugal [17], but lower than the one (9.9 %) obtained in the south of the country [19], reinforcing that the rate of *Leishmania* infection is dynamic over time, depending on the density of proven vector population and on the number of infected vertebrate hosts.

Antibodies to *Leishmania* were detected by IFAT in 3 cats (0.9 %), which is also in agreement with previous studies performed in domestic and stray cats from the Lisbon Metropolitan Area [18, 20] but lower than the 3.8 % of seropositivity obtained in cats

from the Algarve region [31]. This strengthens the assumption that IFAT might not be sensitive enough to detect *Leishmania* infection in cats, or at least in those subclinically infected [2, 32].

## 6. Conclusions

To our knowledge, this is the first study demonstrating the development of anti-*P. perniciosus* saliva antibodies in cats. Due to the potential role of this animal species in sustaining and spreading *L. infantum* infection, the evaluation of the contact of cats with the vector is important in the development of prophylactic measures directed to cats with the aim of reducing the prevalence of infection in an endemic area. Further studies are needed to evaluate if there is a correlation between the number of phlebotomine sand fly bites and the dynamics of antibody production and if the use of imidacloprid/flumethrin collars reduces the frequency of *P. perniciosus* bites and *L. infantum* positivity in cats.

*Ethics statement*

The procedures were approved by the Ethical Committee of IHMT and for the Portuguese veterinary authorities as complying with Portuguese legislation for the protection of animals (Decree-Law no. 113/2013). Consent was obtained from the legal detainer, *i.e.*, the owner of the cat or the person in charge of the rescue associations for stray cats.

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# CHAPTER 3

## Feline leishmaniosis: clinical and parasitological follow-up

**This chapter is based on the research article:**

Pereira, A., Valente, J., Parreira, R., Cristóvão, J.M., Azinheira, S., Campino, L. & Maia, C. (2019) An unusual case of feline leishmaniosis with involvement of the mammary glands. *Topics in Companion Animal Medicine*. 37, 100356. https://doi.org/10.1016/j.tcam.2019.100356.

## 1. Abstract

We report an unusual case of leishmaniosis with the involvement of mammary glands in an old cat with what seemed to be a concurrent regressive feline leukaemia virus infection. *Leishmania donovani* complex parasites were identified for the first time in inflammatory breast fluid during a clinical recurrence manifested about four years after the first diagnosis of feline leishmaniosis. Combined treatment with allopurinol and meglumine antimoniate resulted in clinical cure of mammary lesion and a concurrent uveitis.

**Keywords**: cat, leishmaniosis, *Leishmania donovani* complex, mammary glands, feline leukaemia virus.

## 2. Introduction

Feline leishmaniosis (FeL) caused by *Leishmania donovani* complex parasites is a zoonotic vector-borne disease regarded as emergent in Southern European countries.[1] Most of the infected cats are asymptomatic, and the clinical disease has been commonly associated with immunological disorders due to concomitant retroviral infections [*i.e.*, feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV)], iatrogenic immunosuppression, neoplasia or diabetes mellitus.[2] Generalized lymphadenomegaly and mucocutaneous lesions are the most frequent clinical features found in FeL cases, whereas clinicopathological changes typically include hyperproteinaemia with hypergammaglobulinemia.[1] Nonetheless, unusual forms of FeL have been reported in the last years,[3,4] making diagnosis and treatment of this disease a further challenge to veterinarians. Here, we describe a case of FeL in which *Leishmania* parasites were detected in fine-needle aspiration (FNA) inflammatory breast fluid, during long-term administration of allopurinol.

## 3. Case presentation

An 8-year-old female neutered domestic shorthair cat was presented in February 2014 to a private veterinary hospital in the Lisbon Metropolitan Area, Portugal, due to the presence of palpebral nodules in both eyes. The cat had been adopted from a shelter 1 month before, and the past medical history was unknown. General physical examination

was unremarkable apart from the nodules. Body condition score (BCS) was characterized as ideal (BCS 4/9; weight 3.7 kg).[5] Differential diagnosis included infectious disease (eg. histoplasmosis, leishmaniosis, or mycobacteriosis), neoplasia (eg. apocrine cystadenoma, mastocytoma, or melanoma), sterile nodular granuloma, and xanthoma. A peripheral blood sample was obtained for complete blood cell count, serum biochemistry profile (*i.e.*, alkaline phosphatase, alanine aminotransferase, creatinine, glucose, and urea), serum protein electrophoresis, FeLV p27 antigen, and anti-FIV antibodies detection (Uranotest FeLV/FIV, Urano Vet SL, Spain), and anti-*Leishmania* antibodies [immunofluorescence antibody test (IFAT)] detection. A FNA of the nodular lesions was carried out for cytologic assessment.

Abnormalities found included thrombocytopenia [95 x $10^3$ cell/L, reference range (RR) 150-500 x $10^3$ cell/L] and mild hyperproteinemia (8.0 g/dL, RR 5.7-7.9 g/dL) with hypergammaglobulinemia (3.9 g/dL, RR 1.3-2.2 g/dL). The rapid immunochromatographic test was negative for FIV but positive for FeLV. However, this positive result for P27 antigenemia was not supported by an ELISA test performed by an external laboratory in the same blood sample. This negative p27 antigen (Enzyme-linked immunosorbent assay) ELISA test result, combined with FeLV proviral genome detection by real-time PCR (qPCR), confirmed a regressive FeLV infection.[6] Serological testing for *Leishmania* was positive (titer 320; cut-off ≥80), and the FNA cytology revealed the presence of several basophilic structures compatible with *Leishmania* amastigotes (*L.* amastigotes), not only in the cytoplasm of macrophages but also free from the cells (Figure 1).

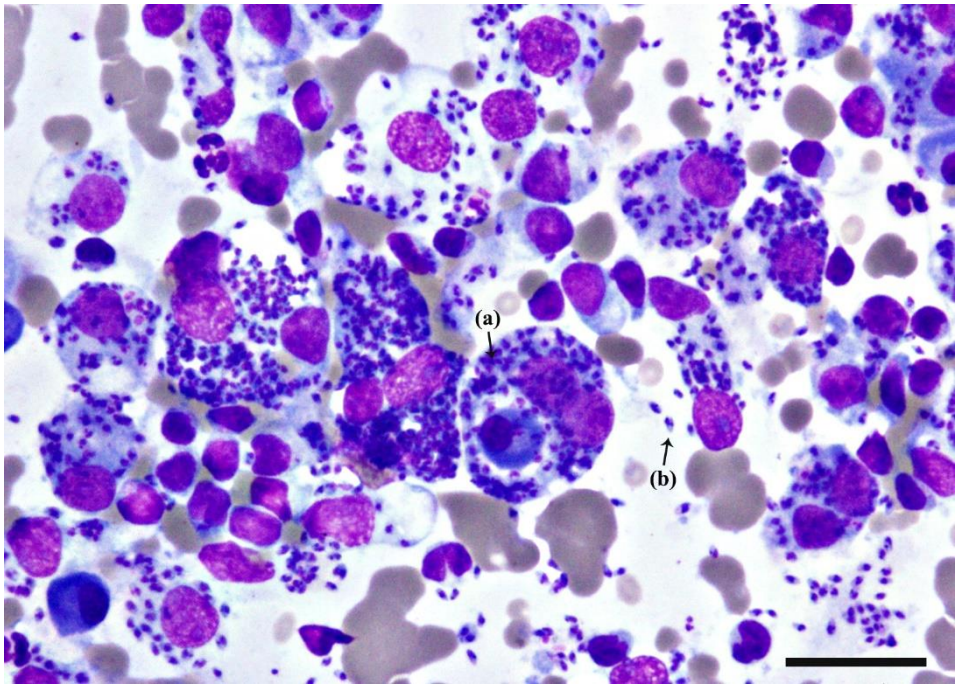


**Figure 1.** Cytology of the fine-needle aspirate from an eyelid nodule. Macrophages with numerous intracellular (A) and extracellular (B) organisms compatible with *Leishmania* amastigotes (Giemsa, scale bar = 50 µm).

Further, a bacteriological analysis was suggested but, unfortunately, declined by the owners. Treatment with allopurinol was initiated (approximately 10 mg/kg PO q12h; Zyloric, Faes Farma, Spain). Two months later, the nodular lesions were resolved, but the total protein concentration increased (9.3 g/dL), including the gamma-globulin fraction (4.1 g/dL). The above monotherapy regimen was maintained. No follow-ups were done due to the absence of the owners' compliance, but in December 2017 the cat was presented for assessment of ocular lesions. According to the owners, the allopurinol treatment had been maintained, but only with half of the prescribed dosage (approx. 5 mg/kg PO q12h). On ophthalmic examination, clinical signs compatible with bilateral anterior uveitis were found, including blepharospasm, photophobia, seromucous discharge, mild conjunctival hyperemia, protrusion of the nictitating membrane, diffuse corneal oedema, diffuse iris thickening, *rubeosis iridis* and keratic precipitates in the anterior chamber of both eyes. Fluorescein dye test was negative. Abnormal blood cell count results included mild microcytic normochromic anemia [red blood cell 4.9 x $10^{12}$ cell/L, RR 4.6-10.2 x $10^{12}$ cell/L; haematocrit 18.4 %, RR 26.0-47.0 %; haemoglobin 6.2 g/dL, RR 8.5-15.3 g/dL; mean corpuscular volume 37.7 fL, RR 38.0-54.0 fL; mean corpuscular haemoglobin concentration 335 g/L, RR 290.0-360.0 g/L; red blood cell

volume distribution width – standard deviation 32.1 fL, RR 26.4-43.1 fL], thrombocytopenia (39 x $10^9$ cell/L, RR 100-515 x $10^9$ cell/L), as well as leukopenia (2.7 x $10^9$ cell/L, RR 5.5-19.5 x $10^9$ cell/L) with neutropenia (.5 x $10^9$ cell/L, RR 3.1-12.5 x $10^9$ cell/L) and monocytopenia (.05 x $10^9$ cell/L, RR .07-1.3 x $10^9$ cell/L). The protein profile analysis revealed that the hyperproteinaemia (10.1 g/dL) worsened with a slight increase of gamma-globulins (4.2 g/dL). FeLV infection, monitored by both ELISA and qPCR assays, remained as regressive (*i.e.*, positive FeLV proviral DNA detection and negative p27 antigen ELISA test[6]) and the IFAT anti-*Leishmania* serum antibody titer continued to be positive (512, cut-off ≥64). The following treatment was prescribed to the cat: prednisolone acetate 1 % (2 drops OU q12h for 30 days; Frisolona Forte, Allergan, Ireland), meloxicam (.05 mg/kg PO q24h for 15 days; Meloxidyl, Ceva, France) and allopurinol (approx. 10 mg/kg PO q12h). The owners did not attend to the programmed clinical re-evaluations, but according to them (by telephone), the cat condition had improved. In January 2018, the cat was presented for prostration. The bilateral uveitis persisted. On physical examination, clinical signs suggestive of a right inguinal mammary gland inflammation were identified. Breast inspection was carried out with the cat in lateral recumbency. The skin was oedematous, slightly hot and with colour change (*i.e.*, bluish). On palpation, the mammary gland was enlarged and softly consistent. No nipple discharge was noted. Approximately 5 ml of a breast yellow-brown coloured fluid was collected by FNA (Figure 2A). The cytology analysis of this fluid showed the presence of basophilic forms compatible with *L.* amastigotes in the cytoplasm of macrophages (Figure 2B).

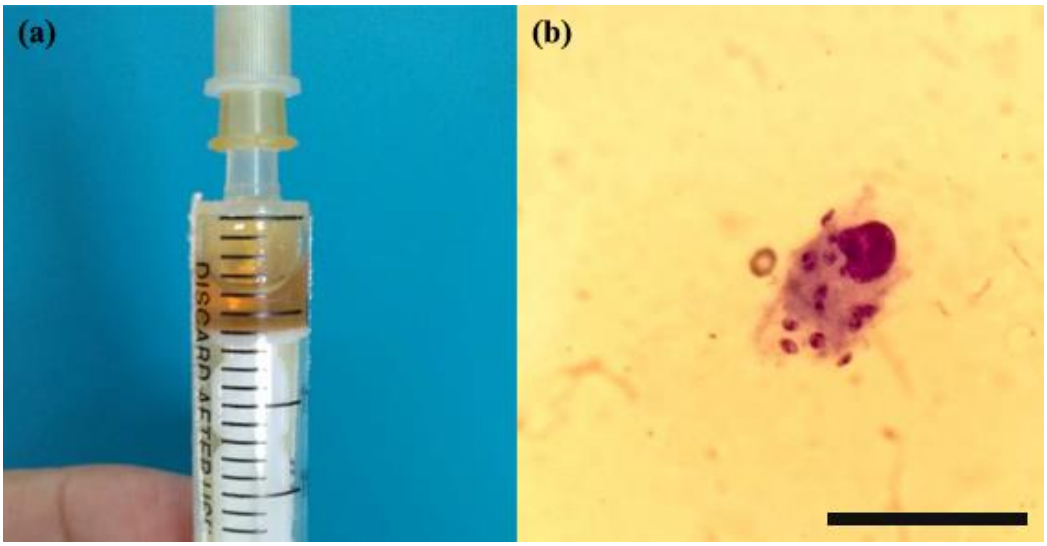


**Figure 2.** (A) Breast yellow-brown coloured fluid collected by fine-needle aspiration. (B) Cytology of the breast fluid. Macrophage with numerous intracellular organisms compatible with *Leishmania* amastigotes (Giemsa, scale bar = 50 µm).

Genomic DNA was extracted from both buffy coat and inflammatory breast fluid samples. *Leishmania* DNA was detected in both samples using a nested PCR assay with genus-specific primers for the small ribosomal DNA (*SSU*-rDNA).[7] For further parasite characterization, 3 additional nested-PCR assays were performed, using specific primers targeting sections of the *Leishmania* cytochrome b,[8] heat shock protein 70[9] and internal transcribed spacers and 5.8 ribosomal DNA.[10] The obtained PCR amplicons were sequenced, and their nucleotide sequences (analyzed with the BLASTn tool - http://blast.ncbi.nlm.nih.gov/Blast.cgi), revealed 100 % identity (covering 100 % of both queries) with homologues belonging to the *L. donovani* complex. Additionally, phylogenetic inferences following a maximum likelihood approach run under the GTR+Γ+I evolutionary model were carried out using MEGA v6.[11] These analyses undeniably placed the obtained sequences in monophyletic clusters composed only by sequences of *L. donovani* complex reference strains, supported by high bootstrap values (Figure 3).

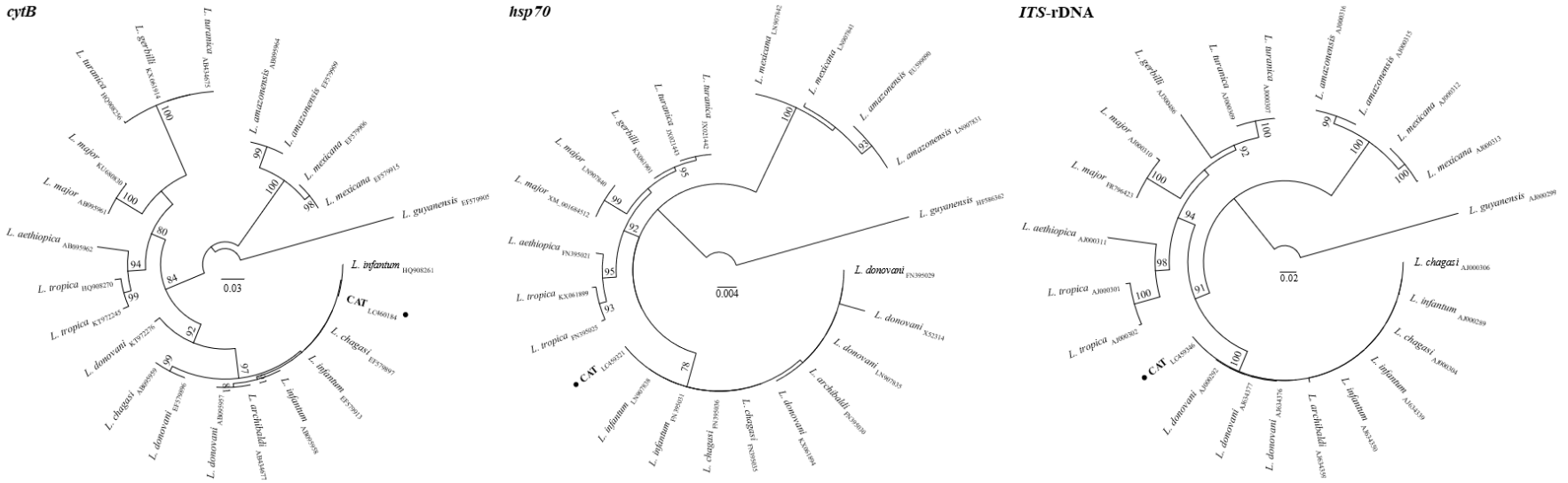


**Figure 3.** Maximum likelihood phylogenetic unrooted trees based on unambiguous *cytB*, *hsp70* and *ITS*-rDNA sequences alignments. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥75 % are shown. The size bar in the number of nucleotide substitutions per site. The trees were rooted using *Leishmania guyanensis* sequences (outgroup). Sequences of reference strains are identified by their accession number (underscore). The sequences obtained in this study are indicated with "●" and their accession number underscored.

Treatment with meglumine antimoniate (50 mg/kg SC q24h for 30 consecutive days; Glucantime, Merial, France) was started and the allopurinol posology (approx. 10 mg/kg PO q12h) maintained. During the treatment, no adverse reactions or renal function abnormalities (evaluated by the measurement of creatinine and urea concentrations, urine protein to creatinine ratio, and urine specific gravity) were observed. Six months after the end of the meglumine antimoniate treatment the cat was re-evaluated. Both mastitis and uveitis were cured, the *SSU*-rDNA PCR from buffy coat was negative, and the IFAT antibody titer decreased (64, cut-off ≥64). Long-term therapy with allopurinol (approx. 10 mg/kg PO q12h) was adopted for the maintenance of remission of FeL.

## 4. Discussion

To the author's knowledge, this is the first detection of *L. donovani* complex parasites in inflammatory breast fluid of a cat. The FeL diagnosis was supported by cytology, IFAT and PCR. Additionally, the phylogenetic analysis of cytochrome b, heat shock protein 70 and internal transcribed spacers and 5.8 ribosomal DNA sequences resulted in the unequivocal implication of the *L. donovani* complex. The parasites within this complex, which were recently considered as a single species, *L. donovani*,[12] have been responsible for all FeL cases reported in Europe.[2]

Coexistence of leishmaniosis and retroviral infections has also been reported in cats and humans.[1,13] Viremic FeLV-infected cats may develop immunosuppression like that observed in human immunodeficiency virus-infected individuals,[14] where the reported rates of treatment failure and recurrence of leishmaniosis are high.[15] Although the impact of regressive FeLV infections on the health of cats is largely unknown,[6] it has been demonstrated that this latent infection can also be responsible for a variety of hematopoietic disorders, including myelosuppression, as a direct result of FeLV provirus integration into the genome of the host cell.[14] However, these disorders were not observed in this case. Clinical FeL is sometimes associated with potentially impaired immunocompetence, and in approximately half of the cases mucocutaneous lesions, lymphadenomegaly, and/or hypergammaglobulinemia are present.[2]

Despite the absence of data to allow a reliable comparison between the clinical presentation of leishmaniosis in cats with/without related concurrent disorders, the

coexistence of infectious/noninfectious diseases seem to contribute to a misrepresentation of the underlying disease.[1] In this case, *Leishmania* parasites were implicated in both eyelid nodules and mammary gland inflammation, as revealed by cytological and PCR evaluations. In cats, lesions associated with the presence of the parasites were previously reported in several tissues.[1,3] The detection of parasites in mammary glands was only described in naturally infected dogs [16] and in humans.[17] In this case, a mastitis either secondary to neoplasia or to bacterial infection with migration of inflammatory cells infected with *Leishmania* cannot be ruled out. However, and despite no bacterial culture was performed, neither structures compatible with bacteria nor the presence of polymorphonuclear neutrophils were observed in the FNA sample. Additionally, no cells with malignant characteristics were present in the cytology. The ocular and hematological abnormalities found in the present case cannot be caused by *Leishmania* infection due to the concurrent FeLV infection.[14] As FeLV-infected cats usually do not show hypergammaglobulinemia,[14] the increased level of gamma-globulins observed in serum protein electrophoresis was probably induced by *Leishmania* infection.[2] Nonetheless, the presence of other concurrent diseases such as feline bartonellosis or feline infectious peritonitis cannot be formally excluded. In fact, these diseases can also result in hypergammaglobulinemia and uveitis.[18,19] However, ex juvantibus, we confirmed a causative role of *Leishmania* infection in the development not only of eyelid nodules in 2014 but also of uveitis and mastitis about four years later.

Empirical treatment of FeL with allopurinol has been effective in most cases.[2] The initial monotherapy prescribed was well tolerated but, as previously reported,[2,20,21] did not result in clearance of infection. Although there are no studies regarding the susceptibility of parasites of *Leishmania donovani* complex isolated from cats to allopurinol, the resistance to this compound associated with disease relapse has been demonstrated in strains isolated from dogs.[22] Additionally, FeL clinical recurrence as a result of halved allopurinol posology, which was given to the cat during an approximately 3-year period, cannot be formally ruled out.

On the other hand, and despite side effects such as dermatological reactions caused by allopurinol and renal injury caused by both allopurinol and meglumine antimoniate have already been described,[1,3,21] the combined use of these two compounds resulted in a decrease in antibody titer and in clinical cure of uveitis and mastitis with no

overt adverse effects. Indeed, compounds with leishmanicidal activity such as meglumine antimoniate have been given in combination with allopurinol for the treatment of FeL refractory cases.[20]

## 5. Conclusion

A four year follow up of a cat with FeL confirms the chronic course of the disease and the possibility of clinical recurrence with manifestations other than those seen at disease onset. Interestingly the parasite can be found associated with mastitis.

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# CHAPTER 4

## Genetic characterisation of *Leishmania* detected in cats

**This chapter is based on the research article:**

Pereira, A., Parreira, R., Cristóvão, J.M., Castelli, G., Bruno, F., Vitale, F., Campino, L. & Maia, C. (2020) Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers. *Infection, Genetics and Evolution*. 77, 104069. https://doi.org/10.1016/j.meegid.2019.104069.

## 1. Abstract

Cats have been found infected by the same *Leishmania* species that also infect dogs and humans in both the New and Old Worlds, and their role as additional reservoir hosts of *L. infantum* has been previously suggested. Currently, the genetic diversity of *Leishmania* spp. detected in cats is poorly understood. In this cross-sectional study, the partial nucleotide sequences of four gene markers (*cytB*, *g6pdh*, *hsp70* and *ITS*-rDNA) were explored to investigate the genetic diversity and the phylogenetic relationships of *Leishmania* parasites detected in cats. A total of 25 cat buffy coat samples where the presence of *Leishmania SSU*-rDNA was revealed by PCR (from a convenience sample of 465 cats screened), as well as six *Leishmania* strains previously isolated from cats, were included in this study. Phylogenetic analyses showed that the majority of *Leishmania* parasites detected in cats did not display distinctive genetic features, sharing the same genetic types with *L. infantum* strains isolated from humans, dogs and phlebotomine sand flies. Unexpectedly, DNA of *L. major* and/or of a *L. major/L. donovani* sensu lato hybrid was detected in buffy coat samples of two cats from different regions of Portugal. However, a mix infection hypothesis cannot be formally excluded. To our knowledge, this study represents the first evidence for the presence of DNA of *Leishmania* hybrid parasites in cats. The results reported here not only reinforce the idea that cats play a role in the epidemiology of zoonotic leishmaniosis but also indicate the circulation of *L. major* and/or *L. major*/*L. donovani* s.l. hybrid parasites in Portugal. Also, whenever sequencing of whole *Leishmania* genomes regularly cannot be accomplished, and while their complete genomes remain under-represented in the nucleotide sequence databases, the combined use of multiple genetic markers, including kinetoplast maxicircle DNA, seems to be essential for typing of *Leishmania* parasites.

**Keywords:** cat, *Leishmania* species, genetic diversity, molecular epidemiology, phylogeny.

## 2. Introduction

Leishmaniosis is a widespread neglected disease caused by kinetoplastid protozoa of the genus *Leishmania*, which is typically transmitted to humans and other mammals by the bite of infected female phlebotomine sand flies (WHO, 2010). The clinical manifestations of the disease are not only largely diverse but also partially correlated with the causative species, ranging from a benign cutaneous form to a lethal visceral illness (WHO, 2010). At least 21 *Leishmania* species have been recognised as being pathogenic to humans (Akhoundi et al., 2016). Additionally, the occurrence of natural infecting hybrids has also been reported (Delgado et al., 1997; Odiwuor et al., 2011; Ravel et al., 2006).

In the Old World, parasites belonging to the so-called *L. donovani* species complex [synonym sensu lato (s.l.)] are mainly associated with visceral leishmaniosis (VL), while both *L. tropica* and *L. major* parasites are associated with cutaneous leishmaniosis (CL) (WHO, 2010). Apart from dogs, cats also seem to play a role in the maintenance, and dissemination, of human and animal leishmaniosis due to *L. infantum* (Maia and Campino, 2011). The incidence of cases of feline leishmaniosis has increased during the last years, to the point where it is now regarded as an emerging feline disease in endemic areas of *L. infantum* (Pennisi and Persichetti, 2018).

Since the 1980s, the multilocus enzyme electrophoresis (MLEE) typing method has been widely used for classification of *Leishmania* parasites, remaining, up to the present day, the gold standard genetic typing approach for these parasites (WHO, 2010). However, this method has several limitations, from requiring bulk cultures of parasites to providing insufficient information on their evolution and genomic plasticity, namely deterring the identification of hybridisation events. As a result, over the last years, several molecular-based methods have become increasingly relevant, not only for diagnosis of infections caused by *Leishmania* but also for assessing their genetic diversity, through a combination of both high sensitivity and species-specificity (Schönian et al., 2011). More recently, the development of molecular typing systems, such as multilocus sequence typing (MLST), have provided new insights into the classification of *Leishmania* and a clarification of their phylogenetic relationships.

Based on these methods, several cases of species-synonymy have been revealed (Akhoundi et al., 2017), including the four species previously recognised as belonging to *L. donovani* complex (*i.e.*. *L. archibaldi*, *L. chagasi*, *L. donovani* and *L. infantum*) which were recently considered as a single species, *L. donovani* (Maurício, 2018).

The bulk of phylogenetic studies have focused on strains of human or canine origin (Cortes et al., 2014), while very few of them involve the analysis of *Leishmania* genetic material obtained from cats (Ceccarelli et al., 2018).

Therefore, the aim of this study was the characterisation and assessment of the phylogenetic relationships between strains of *Leishmania* on the basis of the analysis of partial cytochrome b (*cytB*), glucose-6-phosphate dehydrogenase (*g6pdh*), heat-shock protein 70 (*hsp70*) and internal transcribed spacers-5.8 ribosomal DNA sequences (*ITS*-rDNA) using different analytical approaches. These sequences were either PCR-amplified directly from cat biological samples, or laboratory strains of feline origin, previously isolated in vitro.

## 3. Material and methods

### 3.1. Cats

Between February 2017 and August 2018, a total of 465 cats (convenience sampling) from veterinary medical centres, animal shelters and colonies (captured under the scope of trap-neuter-return programs) from three distinct regions of mainland Portugal [*i.e.*, Center, $n = 73$; the Lisbon Metropolitan Area (LMA), $n = 344$; and the Algarve, $n = 48$], were studied. Peripheral blood samples were obtained from each animal by cephalic or jugular venipuncture, and the blood collected (1-2 ml) placed into tubes with EDTA. After centrifugation, the buffy coat samples were separated from the liquid fraction, and then stored at -20ºC until DNA extraction. This study also included the use of DNA extracts prepared in a previous epidemiological study focused on the assessment of exposure of cats to the bites of phlebotomine sand flies (Pereira et al., 2019).

### 3.2. Laboratory strains

The DNA of a total of 21 strains previously classified by isoenzymatic and/or molecular typing methods as *L. infantum* ($n = 15$), *L. donovani* ($n = 2$), *L. major* ($n = 2$), and *L. tropica* ($n = 2$) was included in this study (Table 1).

**Table 1.** List of nucleotide sequences included in phylogenetic analyses.

| Taxon[a] | Laboratory code | International code | Zymodeme | Country | Host | Gene (accession numbers) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ***cytB*** | ***g6pdh*** | ***hsp70*** | ***ITS*-rDNA** |
| *L.* (*L.*) *donovani* complex | G322[b,c] | | | Portugal | *Felis catus* | LC460182 | | | |
| | G338[b] | | | Portugal | *Felis catus* | LC460183 | | LC459320 | LC459345 |
| | G435[b] | | | Portugal | *Felis catus* | LC460184 | | LC459321 | LC459346 |
| | IMT422 | MFEL/PT/18/IMT422 | | Portugal | *Felis catus* | LC460175 | LC459291 | LC459313 | LC459338 |
| *L. archibaldi* | GEBRE1 | MHOM/ET/72/GEBRE1 | MON-82 | Ethiopia | *Homo sapiens* | AB434677 | DQ449780 | | |
| | LEM3429 | MHOM/SD/97/LEM3429 | MON-35 | Sudan | *Homo sapiens* | | DQ449793 | | AJ634358 |
| | LEM3463 | MHOM/SD/97/LEM3463 | MON-258 | Sudan | *Homo sapiens* | | DQ449794 | FN395030 | AJ634359 |
| *L. chagasi* | ARL | MHOM/BR/07/ARL | | Brazil | *Homo sapiens* | | | FN395037 | |
| | LEM590 | MHOM/BR/74/LEM590 | | Brazil | *Homo sapiens* | EF579897 | | | |
| | M9702 | MHOM/BR/85/M9702 | MON-1 | Brazil | *Homo sapiens* | | | | AJ000306 |
| | MAIKE | MCAN/BR/06/MAIKE | IOC/Z1 | Brazil | *Canis familiaris* | | | FN395035 | |
| | PP75 | MHOM/BR/74/PP75 | MON-1 | Brazil | *Homo sapiens* | AB095959 | | | AJ000304 |
| | WC | MHOM/BR/07/WC | IOC/Z1 | Brazil | *Homo sapiens* | | | FN395036 | |
| *L. donovani* | 1S | MHOM/SD/68/1S | | Sudan | *Homo sapiens* | | | FN395027 | AJ000293 |
| | 2S-25M-C2 | MHOM/SD/62/2S-25M-C2 | | Sudan | *Homo sapiens* | AB095957 | | | |
| | BPK275 | MHOM/NP/03/BPK275 | | Nepal | *Homo sapiens* | LC460189 | LC459301 | LC459326 | LC459351 |
| | DD8 | MHOM/IN/80/DD8[d] | MON-2 | India | *Homo sapiens* | EF579896 | DQ449795 | KX061894 | AJ000292 |
| | DEVI | MHOM/IN/00/DEVI | MON-2 | India | *Homo sapiens* | | DQ449778 | FN395028 | AJ634376 |
| | GILANI | MHOM/SD/82/GILANI | MON-30 | Sudan | *Homo sapiens* | | DQ449781 | FN395029 | AJ634369 |
| | HU3 | MHOM/ET/67/HU3 | MON-18 | Ethiopia | *Homo sapiens* | KT972276 | | X52314 | |
| | IMT180 | MHOM/PT/92/IMT180 | MON-18 | Portugal | *Homo sapiens* | LC460166 | LC459282 | LC459304 | LC459329 |
| | LEM3946 | MCAN/SD/00/LEM3946 | MON-274 | Sudan | *Canis familiaris* | | DQ449786 | | AJ634356 |
| | LRC-L51 | MHOM/IN/--/LRC-L51 | MON-18 | India | *Homo sapiens* | | | LN907834 | |
| | LRC-L53 | MHOM/KE/55/LRC-L53 | MON-36 | Kenya | *Homo sapiens* | | | LN907835 | |
| | SC23 | MHOM/IN/54/SC23 | MON-38 | India | *Homo sapiens* | | DQ449785 | | AJ634375 |
| | THAK35 | MHOM/IN/96/THAK35 | MON-2 | India | *Homo sapiens* | | DQ449779 | | AJ634377 |
| *L. infantum* | 2147 | MFEL/IT/10/2147 | | Italy | *Felis catus* | LC460176 | LC459292 | LC459314 | LC459339 |
| | 6827 | MFEL/IT/08/6827 | | Italy | *Felis catus* | LC460177 | LC459293 | LC459315 | LC459340 |
| | 10816 | MFEL/IT/02/10816 | MON-1 | Italy | *Felis catus* | LC460178 | LC459294 | LC459316 | LC459341 |
| | 12022 | MFEL/IT/99/12022 | | Italy | *Felis catus* | LC460179 | LC459295 | LC459317 | LC459342 |
| | 33861 | MFEL/IT/05/33861 | | Italy | *Felis catus* | LC460180 | LC459296 | LC459318 | LC459343 |
| | 3S | MHOM/SD/62/3S | MON-81 | Sudan | *Homo sapiens* | | DQ449787 | | AJ634361 |
| | BUCK | MHOM/MT/85/BUCK | MON-78 | Malta | *Homo sapiens* | | DQ449784 | FN395031 | AJ634350 |
| | CRE69 | MCAN/GR/94/CRE69 | | Greece | *Canis familiaris* | EF579913 | | | |
| | GS7 | MHOM/CN/93/GS7 | | China | *Homo sapiens* | HQ908261 | | | |
| | IMT163 | MHOM/PT/89/IMT163 | MON-1 | Portugal | *Homo sapiens* | LC460164 | LC459280 | LC459302 | LC459327 |
| | IMT169 | IARI/PT/89/IMT169 | MON-1 | Portugal | *Phlebotomus ariasi* | LC460165 | LC459281 | LC459303 | LC459328 |
| | IMT181 | MHOM/PT/92/IMT181 | MON-24 | Portugal | *Homo sapiens* | LC460167 | LC459283 | LC459305 | LC459330 |
| | IMT189 | IPER/PT/93/IMT189 | MON-1 | Portugal | *Phlebotomus perniciosus* | LC460168 | LC459284 | LC459306 | LC459331 |
| | IMT202 | MHOM/PT/94/IMT202 | MON-29 | Portugal | *Homo sapiens* | LC460169 | LC459285 | LC459307 | LC459332 |
| | IMT205 | MCAN/PT/94/IMT205 | MON-1 | Portugal | *Canis familiaris* | LC460170 | LC459286 | LC459308 | LC459333 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | IMT229 | MCAN/PT/97/IMT229 | MON-1 | Portugal | *Canis familiaris* | LC460171 | LC459287 | LC459309 | LC459334 |
| | IMT241 | MHOM/PT/98/IMT241 | MON-1 | Portugal | *Homo sapiens* | LC460172 | LC459288 | LC459310 | LC459335 |
| | IMT276 | MCAN/PT/01/IMT276 | MON-1 | Portugal | *Canis familiaris* | LC460173 | LC459289 | LC459311 | LC459336 |
| | IMT405 | MFEL/PT/13/IMT405 | MON-1 | Portugal | *Felis catus* | LC460174 | LC459290 | LC459312 | LC459337 |
| | IPT1 | MHOM/TN/80/IPT1[d] | MON-1 | Tunisia | *Homo sapiens* | AB095958 | | | AJ000289 |
| | ISS800 | MHOM/IT/1993/ISS800 | MON-188 | Italy | *Homo sapiens* | | DQ449791 | | AJ634354 |
| | ITM-AP263 | MHOM/MA/67/ITMAP263 | MON-1 | Morocco | *Homo sapiens* | | | FN395033 | |
| | LEM75 | MHOM/FR/78/LEM75 | MON-1 | France | *Homo sapiens* | | DQ449770 | LN907838 | AJ634339 |
| | LEM189 | MHOM/FR/80/LEM189 | MON-11 | France | *Homo sapiens* | | DQ449783 | | AJ634351 |
| | LEM935 | MCAN/ES/86/LEM935 | MON-77 | Spain | *Canis familiaris* | | DQ449797 | | AJ634355 |
| | LEM3472 | MHOM/SD/97/LEM3472 | MON-267 | Sudan | *Homo sapiens* | | DQ449792 | | AJ634370 |
| | LRC-L720 | MCAN/IL/97/LRC-L720 | | Israel | *Canis familiaris* | | | HF586393 | |
| *L.* (*L.*) *major* complex | | | | | | | | | |
| *L. arabica* | JISH220 | MPSA/SA/83/JISH220[d] | MON-99 | Saudi Arabia | *Psammomys obesus* | AB434685 | | | |
| *L. gerbilli* | E-11 | MRHO/SU/87/E-11 | MON-272 | EX-USSR | *Rhombomys opimus* | | | HF586355 | |
| | GERBILLI | MRHO/CN/60/GERBILLI[d] | MON-22 | China | *Rhombomys opimus* | KX061914 | DQ449800 | KX061901 | |
| | KD-87555 | MRHO/UZ/87/KD-87555 | | Uzbekistan | *Rhombomys opimus* | | | | AJ300486 |
| *L. major* | 5-ASKH | MHOM/SU/73/5-ASKH[d] | MON-4 | EX-USSR | *Homo sapiens* | AB095961 | | | AJ000310 |
| | CLONE31 | MHOM/IR/16/CLONE31 | | Iran | *Homo sapiens* | | MF109351 | | |
| | CRE1 | MHOM/IQ/86/CRE1 | MON-26 | Iraq | *Homo sapiens* | | | LN907840 | |
| | FRIEDLIN | MHOM/IL/80/FRIEDLIN | MON-103 | Israel | *Homo sapiens* | KU680830 | XM_001686045 | XM_001684512 | FR796423 |
| | G240[b] | | | Portugal | *Felis catus* | LC460181 | | | LC459344 |
| | G322[b,c] | | | Portugal | *Felis catus* | | | LC459319 | |
| | LCB33 | MHOM/SD/03/LCB33 | MON-74 | Sudan | *Homo sapiens* | | | HF586346 | |
| | LV561 | MHOM/IL/67/LV561 | MON-26 | Israel | *Homo sapiens* | LC460185 | LC459297 | LC459322 | LC459347 |
| | NEAL-P | MRHO/SU/59/NEAL-P | MON-4 | EX-USSR | *Rhombomys* sp. | LC460186 | LC459298 | LC459323 | LC459348 |
| | PT-115 | MHOM/EC/88/PT-115 | | Ecuador | *Homo sapiens* | AB095970 | | | |
| | VIN | MHOM/TM/--/VIN | | Turkmenistan | *Homo sapiens* | | | | AJ272383 |
| *L. turanica* | CLONE3720 | MRHO/SU/80/CLONE3720 | LON-59 | EX-USSR | *Rhombomys* sp. | AB434675 | | | |
| | KXG-2 | MRHO/CN/88/KXG-2 | | China | *Rhombomys* sp. | HQ908256 | | | |
| | KXG-11 | MRHO/CN/87/KXG-11 | | China | *Rhombomys* sp. | | JX970982 | JX021443 | |
| | MNR-3 | MRHO/MN/84/MNR-3 | | Mongolia | *Rhombomys* sp. | | | | AJ000307 |
| | MNR-13 | MRHO/MN/84/MNR-13 | | Mongolia | *Rhombomys* sp. | | | | AJ000309 |
| | QITAI-15 | MRHO/CN/92/QITAI-15 | | China | *Rhombomys* sp. | | JX021341 | JX021442 | |
| *L.* (*L.*) *mexicana* complex | | | | | | | | | |
| *L. amazonensis* | LAV003 | MHOM/GF/02/LAV003 | MON-41 | French Guiana | *Homo sapiens* | | | LN907831 | |
| | M1841 | MPRO/BR/72/M1841 | | Brazil | *Proechimys* sp. | EF579909 | | | |
| | M1845 | MPRO/BR/72/M1845 | | Brazil | *Proechimys* sp. | HM439238 | | | |
| | M2269 | MHOM/BR/73/M2269[d] | MON-132 | Brazil | *Homo sapiens* | AB095964 | AY099298 | EU599090 | AJ000316 |
| | WR369 | MHOM/PA/80/WR369 | | Panama | *Homo sapiens* | | | | AJ000315 |
| *L. aristidesi* | GML | MORY/PA/69/GML | MON-133 | Panama | *Oryzomys* sp. | AB434678 | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *L. garnhami* | JAP78 | MHOM/VE/76/JAP78[d] | MON-41 | Venezuela | *Homo sapiens* | AB095965 | | EU599092 | |
| *L. mexicana* | BEL21 | MHOM/BZ/82/BEL21[d] | MON-156 | Belize | *Homo sapiens* | EF579906 | | LN907841 | |
| | CRE47 | MHOM/MX/93/CRE47 | | Mexico | *Homo sapiens* | EF579915 | | | |
| | EC103-CL8 | MHOM/EC/87/EC103-CL8 | MON-110 | Ecuador | *Homo sapiens* | | | LN907842 | |
| | GO22 | MHOM/GT/86/GO22 | | Guatemala | *Homo sapiens* | | | | AJ000312 |
| | SOLIS | MHOM/MX/85/SOLIS | MON-152 | Mexico | *Homo sapiens* | | | | AJ000313 |
| | U1103 | MHOM/GT/2001/U1103 | | Guatemala | *Homo sapiens* | | FR799586 | | |
| *L. pifanoi* | LL1 | MHOM/VE/57/LL1[d] | MON-40 | Venezuela | *Homo sapiens* | AB434679 | | | |
| *L.* (*L.*) *tropica* complex | | | | | | | | | |
| *L. aethiopica* | 1470 | MHOM/ET/94/1470 | | Ethiopia | *Homo sapiens* | | | | AJ000311 |
| | L100 | MHOM/ET/72/L100[d] | MON-14 | Ethiopia | *Homo sapiens* | AB095962 | | FN395021 | |
| | NLB107-08 | MHOM/KE/--/NLB107-08 | | Kenya | *Homo sapiens* | | | FN395019 | |
| *L. killicki* | LEM163 | MHOM/TN/86/LEM163 | MON-8 | Tunisia | *Homo sapiens* | AB434676 | | | |
| *L. tropica* | DD7 | MHOM/IN/79/DD7 | | India | *Homo sapiens* | | | FN395025 | |
| | K27 | MHOM/SU/74/K27[d] | MON-60 | EX-USSR | *Homo sapiens* | HQ908270 | DQ449801 | KX061899 | |
| | LRC-L747 | ISER/IL/02/LRC-L747 | | Israel | *Phlebotomus sergenti* | LC460188 | LC459300 | LC459325 | LC459350 |
| | NLB297 | MHOM/KE/84/NLB297 | | Kenya | *Homo sapiens* | | | | AJ000301 |
| | ROSSI-II | IROS/NA/76/ROSSI-II | | Namibia | *Phlebotomus rossi* | KT972245 | | | AJ000302 |
| | SINGER | MHOM/IL/80/SINGER | MON-54 | Israel | *Homo sapiens* | | | LN907846 | |
| | VEDHA | MHOM/TR/99/VEDHA | MON-53 | Turkey | *Homo sapiens* | LC460187 | LC459299 | LC459324 | LC459349 |
| *L.* (*V.*) *braziliensis* complex | | | | | | | | | |
| *L. braziliensis* | M2904 | MHOM/BR/75/M2904[d] | MON-165 | Brazil | *Homo sapiens* | AB095966 | | | |
| *L. peruviana* | LC39 | MHOM/PE/84/LC39[d] | MON-128 | Peru | *Homo sapiens* | AB433282 | | | |
| *L.* (*V.*) *guyanensis* complex | | | | | | | | | |
| *L. guyanensis* | LEM85 | MHOM/GF/79/LEM85[d] | MON-45 | French Guiana | *Homo sapiens* | | | HF586362 | |
| | M4147 | MHOM/BR/75/M4147 | | Brazil | *Homo sapiens* | | AY099300 | | AJ000299 |
| *L. panamensis* | LS94 | MHOM/PA/71/LS94[d] | MON-47 | Panama | *Homo sapiens* | | | EU599094 | |
| | NEL3 | MHOM/CR/87/NEL3 | | Costa Rica | *Homo sapiens* | | | | AJ000298 |
| | PSC-1 | MHOM/PA/94/PSC-1 | | Panama | *Homo sapiens* | | CP009389 | | |

[a] Species as defined by the depositors; [b] Strain not isolated; [c] Putative hybrid; [d] WHO reference strain.

Abbreviations: *cytB*, cytochrome *b*; g6pdh, glucose-6-phosphate dehydrogenase; *hsp70*, heat-shock protein 70; *ITS*-rDNA, internal transcribed spacers-5.8 ribosomal DNA; *SSU*-rRNA, small subunit ribosomal DNA; bp, base pars; *L.*, *Leishmania*; *V.*, *Viannia*.

### 3.3. DNA extraction, amplification and sequencing

Genomic DNA was extracted from buffy coat samples using the High Pure Template Preparation Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The presence of *Leishmania* DNA in cat samples was firstly screened using a nested-PCR protocol with primers targeting the small ribosomal DNA (*SSU*-rDNA) (Table 2). For further molecular characterisation of both *SSU*-rDNA positive samples and laboratory-isolated feline *Leishmania* strains, multiple PCR-based assays using specific primers targeting sections of *cytB*, *g6pdh*, *hsp70*, and *ITS*-rDNA, were performed (Table 2). In all amplification reactions, positive (*L. infantum* MON-1; MHOM/PT/88/IMT318) and negative (without DNA) controls were included. PCR products were visualised under UV illumination after electrophoresis on 1.5 % agarose gels stained with GreenSafe Premium (Nzytech, Portugal), and their migration compared to that of a 100 bp DNA ladder (Nzytech, Portugal). The obtained amplicons were purified and sequenced by Sanger's method (STABVida, Portugal), using as sequencing primers those used for DNA amplification.

**Table 2.** PCR protocols performed for detection and characterisation of *Leishmania* DNA.

| Target gene | Primer sequence (5'-3') | Amplicon size | Reaction setup | Thermocycling conditions | Reference |
|---|---|---|---|---|---|
| *cytB*[a] | 1st PCR<br>Fw: AGCGGAGAGRARAGAAAAGG<br>Rev: CTACAATAAACAAATCATAATATRCAATT | 919 bp | 25 µl reaction: 5 µl of DNA; 0.8 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 3 min; 45 cycles [94 ºC - 1 min; 48 ºC - 1 min; 72 ºC - 1 min]; 72 ºC - 5 min | (Kato, Cáceres & Hashiguchi, 2016; Herrera *et al.*, 2017) |
| | 2nd PCR<br>Fw: GGTGTAGGTTTTAGTYTAGG<br>Rev: GYTCRCAATAAAATGCAAATC | 568 bp | 25 µl reaction: 5 µl of 1st PCR product[b]; 0.8 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 3 min; 45 cycles [94 ºC - 1 min; 48 ºC - 1 min; 72 ºC - 1 min]; 72 ºC - 5 min | |
| *g6pdh* | Fw: ATGTCGGAAGAGCAGTCT<br>Rev: TCACAGCTTATTCGAGGGAA | 1689 bp | 25 µl reaction: 5 µl of DNA; 0.4 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 96 ºC - 5 min; 45 cycles [96 ºC - 1 min; 50 ºC - 1 min; 72 ºC - 90 s]; 72 ºC - 10 min | (Zemanová *et al.*, 2007) |
| *hsp70* | 1st PCR<br>Fw: GGACGCCGGCACGATTKCT<br>Rev: CCTGGTTGTTGTTCAGCCACTC | 1286 bp | 25 µl reaction: 5 µl of DNA; 0.8 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 5 min; 45 cycles [94 ºC - 40 seg; 61 ºC - 1 min; 72 ºC – 2 min]; 72 ºC - 10 min | (Van der Auwera *et al.*, 2013) |
| | 2nd PCR<br>Fw: GACAACCGCCTCGTCACGTTC<br>Rev: GTCGAACGTCACCTCGATCTGC | 741 bp | 25 µl reaction: 5 µl of 1st PCR product[b]; 0.4 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 5 min; 45 cycles [94 ºC - 40 s; 65 ºC - 1 min; 72 ºC - 1 min]; 72 ºC - 10 min | |
| *ITS*-rDNA | 1st PCR<br>Fw: GCTGTAGGTGAACCTGCAGCAGCTGGATCATT<br>Rev: GCGGGTAGTCCTGCCAAACACTCAGGTCTG | Variable | 25 µl reaction: 5 µl of DNA; 0.2 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 94 ºC - 3 min; 45 cycles [94 ºC - 30 s; 58 ºC - 30 s; 72 ºC - 90 s]; 72 ºC - 10 min | (Parvizi *et al.*, 2005) |
| | 2nd PCR<br>Fw: GCAGCTGGATCATTTTCC<br>Rev: AACACTCAGGTCTGTAAAC | Variable | 25 µl reaction: 5 µl of 1st PCR product[b]; 0.2 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 94 ºC - 3 min; 45 cycles [94 ºC - 30 s; 58 ºC - 30 s; 72 ºC - 90 s]; 72 ºC - 10 min | |
| *SSU*-rDNA | 1st PCR<br>Fw: GGTTCCTTTCCTGATTTACG<br>Rev: GGCCGGTAAAGGCCGAATAG | 603 bp | 30 µl reaction: 10 µl of DNA; 0.5 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 94 ºC - 5 min; 35 cycles [94 ºC - 30 s; 60 ºC – 30 s; 72 ºC – 30 seg]; 72 ºC - 10 min | (Cruz *et al.*, 2002) |
| | 2nd PCR<br>Fw: TCCATCGCAACCTCGGTT<br>Rev: AAAGCGGGCGCGGTGCTG | 358 bp | 25 µl reaction: 5 µl of 1st PCR product[c]; 0.6 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 94 ºC - 5 min; 32 cycles [94 ºC - 30 s; 65 ºC - 30 min; 72 ºC – 30 seg]; 72 ºC - 10 min | |

[a] PCR protocol designed in the course of this study; [b] PCR product was previously diluted 1:50 in nuclease-free water; [c] PCR product was previously diluted 1:200 in nuclease-free water.

Abbreviations: *cytB*, cytochrome b; *g6pdh*, glucose-6-phosphate dehydrogenase; *hsp70*, heat-shock protein 70; *ITS*-rDNA, internal transcribed spacers-5.8 ribosomal DNA; *SSU*-rRNA, small subunit ribosomal DNA; bp, base pars.

### 3.4. Sequence analysis and phylogenetics

Nucleotide (nt) sequence similarity searches were performed with the BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments of nt datasets were carried out using the iterative G-INS-i refinement method as implemented in MAFFT v7 (Katoh and Standley, 2013). The resulting alignments were treated via Gblocks (Castresana, 2000) with the most stringent options selected (except for *ITS*-rDNA alignment where more permissive edition conditions were used), followed by their manual correction taking into account the encoding reading frame (except for *ITS*-rDNA). The alignments of coding genes sequences (*i.e.*, *cytB*, *g6pdh* and *hsp70*) were also concatenated using FaBox (Villesen, 2007). The evolutionary information contained in each aligned dataset (phylogenetic signal) was assessed by likelihood-mapping (Strimmer and von Haeseler, 1997) using TREE-PUZZLE v5.3 (Schmidt et al., 2002). Phylogenetic trees were inferred by Maximum Likelihood (ML) and Bayesian methods under fitted evolutionary models, selected based on the corrected Akaike information criterion, as implemented in jModelTest v2 (Darriba et al., 2012). Maximum likelihood phylogenetic trees were constructed using MEGA v6 (Tamura et al., 2013), and the stability of the obtained trees topologies assessed by the bootstrap test with 1000 replicates. For Bayesian phylogenetic inference, the BEAST v1.10.4 (Suchard et al., 2018) software was used to estimate the posterior probability distribution through Markov chain Monte Carlo (MCMC) sampling. Two independent MCMC were run until 1 x $10^8$ generations were sampled, and the first 10 % sampled trees discarded as burn-in before Maximum Clade Credibility trees were constructed. For each case, convergence was assessed with Tracer v1.7.1 (available at http://beast/bio/ed.ac.uk/Tracer), ensuring that all effective sample size (ESS) values were above 200, after the burn-in removal step. Default priors were considered, except for the molecular clock models. The clock hypothesis of each dataset was analysed through the molecular clock likelihood-ratio test, as implemented in MEGA v6 (Tamura et al., 2013). The generated trees were edited for display using FigTree v1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/). Further, alternative potential evolutionary paths were also investigated by the analyses of phylogenetic networks inferred from NeighborNet methods. Split-networks were produced from Kimura-2 parameter distance matrices using SplitsTree v4.14.8 (Kloepper and Huson, 2008). Intraspecific phylogenies of *L. donovani* complex concatenated data was additionally

explored through the construction of median-joining networks obtained using Network 5.0.1.1 (available at http://www.fluxus-engineering.com/sharenet_rn.htm). Finally, principal coordinate analyses were carried out using the PCOORD software (available at http://www.hiv.lanl.gov/content/sequence/PCOORD/PCOORD.html). Small differences between the analysed sequences were emphasised with the use of unedited alignments. This tool yields 2D plots in which the main patterns of evolutionary affinities are visualised on two orthogonal axes.

The nt sequences obtained during this study were deposited in the DDBJ/ENA/GenBank under the accession numbers LC459280-LC459351 and LC460164-LC460189. The additional sequences of reference strains used in phylogenetic analyses were downloaded from public sequence databases, and their respective accession numbers are listed in Table 1.

## 4. Results

A PCR product of the expected size was amplified from buffy coat samples of 25 (5.4 %; *n* = 465) cats using genus-specific primers targeting *Leishmania SSU*-rDNA. However, successful amplification by PCR of the sequences of any of the other genetic markers under analysis (*cytB*, *g6pdh*, *hsp70* and *ITS*-rDNA) could merely confirm the presence of the parasite's DNA in five of them (20.0 %; *n* = 25). These included *n* = 5 *cytB*, *n* = 4 *hsp70*, *n* = 4 *ITS*-rDNA, while only a *g6pdh* segment-specific amplicon was obtained from one of the analysed buffy coats. Sequences derived from three cats from LMA were identified as belonging to *L. donovani* complex, based on *g6pdh* and/or *cytB*, *hsp70* and *ITS*-rDNA sequence analysis using MegaBlast. Additionally, one single sequence amplified from another cat from LMA was identified as *L. major* based on *cytB* and *ITS*-rDNA BLASTn sequence searches. Finally, the *cytB* and *hsp70* sequences obtained from a cat from the Algarve showed 100 % identity (covering 100 % of both query sequences) with homologous sequences of *L. donovani/infantum* and *L. major*, respectively. For each of 21 reference strains used in this study, *cytB*, *g6pdh*, *hsp70* and *ITS*-rDNA target regions were successfully amplified and sequenced. The respective accession numbers are listed in Table 1.

To further extend the characterisation of these sequences, phylogenetic analyses were performed using ML, Bayesian and NeighborNet methods. An initial cladistic analysis of each gene fragment showed that all the *Leishmania* sequences obtained from cats (indicated with "*") segregating within the subgenus *Leishmania* (Supplementary Figures 1, 2, 3 and 4). The majority of these sequences segregated together with those of *Leishmania donovani* complex reference-strains in a monophyletic cluster with high statistical support, regardless of the target gene or phylogenetic inference method used. Within the *Leishmania donovani* complex, the existence of intra-groups was suggested in all trees, but none of them evidenced monophyly for the *L. archibaldi*/*donovani* and *L. chagasi*/*infantum* species. In contrast, both *cytB* and *ITS*-rDNA sequences amplified from a cat from the LMA segregated in a robust monophyletic cluster, exclusively composed by sequences of reference strains of *L. major* (Supplementary Figures 1 and 4). Nonetheless, the paraphyletic structure of the *L. major* species complex was suggested by the analysis of *cytB* and *ITS*-rDNA datasets (Supplementary Figures 1 and 4). Contradictory topological tree-segregation results were observed for the sequences obtained from a cat from the Algarve. The trees based on *cytB* and *hsp70* data supported their segregation in two different phylogenetic lineages, where the obtained sequences clustered together within the *L. donovani* complex and the *L. major* radiation, respectively (Supplementary Figures 1 and 3). To better understand the phylogenetic relationships shared by *L. donovani* complex sequences derived from cats, additional analyses were inferred from a concatenated alignment (2061 bp-long) of partial sequences of three different protein-coding genes (*i.e.*, *cytB*, *g6pdh* and *hsp70*). The concatenated tree based on ML and Bayesian methods highly supported the monophyly of *L. donovani* complex (Figure 1A).

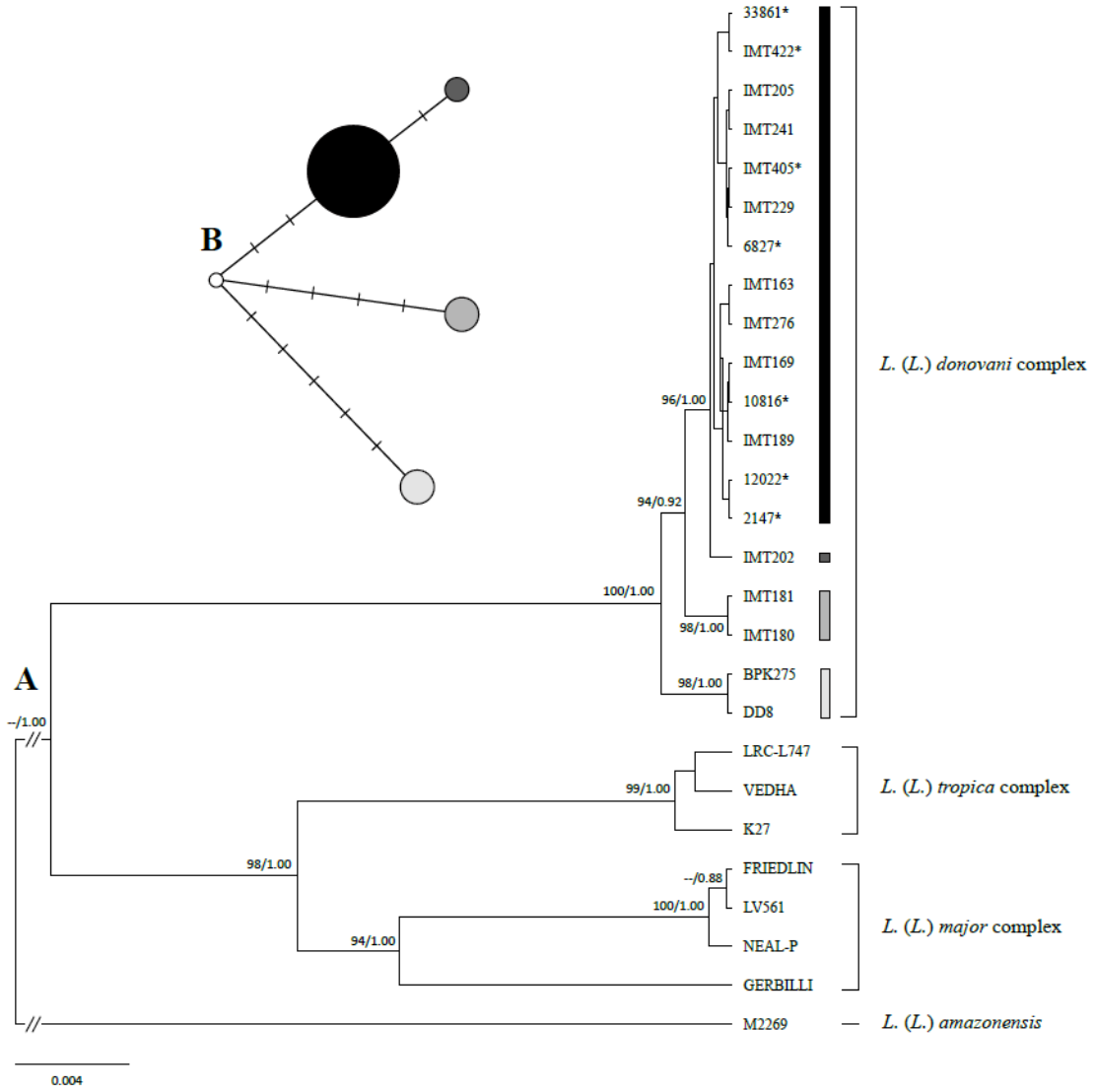


**Figure 1.** Phylogenetic analysis based on unambiguous *cytB*, *g6pdh* and *hsp70* concatenated sequences alignment (2061 nt). (A) Phylogenetic unrooted tree generated using the GTR+G+I model of evolution, assuming a strict molecular clock. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥ 75 % and/or posterior probabilities ≥ 0.80 are shown. Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Leishmania amazonensis* sequences (outgroup). The sequences of strains detected/isolated from cats are identified with "*". (B) Median-joining network of *L. donovani* complex. The area of circles is proportionally related to the frequency of the gene type. Each gene type is indicated with different levels of grey shading. The gene polymorphisms are indicated in the branches by dashes. The median vector is indicated by a white circle and represents a hypothetical ancestor.

Within this, three distinct groups were formed, two of them exclusively composed by sequences of reference strains of *L. donovani* and *L. infantum*, respectively. The last one included all sequences amplified from cat samples. Further, phylogenetic networks were obtained from the same gene-segments/concatenated datasets. The *Leishmania* relationships consistently displayed by cladistics analyses were also recovered from the networks (data showed only for the concatenated dataset; Figure 2).

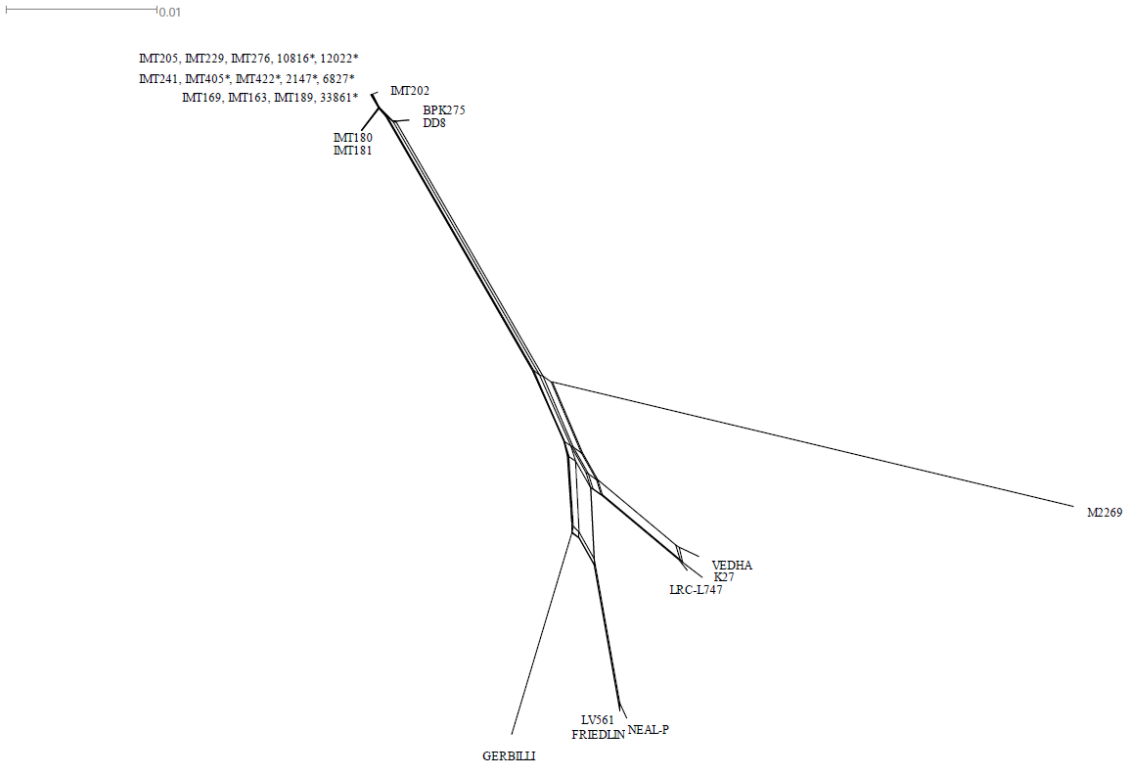


**Figure 2.** Split-network based on unambiguous *cytB*, *g6pdh* and *hsp70* concatenated sequences alignment (2061 nt). Distances were calculated using the K2P model of evolution. The sequences of strains detected/isolated from cats are identified with "*".

Moreover, based on the concatenated median-joining network of *L. donovani* complex sequences, four distinct gene types were recognized, all of them connected by a median vector (Figure 1B). The most frequent gene type had the majority of sequences of *L. infantum* reference strains used in this study and all sequences obtained from cats. This gene type occupied an intermediate position between IMT202 and IMT180/IMT181 gene types and was separated by seven mutational steps from the BPK275/DD8 gene type.

Despite this analysis, it should be stated that all datasets used were somewhat limited in the number of sequences they contain, especially in what regards the number of operational taxonomic units included in the *L. donovani* complex (for which a sequence for the chosen genetic markers could be obtained or found in the public nucleotide sequence databases). Moreover, analysis of the phylogenetic signal for each of these datasets was also shown to be sub-optimal. Indeed, likelihood mapping showed that the percentage of totally resolved sequence quartets (of the total number of possible quartets in 1000 replicates of the sequence data used) concerning the analysis of the *cytB*, *g6pdh*, *hsp70*, *ITS*-rDNA, and *cytB-g6pdh-hsp70* concatenated datasets corresponded to 78.9 %, 64.8 %, 64.6 %, 58.4 %, and 56.7 %, respectively. While these values did not usually limit the segregation of the sequences used into the expected main genetic lineages, they may explain the paraphyletic structure of the *L. major* complex cluster in the *cytB* and *ITS*-rDNA trees and the inability to resolve the *L. donovani* complex into clear subclusters. In fact, the low resolution of this species complex is also evident when, in addition to phylogenetic inference, the sequence data were analysed by multivariate statistics using principal coordinate analysis (Supplementary Figure 5), which allows the identification of meaningful patterns in sequence data without a priori knowledge about them, summarizing sequence variation in a limited number of axis (or dimensions).

Once again, PCOORD revealed limited resolving power to separate the analysed sequences into distinct subsets. Moreover, regardless of the dataset used, all the sequences amplified from cats clustered with all the other. Therefore, and as far as the analysed genetic markers could reveal, feline strains do not evidence any particular genetic feature setting them apart from those of vector, human, or canine origin.

## 5. Discussion

The MLEE has been considered the baseline of *Leishmania* species identification, however, due to a few technical limitations, only a small number of strains isolated from cats were typed by this method (Maia et al., 2015; Pennisi et al., 2015). Indeed, the classification of *Leishmania* parasites at the species level in both clinical cases of feline infection and epidemiological studies, has been commonly based on the detection of species-specific antibodies and/or DNA using species-specific primers (Can et al., 2016; Leal et al., 2018; Pennisi et al., 2015). In this study, regions of four different genetic

markets (*cytB*, *g6pdh*, *hsp70* and *ITS*-rDNA) were analyzed to investigate the phylogenetic relationships and the genetic diversity of *Leishmania* strains detected/isolated from cats.

The *SSU*-rDNA PCR assay performed to screen cat blood samples for the presence of *Leishmania* DNA showed to be more sensitive than the remaining PCR assays used for characterisation of these parasites. In fact, PCR assays targeting conserved regions of *SSU*-rDNA and kinetoplast minicircle DNA have been demonstrated to be among the most sensitive methods for detection of *Leishmania* DNA, mainly as a result of their high gene copy number per parasite cell (Akhoundi et al., 2017; Albuquerque et al., 2017; Lachaud et al., 2002). However, these targets display only a few phylogenetically informative sites, allowing the identification of leishmanial parasites only at the genus and/or subgenus level (Schönian et al., 2011). On the other hand, *cytB*, *g6pdh*, *hsp70* and/or *ITS*-rDNA genes have been suggested to be used for better discrimination of *Leishmania* species worldwide (Asato et al., 2009; Dávila and Momen, 2000; Fraga et al., 2010; Zemanová et al., 2007). The *hsp70* and *ITS*-rDNA have been identified as the markers with the highest discriminative power at both interspecies and intraspecies levels, and the *cytB* as one of the most specific and sensitive for detection/identification of *Leishmania* spp. (Akhoundi et al., 2017; Kuhls and Mauricio, 2019). Although less commonly analysed than the markers referred above, *g6pdh* has, nonethless, already been used in MLST schemes (Herrera et al., 2017; Zemanová et al., 2007; Zhang et al., 2013), including those devised for inferring the phylogenetic structure of the *Leishmania donovani* complex (Zemanová et al., 2007).

Herein, the PCR-assays employed for partial amplification of these genes showed to be 100 % sensitive with template DNA obtained directly from several cultured strains of *L. donovani* complex, *L. major* and *L. tropica*. On the other hand, the sensitivity of these PCR-protocols was considerably lower using cat buffy coat samples considered positive to *Leishmania* based on the amplification of *SSU*-rDNA by PCR. In this study, the results obtained for *cytB* are most probably due to a combination of a high copy number template with a two-step PCR protocol, while a lower copy number template and a less sensitive amplification protocol (one-step PCR) explain those obtained for *g6pdh*. These observations reinforce the importance of combining, whenever possible, two-step PCR assays with primers targeting multicopy genes especially in samples, such as blood,

where the parasite load tends to be low (Sundar and Singh, 2018). The detection of *Leishmania* DNA was tentatively optimised using buffy coat samples since, as far as dogs are concerned, the detection of *Leishmania* nucleic acids has been shown to be more efficient from the latter than from whole blood (Maia et al., 2010).

In general, phylogenetic relationships reconstructed from both singular gene and concatenated nucleotide sequences alignments are topologically congruent, and in agreement with the main taxonomic groups comprised in the classical classification of *Leishmania* parasites (Akhoundi et al., 2016). As previously demonstrated, the genetic targets used in this study showed to be polymorphic enough to allow clear discrimination between *Leishmania* parasites at the complex level (Asato et al., 2009; Dávila and Momen, 2000; Fraga et al., 2010; Zemanová et al., 2007). The exception was the *L. major* complex, where a statistically supported monophyletic origin was consistently demonstrated only for *L. major* sensu stricto, regardless of the genetic markers analysed, as also observed in other studies (Bravo-Barriga et al., 2016; Dávila and Momen, 2000; Van der Auwera et al., 2013). On the contrary, phylogenetic trees based on *cytB*, *hsp70*, and *ITS*-rDNA sequences place *L. gerbilli*, *L. turanica* and *L. arabica* (the other members of the *L. major* complex) in either a paraphyletic position to *L. major* (*cytB* and *ITS*-rDNA trees; Supplementary Figures 1 and 4), or clustering with it in a monophyletic assemblage which is not, however, supported by either bootstrap or posterior probability (*hsp70* tree; Supplementary Figure 3).

Phylogenetic analyses of the concatenated sequences showed that the majority of parasites found in cats belonged to the *L. donovani* complex. In addition, and as far as the sequence data indicated, none of the analysed sequences evidenced any distinctive feature associated with feline *Leishmania* parasites, segregating among those isolated from dogs, humans and phlebotomine sand flies in all kinds of analyses (Figure 1B). This absence of segregation of *Leishmania* genetic markers into feline-specific types was also indirectly demonstrated by principal coordinate analysis (Figure 3).

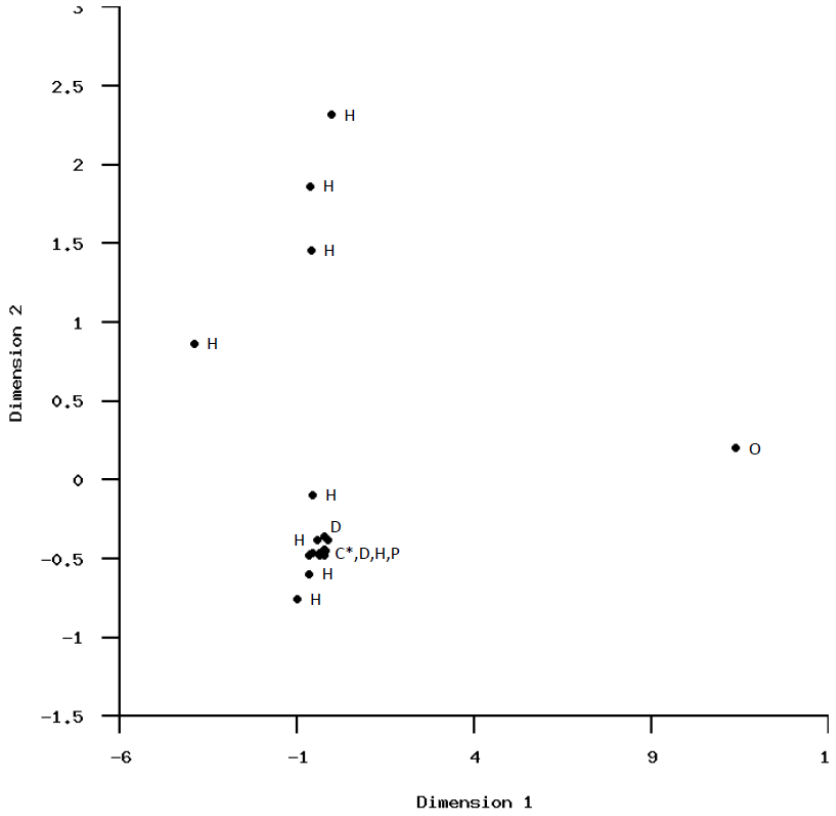


**Figure 3.** Principal coordinate analysis 2D plot of the unedited *cytB*, *g6pdh* and *hsp70* concatenated sequences alignment. The first two axes cover 90.4 % of variation. C*, D, H and P letters corresponding to sequences of *Leishmania donovani* complex strains detected in cats, dogs, humans and phlebotomine sand flies, respectively. Sequences of *L. amazonensis* (strain M2269) were used as outgroup and are indicated by "O".

In the last few years, cats have been regarded as possible additional reservoirs of *L. infantum* (Akhtardanesh et al., 2018; Maia and Campino, 2011). The feline infection with *L. infantum* has been confirmed using molecular tools in several countries of Eurasia (Akhtardanesh et al., 2017; Can et al., 2016; Pennisi et al., 2015).

Although the feline strains of *Leishmania* were not formally identified as *L. infantum*, this study contributes with new insights in the epidemiology of zoonotic visceral leishmaniosis reinforcing the possible role played by cats.

Over the years four different species (*i.e.*, *L. archibaldi*, *L. chagasi*, *L. donovani* and *L. infantum*) have been described as belonging to the *L. donovani* complex, but several molecular studies have supported their synonymy (Maurício, 2018). Additionally, the occurrence of genetic recombination within this complex has been shown (Mauricio

et al., 2006). The recognition of *L. donovani* (syn. *L. archibaldi*) and *L. infantum* (syn. *L. chagasi*) as the only two valid species is now accepted by most researchers, but once again, according to the phylogenetic species concept (Tibayrenc, 2006), their monophyly has not been shown to be consistent (Maurício, 2018). In the present study, the formation of *L. donovani* and *L. infantum* specific-clusters seemed to be influenced by the number of representative sequences included in the phylogenetic analyses. The concatenated tree suggested the monophyly of the *L. donovani* and *L. infantum* clusters, but when the sequences obtained from the IMT180 and IMT181 strains [identified by MLEE as *L. donovani* MON-18 (Campino et al., 1994) and *L. infantum* MON-24 (Cortes et al., 2014), respectively] were included in the analyses, an additional monophyletic cluster, exclusively composed by them, and supported by both high bootstrap and posterior probability values, was formed (Figure 1B). Moreover, the correspondent median-joining network indicated that these strains had the same genetic type (Figure 1A). Due to the absence of consistent phylogenetic or diagnostic markers for any of the recognised *L. donovani* complex species, some researchers have been suggesting a need to rethink the genetic characterisation of *Leishmania* based on whole-genome sequencing data (Van der Auwera et al., 2011). Nonetheless, the recognition of the *L. donovani* complex as a single species (*L. donovani*) as recently proposed by Maurício (2018), is in agreement with the results reported in the present study.

Unexpectedly, the phylogenetic analyses also suggested the presence of both *L.major/ L. donovani* complex in an adult cat from the Algarve and *L. major* in a young cat from the LMA. These results highlighted the employment of *cytB* in MLST schemes for the identification of *Leishmania* parasites, not only due to their specificity and sensitivity but also to its uniparental inheritance feature (Herrera et al., 2017). As hybrids present full genomic complements from both parents, except for the kinetoplast maxicircle DNA (Akopyants et al., 2009), the partial amplification of both *L. donovani* complex *cytB* and *L. major hsp70* from a cat, using non-restrictive primers, highly supports its infection by *L.major*/*L. donovani* s.l. hybrid parasites. Nonetheless, in formal terms, the hypothesis of a *L.major*/*L. donovani* s.l. mix infection cannot be formally ruled out, especially since they have previously been described in humans (Babiker et al., 2014; Badirzadeh et al., 2018). On the other hand, the concordant sequence identity results obtained from both *cytB* and nuclear *ITS*-rDNA targets suggests that the other cat

harboured DNA of *L. major* parasites. Nevertheless, the infection by hybrids should also not be formally excluded because their genetic inheritance can occur with loss of heterozygosity at some loci (Romano et al., 2014).

Zoonotic CL caused by *L. major* is dispersed in several countries from West to Northern Africa, the Middle East and Central Asia (WHO, 2010). Although rodents are the proven reservoirs of *L. major*, the parasite DNA has recently been identified in the blood of cats in Turkey (Paşa et al., 2015). The detection of DNA of *L. major* and/or *L. major*/*L. donovani* s.l. hybrids in cat's buffy coat samples collected out of sand fly seasonal activity (Alten et al., 2016; Branco et al., 2013; Maia et al., 2013) suggests a real infection. Also, taking into account the previous isolation of *L. major*/*L. infantum* hybrids from autochthonous human cases (Ravel et al., 2006), as well as the detection of *L. major* DNA in the phlebotomine sand fly *Sergentomyia minuta* (Campino et al., 2013), the present results reinforce the possible circulation of these parasites in Portugal. Eco-epidemiological and phylogenetic studies are needed to clarify the possible maintenance and transmission of both *L. major* and *Leishmania* hybrids parasites, focusing on their isolation and typing, ideally relying on whole-genome sequences from *Leishmania* strains isolated from both sand flies and vertebrate hosts.

Nevertheless, and from a public health point of view, official notification of all cutaneous and visceral human clinical cases, together with *Leishmania* species identification, should be encouraged by the European health services.

*Ethics statement*

The procedures were approved by the Ethical Committee of IHMT and for the Portuguese veterinary authorities as complying with Portuguese legislation for the protection of animals (Decree-Law no. 113/2013). Consent was obtained from the legal detainer, *i.e.*, the owner of the cat or the person in charge of the rescue associations for stray cats.

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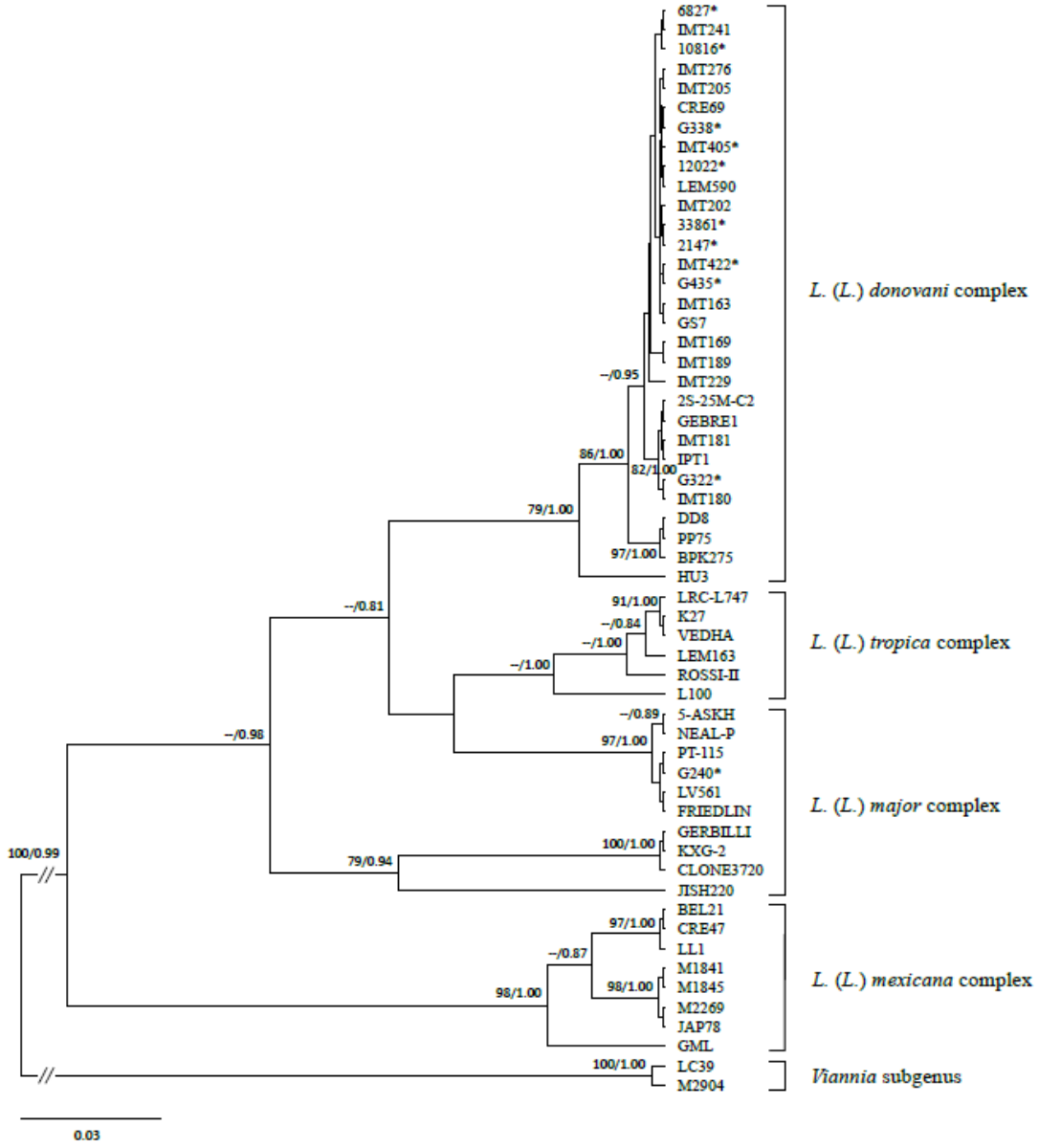
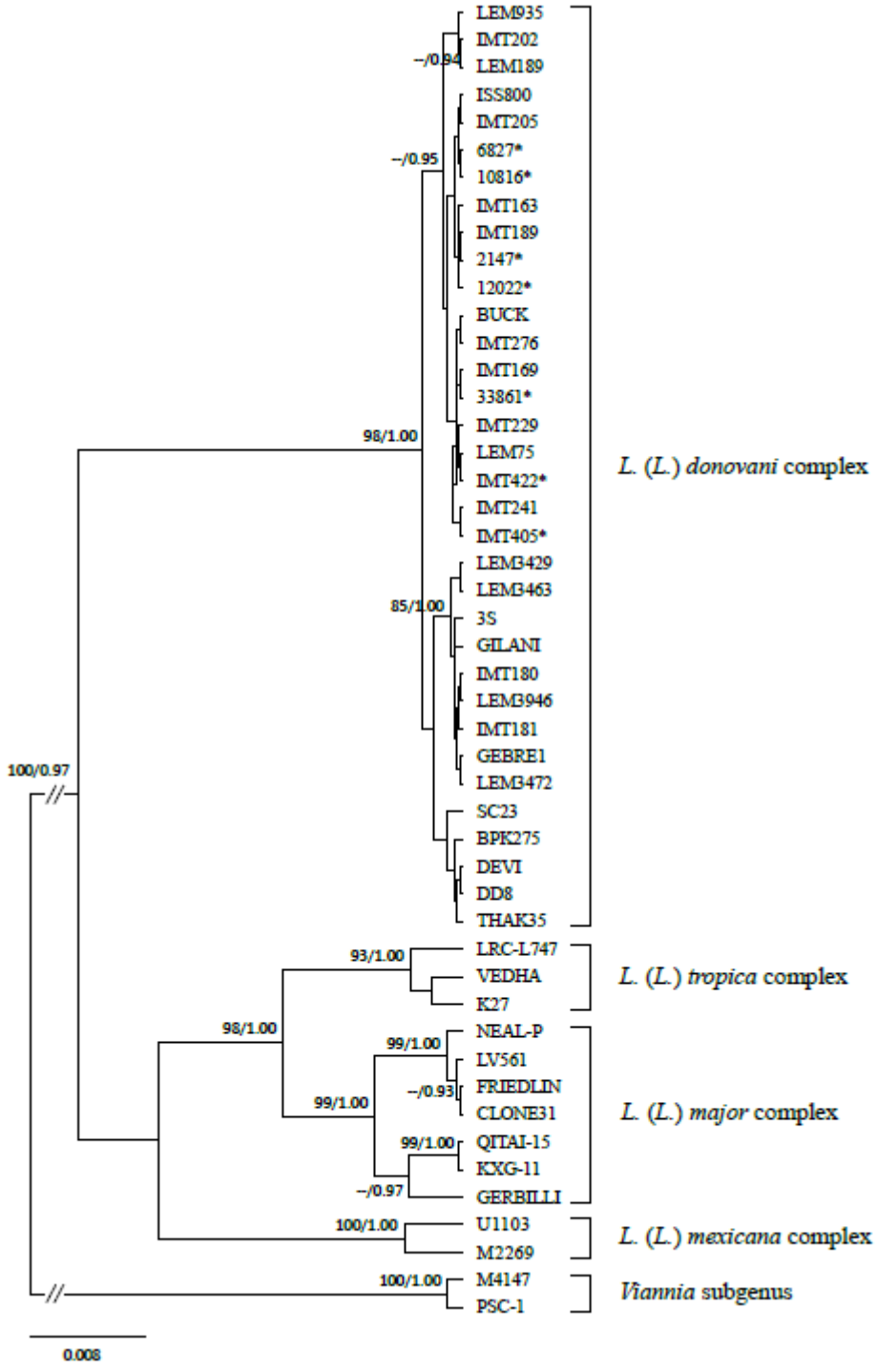
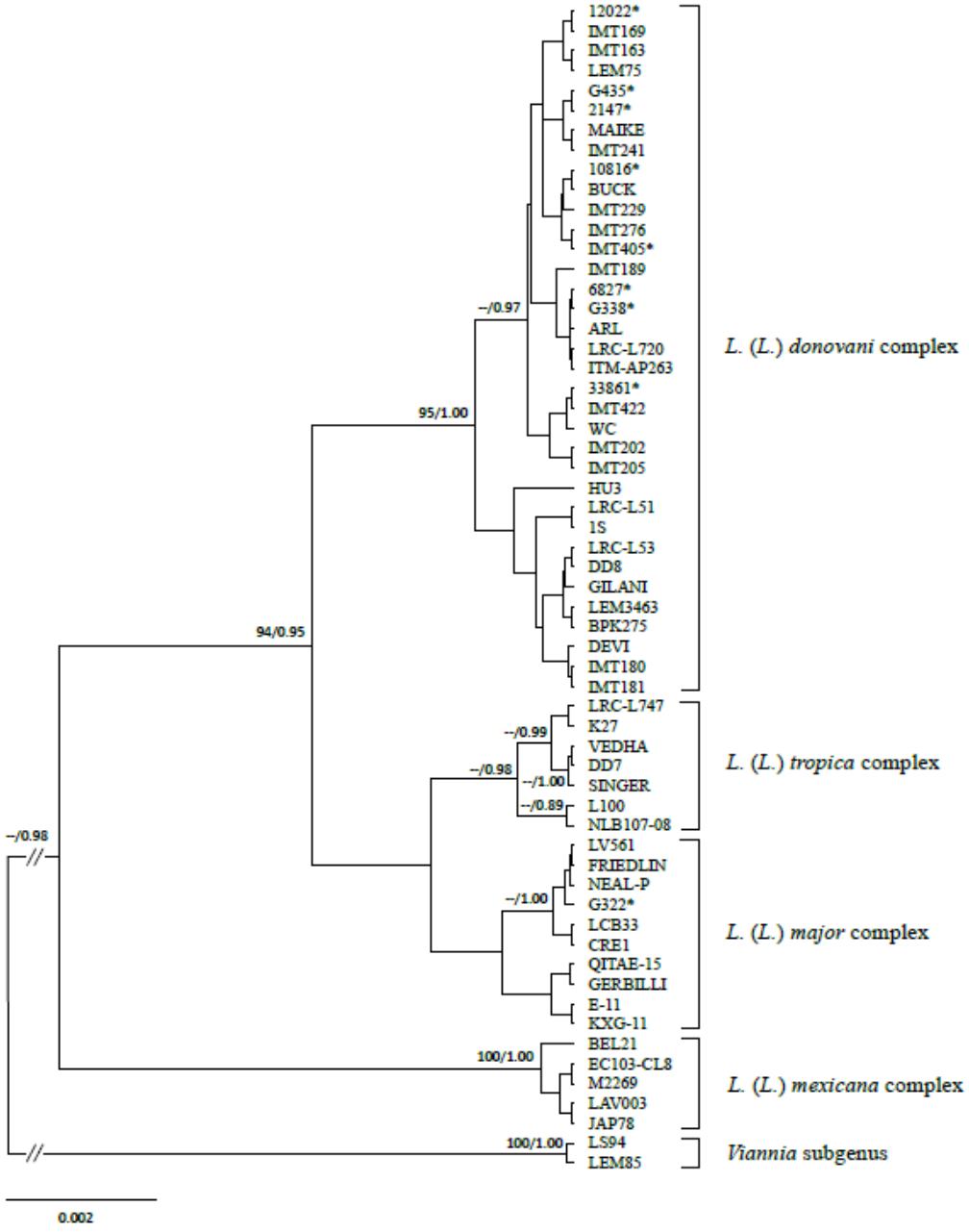
# 8. Supplementary data



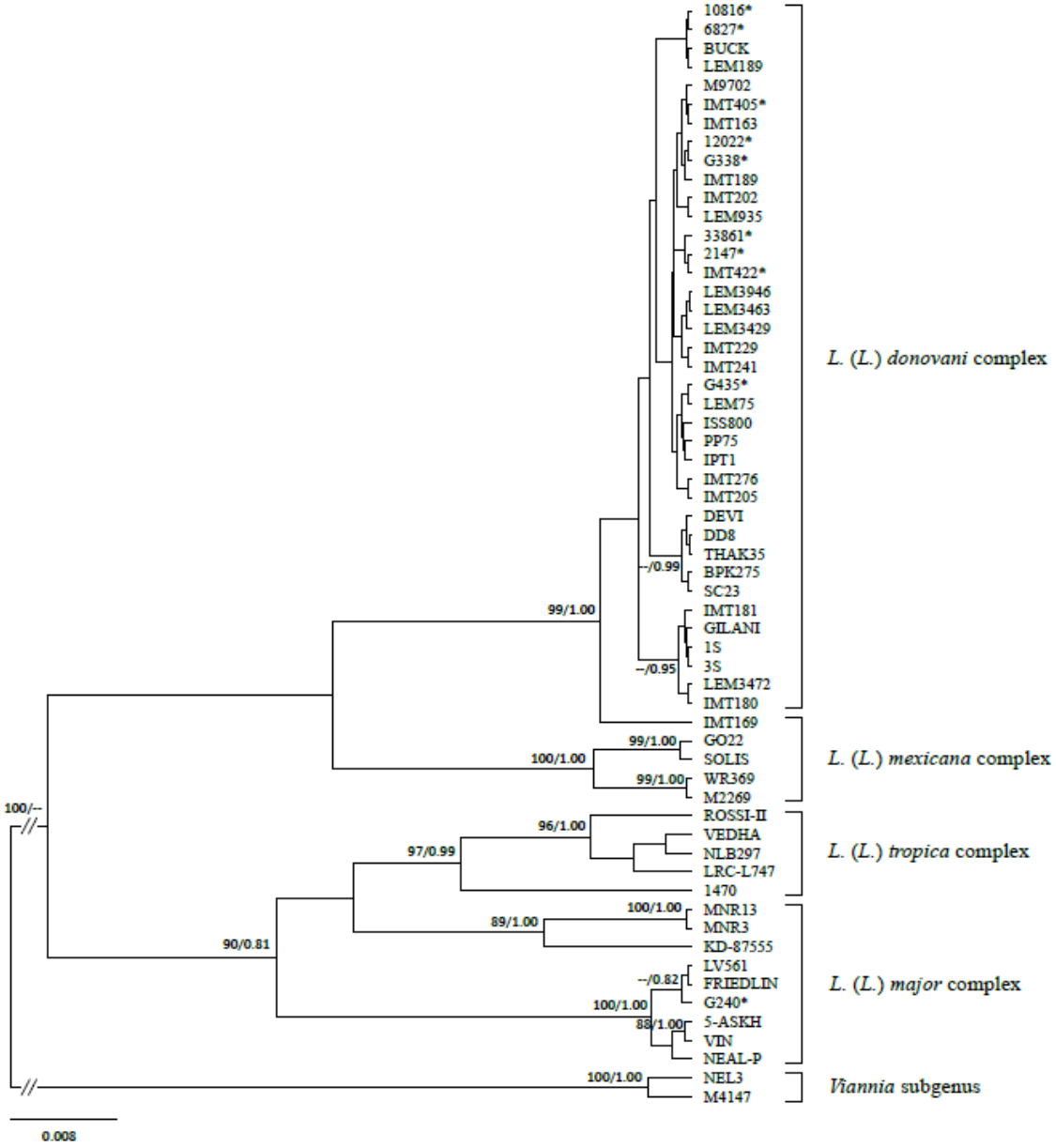

**Supplementary Figure 1.** Phylogenetic unrooted tree based on unambiguous *cytB* sequences alignment (470 nt) using the HKY+G model of evolution, assuming a strict molecular clock. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥ 75 % and/or posterior probabilities ≥ 0.80 are shown. Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Viannia* subgenus sequences (outgroup). The sequences of strains detected/isolated from cats are identified with "*".



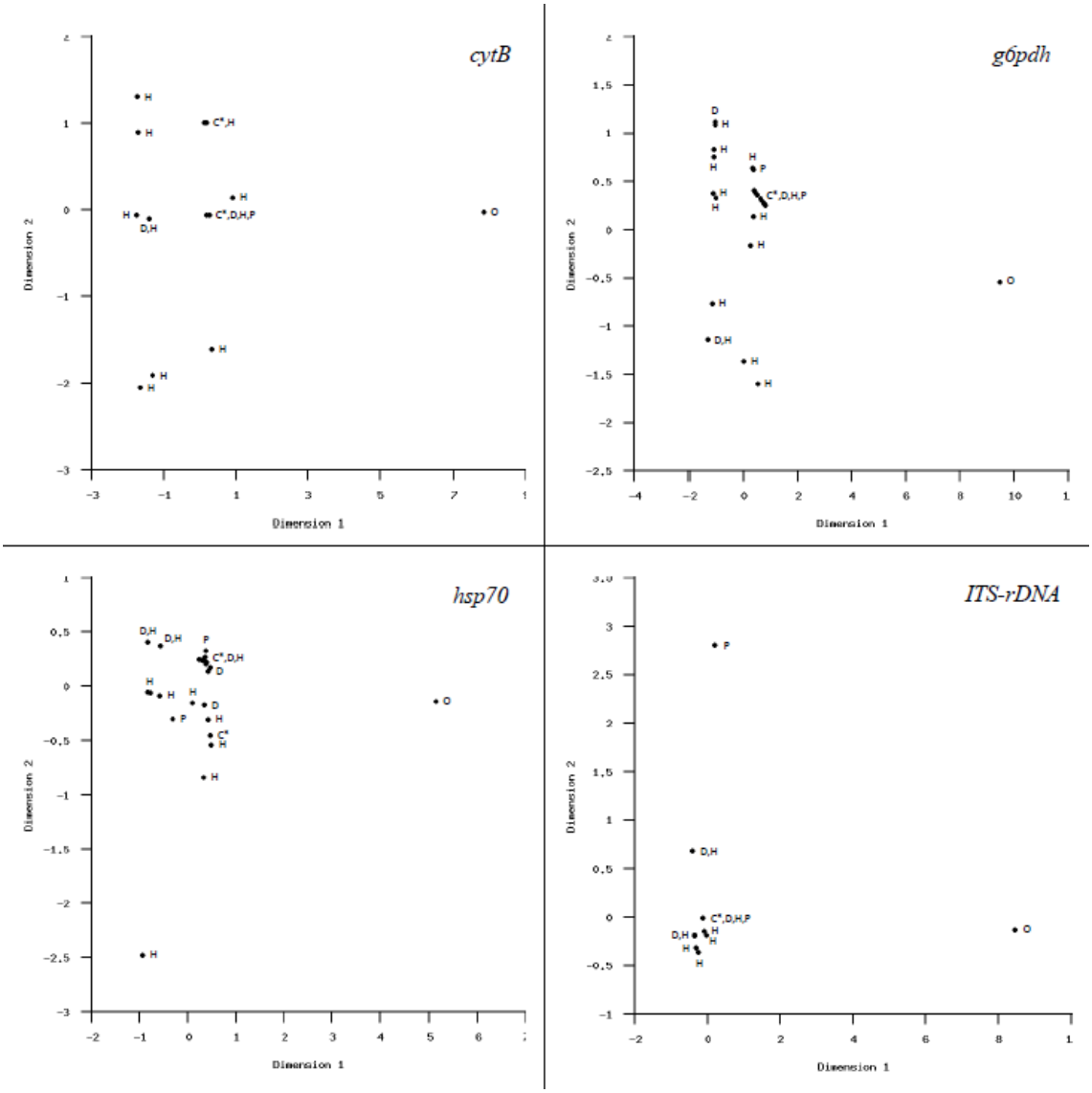

**Supplementary Figure 2.** Phylogenetic unrooted tree based on unambiguous *g6pdh* sequences alignment (942 nt) using the HKY+G model of evolution, assuming a relaxed molecular clock. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥75 % and/or posterior probabilities ≥ 0.80 are shown. Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Viannia* subgenus sequences (outgroup). The sequences of strains detected/isolated from cats identified with "*".




**Supplementary Figure 3.** Phylogenetic unrooted tree inferred based on unambiguous *hsp70* sequences alignment (649 nt) using the TrN+G model of evolution, a strict molecular clock. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥ 75 % and/or posterior probabilities ≥ 0.80 are shown. Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Viannia* subgenus sequences (outgroup). The sequences of strains detected/isolated from cats are identified with "*".




**Supplementary Figure 4.** Phylogenetic unrooted tree inferred based on unambiguous *ITS*-rDNA sequences alignment (918 nt) using the HKY+G model of evolution, assuming a relaxed molecular clock. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) ≥ 75 % and/or posterior probabilities ≥ 0.80 are shown. Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Viannia* subgenus sequences (outgroup). The sequences of strains detected/isolated from cats are identified with "*".




**Supplementary Figure 5.** Principal coordinate analysis 2D plot of the unedited *cytB*, *g6pdh*, *hsp70* and *ITS*-rDNA sequences alignments. The first two axes cover, respectively, 74.2, 90.3, 78.1 and 84.4 % of variation. C*, D, H and P letters corresponding to sequences of *Leishmania donovani* complex strains detected in cats, dogs, humans and phlebotomine sand flies, respectively. Sequences of *L. amazonensis* (strain M2269) were used as outgroup and are indicated by "O".

# CHAPTER 5

## *In vitro* fitness and *ex vivo* infectiousness of feline *Leishmania* strains

**This chapter is based on the research article:**

Pereira, A., Parreira, R., Cristóvão. J.M., Vitale, F., Bastien, P., Campino, L. & Maia, C. (2021) Leishmania infantum strains from cats are similar in biological properties to canine and human strains. *Veterinary Parasitology*. 298, 109531. https://doi.org/10.1016/j.vetpar.2021.109531.

## 1. Abstract

Zoonotic visceral leishmaniosis is a worldwide severe disease caused by *Leishmania infantum*, a protozoan that has phlebotomine sand flies as vectors and dogs as primary reservoir hosts. Over the last few decades, cats have been regarded as an indisputable piece within the ecological system in which *L. infantum* is maintained indefinitely. However, little is known about feline strains, including their phenotypic plasticity and infectivity. In this study, the phenotypic behaviour of seven *L. infantum* feline strains was compared to those of well-characterised counterparts isolated from two dogs and two humans in terms of growth profile, adaptive capacity under several stress conditions, susceptibility to antileishmanial drugs, and infectivity to host cells. Feline strains displayed a similar growth profile, survival capacity, and ability to infect feline, canine, and human monocyte-derived primary macrophages. Furthermore, multivariate cluster analysis suggested that most strains studied did not display distinctive phenotypic features. To our knowledge, this is the first study to analyse the phenotypic behaviour of feline *L. infantum* strains. This study brings new insights into the hypothetical role of cats as reservoir hosts of *L. infantum* since the parasites found in them are phenotypically identical to those of dogs and humans. However, further studies on the transmission dynamics should be encouraged to fully establish the status of cats in the maintenance of *L. infantum* foci.

**Keywords:** cat, disease reservoirs, *Leishmania*, leishmaniosis, parasitic sensitivity tests, phenotype.

## 2. Introduction

Leishmaniosis is a neglected vector-borne disease that results from infection with protozoan parasites of the genus *Leishmania*, which are typically transmitted to mammalian hosts through the bite of phlebotomine sand flies (WHO, 2010).

The infection outcome depends on the invading species of *Leishmania*, host genetics, and immune status, ranging from asymptomatic to fatal systemic disease (Bañuls et al., 2011). In humans, two primary clinical forms are prevalent worldwide: cutaneous leishmaniosis and visceral leishmaniosis (VL) (Burza et al., 2018; Gradoni, 2018). The latter is the most serious form of the disease and is caused by *L. donovani* and *L. infantum* (both grouped in the *L. donovani* complex) (WHO, 2010). In addition, *L. donovani* is typically recognised as anthroponotic and *L. infantum* as zoonotic, with dogs (*Canis lupus familiaris*) acting as the main reservoir hosts (Quinnell and Courtenay, 2009). Nevertheless, an increasing number of clinical cases of feline leishmaniosis (FeL) and subclinical *L. infantum* infections in cats (*Felis catus*) have been reported in recent years, mostly in Southern Europe where canine leishmaniosis is endemic (Pennisi et al., 2015; Spada et al., 2020).

There is growing evidence that these felids may be involved in maintaining *L. infantum* in both domestic and peridomestic habitats (see review Maia et al., 2018). This hypothesis has built on the fact that cats (i) are naturally susceptible to *L. infantum* but usually do not present clinical signs or develop clinicopathological abnormalities; (ii) are a suitable blood source for phlebotomine sand flies; (iii) maintain parasites with genetic and biochemical features similar to those isolated from both humans and dogs; (iv) often have viable parasites in the skin and blood; and (v) are popular household pets. Nonetheless, crucial issues await clarification in order to formally incriminate cats as reservoir hosts of *L. infantum*.

As previously shown, the studies focusing on phenotypic plasticity and infectivity of *Leishmania* spp. may bring further data relating to parasite infectivity, pathogenicity, evolution, and bioecological dynamics. However, most of them have focused on strains isolated from humans, dogs, or phlebotomine sand flies (Vanaerschot et al., 2010; Cortes et al., 2018; Araújo et al., 2020; Mas et al., 2020). Therefore, this study aimed to assess

the phenotypic behaviour of *L. infantum* strains of feline origin when compared to that of well-characterised strains isolated from dogs and humans, in terms of growth profile, adaptive capacity under thermal, nutritional and oxidative stress conditions, and susceptibility to antileishmanial drugs as well as infectivity.

## 2. Materials and Methods

### 2.1. Parasites

A total of 11 *L. infantum* strains isolated from cats, dogs, and humans (identified with the prefix C, D and H, respectively) were studied (Table 1). *Leishmania* strains were provided by the French National Reference Centre for Leishmanioses (*n* = 5) (Pr Patrick Bastien) and the collection of "*Leishmania*" of the Biological Resources Centre of the Academic Hospital (CHU) of Montpellier (BRC-Leish – http://www.parastiologie.univmontp1.fr/cryobanque.htm) (Username BIOBANKS - BB-0033-00052); by the Centro di Referenza Nazionale per le Leishmaniosi (C.Re.Na.L.) – Istituto Zooprofilattico Sperimentale della Sicilia, Italy (*n* = 5) (Dr Fabrizio Vitale); and by the Instituto de Higiene e Medicina Tropical – Universidade NOVA de Lisboa, Portugal (*n* = 1).

Promastigotes were maintained at 24 ºC in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 20 % (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich) (cRPMI[20]). All strains used in this study were sub-cultured for 10-20 passages.

**Table 1.** Characterisation of the eleven *Leishmania* strains included in the study.

| Species, zymodeme | Laboratory code | International code[b] | Host | Geographic origin | Reference |
|---|---|---|---|---|---|
| *L. infantum* | 2147 | MFEL/IT/10/2147 | Cat | Italy | (Pereira et al., 2020) |
| *L. infantum* | 6827 | MFEL/IT/08/6827 | Cat | Italy | (Pereira et al., 2020) |
| *L. infantum*, MON-1 | 10816 | MFEL/IT/02/10816 | Cat | Italy | (Pereira et al., 2020) |
| *L. infantum* | 12022 | MFEL/IT/99/12022 | Cat | Italy | (Pereira et al., 2020) |
| *L. infantum* | 33861 | MFEL/IT/05/33861 | Cat | Italy | (Pereira et al., 2020) |
| *L. infantum*, MON-1 | IMT405 | MFEL/PT/13/IMT405 | Cat | Portugal | (Maia et al., 2015) |
| *L. infantum*[a] | IMT422 | MFEL/PT/18/IMT422 | Cat | Portugal | (Pereira et al., 2020) |
| *L. infantum*, MON-1 | IMT229 | MCAN/PT/97/IMT229 | Dog | Portugal | (Cortes et al., 2014) |
| *L. infantum*, MON-1 | IMT373 | MCAN/PT/05/IMT373 | Dog | Portugal | (Maia et al., 2013) |
| *L. infantum*, MON-1 | IMT184 | MHOM/PT/93/IMT184 | Human | Portugal | (Maia et al., 2013) |
| *L. infantum*, MON-1 | IMT369 | MHOM/PT/2004/IMT369 | Human | Portugal | (Maia et al., 2013) |

[a] Originally classified as *Leishmania donovani* sensu lato; [b] According to the World Health Organization.

### 2.2. *In vitro* growth kinetics and metacyclogenesis

Promastigotes in the logarithmic growth phase (log-phase) were inoculated in cRPMI$^{20}$ at a final density of 1.0 x $10^5$ parasites/mL, plated in quadruplicate in 24-well flat-bottom plates (VWR®, USA) and incubated (ICP 600, Memmert, Germany) at 24 ºC for 12 consecutive days. Growth curves were obtained by daily quantification of viable promastigotes using a Neubauer chamber (Heinz Herenz, Germany). For morphometric characterisation, promastigotes were harvested from day 1 to day 12 and washed with 1x phosphate-buffered saline solution (PBS). After centrifugation (1000 x *g*; 10 min; 4 ºC), the cell pellets were suspended in FBS and spread onto microscope slides. Air-dried smears were fixed with methanol, stained with 5 % (v/v) Giemsa, and examined by bright-field microscopy at 1000 x magnification (Eclipse 80i, Nikon, Japan). Cell body and flagellum lengths of 100 randomly selected promastigotes were measured for each time point using NIS-Elements Basic Research v3.20.00 (Nikon). Metacyclic forms were identified according to Alexandre et al. (2020) (*i.e.*, body length <14 µm and flagellum/cell body length ratio ≥2).

### 2.3. *In vitro* thermal stress assay

Log-phase promastigotes suspended in cRPMI$^{20}$ (1.0 x $10^5$ parasites/mL) were plated in quadruplicate in 24-well flat-bottom plates and incubated at 20, 28, 33, 37, and 40 ºC for 2, 24, 48, and 192 h to assess the effect of temperature on the growth rate of parasites. After incubation under each thermal stress condition, the cultures were kept at 24 ºC until completing a total of 12 days. Parasite density was determined every 24 h from day 0 to day 12, using a Neubauer chamber.

### 2.4. *In vitro* nutritional stress assay

Log-phase promastigotes suspended in PBS with 75, 50, and 25 % (v/v) cRPMI$^{20}$ at a final density of 1.0 x $10^5$ parasites/mL were plated in quadruplicate in 24-well flat-bottom plates and incubated at 24 ºC for 12 consecutive days to evaluate the effect of nutrient depletion on the growth rate of parasites. Parasite density was determined every 24 h using a Neubauer chamber.

### 2.5. *In vitro* oxidative stress assay

Log-phase promastigotes suspended in cRPMI[20] (final density of 5.0 x $10^6$ parasites/mL) were plated in quadruplicate in 96-well flat-bottom plates (VWR) with increasing concentrations (*i.e.*, 0.11, 0.21, 0.43, 0.85, 1.70 and 3.40 mM) of hydrogen peroxide ($H_2O_2$; Merck®, Germany), and incubated at 24 ºC for 24 h, to explore the effect of reactive oxygen species (ROS) on parasite growth. Parasite viability was determined using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (M2128, Sigma-Aldrich) tetrazolium reduction assay as described by Cortes et al. (2018) with some modifications. Briefly, the MTT substrate was prepared in PBS and added to each well at a final concentration of 5 mg/ml. After incubation (37 ºC; 2-4 h), the plates were centrifuged at 1000 x g for 30 mins at 0 ºC. The supernatant was removed, and the precipitated formazan crystals were dissolved by adding dimethyl sulfoxide (200 µL; DMSO; Merck). The quantity of formazan was measured by recording changes in absorbance at 595 nm using a microplate spectrophotometer (TRIAD Multi-Mode Microplate Reader; Dynex Technologies, USA). For each strain, three independent experiments were performed.

### 2.6. *In vitro* drug susceptibility assay

Log-phase promastigotes suspended in cRPMI[20] (final density of 5.0 x $10^6$ parasites/mL) were plated in 96-well flat-bottom plates with increasing concentrations of antileishmanial reference drugs (Table 2). After 48 h of incubation at 24 ºC, MTT was added, and parasite viability was determined as previously described. Three independent assays were carried out to determine the half-maximal inhibitory concentration ($IC_{50}$) of each strain.

**Table 2.** List of drugs used for *in vitro* susceptibility assays.

| Drug (supplier) | Incubation time | Stock solution solvent | Final concentration (range)[a] |
|---|---|---|---|
| Allopurinol (Atral-Cipan, Portugal) | 48 h | Sodium hydroxide, 1N | 0.37-11.8 mM |
| Amphotericin B (Sigma-Aldrich, USA) | 48 h | RPMI-1640 medium | 21.1-865.7 nM |
| Miltefosine (Zentaris, Germany) | 48 h | RPMI-1640 medium | 7.7-245.4 µM |
| Trivalent antimony (Sigma-Aldrich, USA[b]) | 48 h | Hydrochloric acid, 4M | 19.3-616.0 µM |
| Pentavalent antimony (Boehringer Ingelheim Animal Health, Portugal)[c] | 48 h | NA | 12.8-409.9 mM |

[a] Prepared as successive 1:2 serial dilutions of the different drugs in RPMI-1640 complete medium; [b] Obtained from antimony trichloride; [c] Obtained from meglumine antimoniate (Glucantime).

Abbreviations: NA, not applicable.

### 2.7. *Ex vivo* macrophage infection assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heterogeneous groups of feline, canine, and human *Leishmania*-negative [*i.e.*, for which no *Leishmania SSU*-rDNA was detected by nested-PCR (Pereira et al., 2020)] healthy donors using the density gradient separation method described by Dagur and McCoy (2015) with modifications. Briefly, anticoagulated blood in EDTA was diluted 1:2 in PBS, layered onto Histopaque-1077 (Sigma-Aldrich), and centrifuged at 400 x *g* for 30 min at 22 ºC. The PBMCs were harvested, washed once with PBS, and incubated for 5 min at 4 ºC with an ammonium-chloride-potassium lysing buffer to promote erythrocyte lysis. After incubation, the cells were washed twice with PBS and resuspended in RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated FBS, 2 mM L-Glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (cRPMI$^{10}$). Viable cells [identified by the trypan blue exclusion method (Strober, 2015)] were counted using a Neubauer chamber, seeded at a concentration of 1.0 x $10^6$ cells/mL in tissue culture treated flasks (T25; VWR) and incubated at 37 ºC in a humidified atmosphere of 5 % $CO_2$-95 % air (Heracell 150i; Thermo Scientific, USA). On the 3rd day of incubation, non-adherent cells were removed, and the culture medium was replaced. Three days later, the monocyte-derived macrophages were washed twice with PBS, and a non-enzymatic dissociation solution (*i.e.*, ice-cold PBS with 2.5 mM EDTA) was added. Cells were inspected using an inverted microscope and incubated on ice until most of them were detached and individualised (30 min on average). The remaining adherent cells were gently detached by a cell-scraper (VWR). After harvesting, the cells were washed once with PBS and resuspended in cRPMI$^{10}$. Viable macrophages were counted and seeded at a concentration of 2.5 x $10^4$ cells/well in duplicate in a 16 well Nunc Lab-Tek Chamber Slide system (Thermo Scientific). The cells were incubated at 37 ºC in a humidified atmosphere of 5 % CO2–95 % air for 24 h and further infected with stationary phase promastigotes at a 5:1 parasite/host cell ratio for 24 h (Maia et al., 2007). After incubation, the slides were washed twice with PBS to remove non-internalised promastigotes, were fixed with methanol, and stained with 5 % (v/v) Giemsa. The cells were mounted in dibutyl phthalate polystyrene xylene (DPX, Merck®) and counted by bright-field microscopy (CKX41, Olympus, Japan) at 1000 x magnification. The percentage of infected cells and the number of amastigotes per infected cell were assessed as previously described (Maia et

al., 2007). The infection index was determined by the multiplication of both parameters to estimate the overall parasite burden, as described by Cortes et al. (2018). For each strain, two independent experiments were performed.

### 2.8. Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics v26.0 and GraphPad Prism v8.0.1. The coefficient of variation (CV) was used to measure the dispersion of continuous variables. In high dispersion cases, the results were reported as median with interquartile range (Q1-Q3) rather than arithmetic mean with standard deviation (Whitley and Ball, 2002). D'Agostino-Pearson and Shapiro-Wilk tests were performed to analyse normality, while homoscedasticity was assessed by Bartlett's and Brown-Forsythe tests. If assumptions of parametric tests were not valid, non-parametric tests were used. One-way ANOVA, Welch's/Brown-Forsythe or Kruskal-Wallis tests were performed for comparing parasite densities under stress conditions, $IC_{50}$ values between strains, and *ex vivo* infection data. Whenever significant differences ($p < 0.05$) were found, multiple comparisons were conducted using Holme-Sidak, Tamhane T2, or Dunn's tests, respectively.

A multivariate cluster analysis was performed after these initial approaches to identify hidden group structures in the dataset. (*i.e.*, if feline strains display distinct phenotypic features from those of human or canine origin). The variables for describing strains in clusters were standardised and included parasite density/viability under stress conditions (thermal, nutritional, and oxidative), the inhibitory effect of drugs, and infection index. Strains were grouped using the nearest-neighbour (single linkage) hierarchical clustering method, considering the squared Euclidean distance as a dissimilarity measure between strains. The number of distinct clusters to retain in the final model was determined based on the $R^2$ criterion described by Maroco (2014). The classification of each strain in the retained clusters was further refined using the *k*-means algorithm.

# 3. Results

## 3.1. Growth kinetics and metacyclogenesis

Most strains analysed showed similar growth patterns (Fig. 1), entering the log-phase after a day in culture and achieving the stationary phase after the 3rd or 4th day. Strain C-33861 reached the highest parasite density (above 1.0 x $10^6$ parasites/mL between the 3rd and 5th days) among those studied. In contrast, H-IMT369 and D-IMT373 did not exceed 5.0 x $10^5$ parasites/mL/day. Strains D-IMT373, D-IMT229, and C-IMT422, presented a consistent parasitic density during the stationary phase, with the latter showing a considerably higher proportion of viable parasites from day 3 onwards. Metacyclic forms appeared between days 3-6 and were systematically identified (for all strains) until day 12. In the last third of this period, these forms comprised about 20 % of the total number of observed promastigotes. In strains C-10816, C-33861, and H-IMT369, the percentage of metacyclics exceeded 40 % in the last 2-3 days.

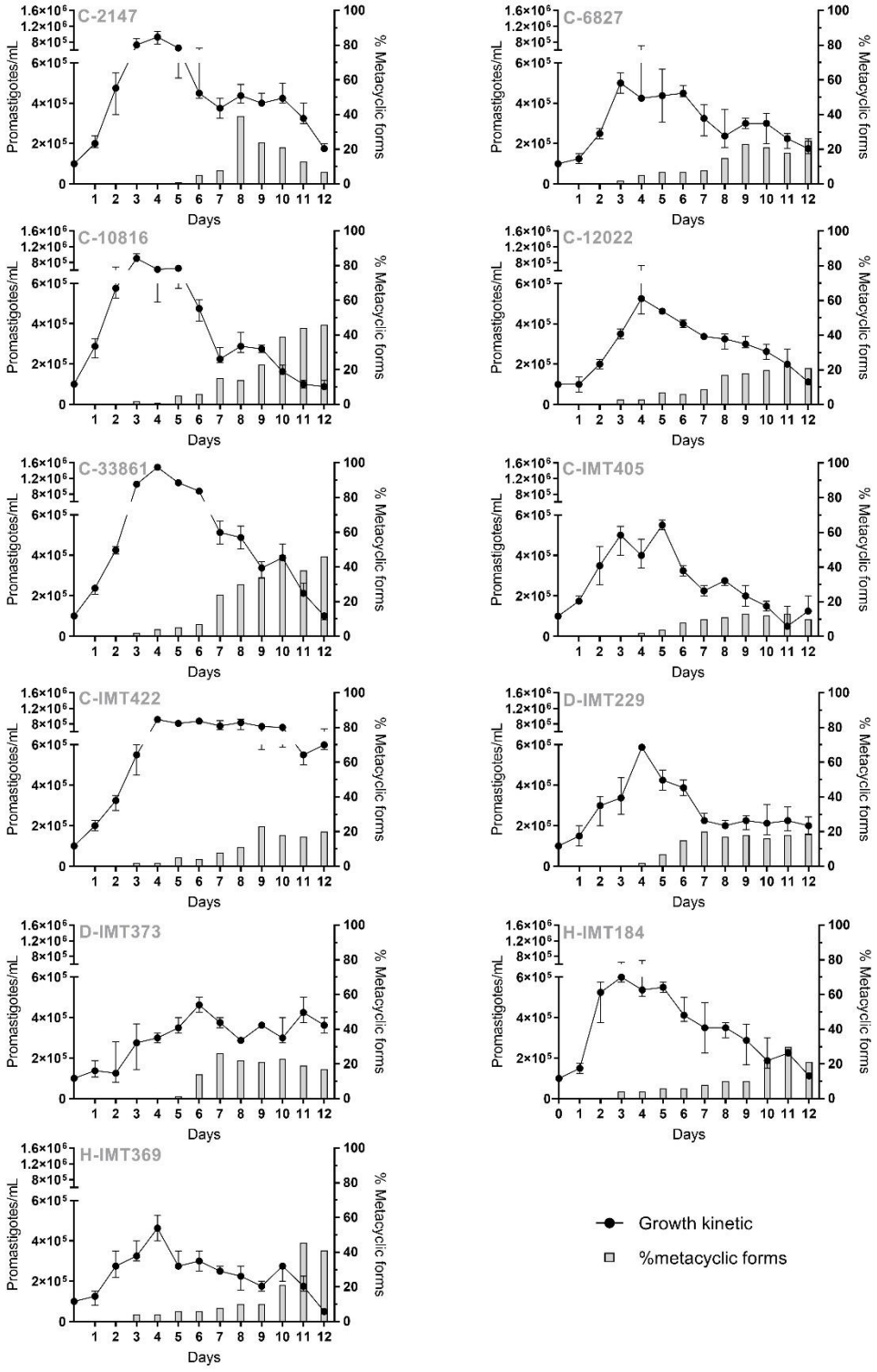



**Figure 1.** Growth kinetics and metacyclogenesis of cultured promastigotes under optimal laboratory conditions. Results are expressed as median values and interquartile range (Q1-Q3) of four replicates. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.

### 3.2. Thermal, nutritional, and oxidative stresses

Based on the results of bivariate statistics, several significant differences were observed between the studied strains under thermal, nutritional, and oxidative stress conditions (Supplementary Figs. S1-S7). However, none of these differences reflected distinctive features associated with the host source (*i.e.*, cat, dog, or human). Overall, a short exposure (*i.e.*, ≤24h) to temperatures ranging from 28-33 ºC promoted log-phase growth and a more rapid entry into the stationary phase (Supplementary Figs. S1 and S2). In contrast, after 192 h at 20 ºC, all strains entered the log-phase later but remained viable until the end of each assay (Supplementary Fig. S3). Strain C-10816 showed a significantly higher ability to grow at lower temperatures when compared to several others (namely C-6827, $p = 0.046$; C-12022, $p < 0.001$; C-IMT405, $p < 0.001$; D-IMT229, $p < 0.001$; D-IMT373, $p < 0.001$; and H-IMT369, $p < 0.001$; Supplementary Fig. S3). Most strains were not substantially affected by extreme nutrient depletion (*i.e.*, 25 % of cRPMI[20]; Supplementary Fig. S6), although D-IMT229 and C-IMT405 were significantly more susceptible to nutrient depletion than C-2147 ($p < 0.001$), C-10816 ($p < 0.001$), C-33861 ($p = 0.037$ and 0.010, respectively), C-IMT422 ($p < 0.001$) and D-IMT373 ($p = 0.003$ and 0.008, respectively). Furthermore, the feline strain C-IMT422 systematically reached the highest parasitic densities, regardless of the percentage of growth medium available (*i.e.*, 25-75 % cRPMI[20]). Data from the analysis of the impact of oxidative stress on cellular growth revealed that all strains had a similar susceptibility to exogenously added $H_2O_2$ in a concentration-dependent manner (Supplementary Fig. S7). Strain C-2147 was significantly less susceptible ($p = 0.049$) than H-IMT369 at 0.85 mM. At the highest concentrations (*i.e.*, ≥1.70 mM), no strain presented cellular viability higher than 15 %.

### 3.3. Drug susceptibility

*Leishmania* strains C-2147 and C-6827 showed a significant higher susceptibility to allopurinol ($IC_{50}$ = 0.72 and 0.64 mM, respectively) than C-10816 ($IC_{50}$ = 1.41 mM; $p = 0.008$), C-12022 ($IC_{50}$ = 1.39 mM; $p = 0.010$), C-IMT405 ($IC_{50}$ = 1.59 mM; $p < 0.001$) and H-IMT184 ($IC_{50}$ = 1.46 mM; p = 0.010) (Supplementary Fig. 8A). On the other hand, strains C-IMT422 and D-IMT229 presented the highest $IC_{50}$ amphotericin values (307.50 and 273.70 nM, respectively) and seemed to be significantly less susceptible ($p = 0.009$

and 0.042, respectively) to this drug than C-10816 ($IC_{50}$ = 129.6 nM; Supplementary Fig. 8B). The $IC_{50}$ values for miltefosine ranged from 8.97 to 47.7 mM. The highest value was obtained for C-2147, while C-IMT422 showed the lowest ($p$ <0.003; Supplementary Fig. 8C). When considering the effect of pentavalent antimony ($Sb^V$) on cell growth, C-6827 disclosed the lowest susceptibility to this compound. This strain presented a significantly higher $IC_{50}$ (73.2 mM) than C-10816 (62.2 mM; $p$ = 0.003), C-12022 (74.3 mM; $p$ = 0.007), C-33861 (62.6 mM; $p$ = 0.008), C-IMT422 (74.7 mM; $p$ = 0.017) and D-IMT229 (84.8 mM; $p$ = 0.016) (Supplementary Fig. 8D). No significant differences were identified between strains exposed to trivalent antimony ($Sb^{III}$; Supplementary Fig. 8E).

### 3.4. Macrophage infection pattern

The percentage of macrophages infected with strains isolated from cats, dogs, and humans was relatively homogenous (Supplementary Fig. 9A). However, H-IMT369 showed a significantly higher ability to infect feline macrophages than H-IMT184 (80.0 % *vs* 56.5 % of infected macrophages, respectively; $p$ <0.037). Strain H-IMT369 was also significantly more infectious to human-derived macrophages (80.0 % of infected cells) than several other strains (*i.e.*, C-IMT405, $p$ = 0.006; C-IMT422, $p$ = 0.009; D-IMT229, $p$ = 0.009; and D-IMT373, $p$ = 0.001; ≤52.5 % of infected macrophages). Among those strains isolated from cats, C-12022 was the most effective regarding the invasion of feline macrophages with a mean of 4.7 ± 1.3 amastigotes per infected cell (Supplementary Fig. 9B). Nevertheless, when the latter was compared with those of canine and human origin, no significant differences were observed at a 5 % significance level. A statistically significant difference ($p$ <0.001) was, however, observed between the mean value of parasite load per infected dog macrophage between strains H-IMT184 (4.4 ± 0.0) and C-33861 (1.7 ± 0.0). On the other hand, strains C-IMT405 and C-12022 showed the highest values of intracellular parasite load (4.0 ± 0.8 and 3.6 ± 0.3 amastigotes per infected cell, respectively) while C-33861 and C-IMT422 showed the lowest (1.6 ± 0.2 and 1.1 ± 0.2 amastigotes per infected cell, respectively). Nevertheless, based on infection index values, no differences were evidenced between the *Leishmania* strains regarding their ability to infect canine and feline macrophages (Supplementary Fig. 9C). Strain H-IMT369 had a significantly higher infection index for human macrophages (270.8 ± 55.3) than several other strains, including C-2147 (124.5 ± 12.9; $p$ = 0.021), C-10816 (99.7 ±

27.1; $p = 0.005$), C-33861 (66.3 ± 18.9; $p < 0.001$), C-IMT422 (44.9 ± 9.6; $p < 0.001$) and D-IMT229 (120.1 ± 17.0; $p = 0.016$).

### 3.5. Clustering in multivariate data

According with $R^2$ criteria, 4 clusters were retained that explained 71 % of the total variance ($R^2 = 0.71$; $k = 3$). Cluster 1 was the largest and comprised most studied strains, namely 3 isolated from cats, 2 from dogs, and 2 from humans (Figure 2). The feline strains C-33861 and C-IMT422 grouped in Cluster 2, while 12022 and 2147 formed the Clusters 3 and 4, respectively.

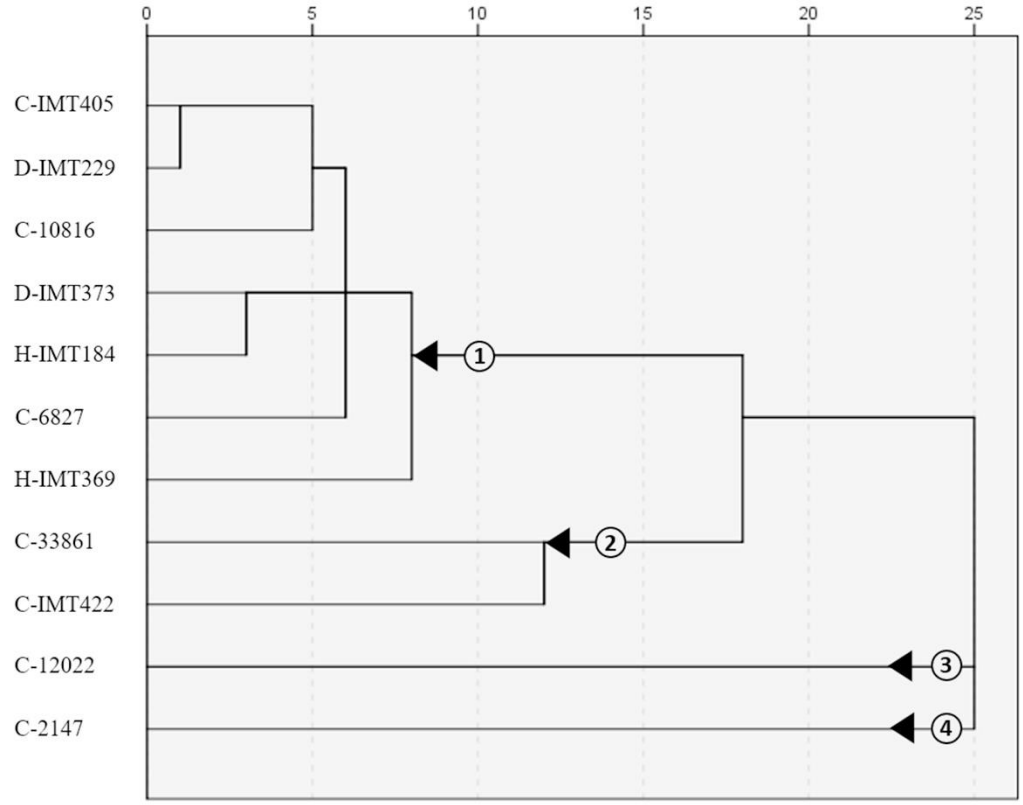



**Figure 2.** Dendrogram of cluster analysis using the nearest-neighbour hierarchical clustering method and considering the squared Euclidean distance as a dissimilarity measure between strains. The horizontal axis represents the distance or dissimilarity between clusters identified at specific branch nodes by a number. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.

## 4. Discussion

Over the last two decades, cats have raised the interest of many researchers due to their potential role in maintaining, and disseminating, human and animal leishmaniosis. According to Asfaram et al. (2019), these felids can hypothetically act as primary or secondary reservoir hosts for human infection caused by *L. infantum*. Nevertheless, several criteria must be fulfilled to formally incriminate a given species as a reservoir host. These include a confirmation that the parasites from an infected animal are indistinguishable from those isolated from humans (Maia et al., 2018).

In the present study, we compared the phenotypic behaviour and infectiousness of several parasitic strains of feline, canine and human origin, previously identified as *L. infantum* (Cortes et al., 2014; Maia et al., 2015; Franssen et al., 2020; Pereira et al., 2020). Recently, Pereira et al. (2020) demonstrated (*i.e.*, based on the analysis of four distinct target genes) that *L. infantum* strains of feline origin share the same genetic type as those isolated from humans, dogs (primary reservoir hosts), and both *Phlebotomus ariasi* and *Phlebotomus perniciosus* (proven vector species). Whilst a phenotype is a detectable expression of a genotype, it may be widely modulated by epigenetic and environmental factors (Mideo and Reece, 2012; Afrin et al., 2019). In this context, the phenotypic characterisation of promastigotes cultured *in vitro* can contribute to a better understanding of the dynamics of leishmaniosis and its clinical outcomes (Cortes et al., 2012; Vanaerschot et al., 2010; Cortes et al., 2018; Mas et al., 2020). In this study, the analysis of growth kinetics and metacyclogenesis profiles showed no apparent differences among promastigotes of feline, canine, and human origin. These results are in agreement with those recently described for *L. infantum* strains isolated from different hosts (*i.e.*, humans, dogs, and *P. perniciosus*) from endemic areas (Araújo et al., 2020; Mas et al., 2020). Moreover, the parasite's ability to proliferate appeared to be unrelated to differentiation to metacyclic forms. Contrary to initial belief, it was previously demonstrated that proliferation, and metacyclogenesis, are independently regulated (Serafim et al., 2012). Overall, the proportion of metacyclic promastigotes increased gradually from day 3-4 onwards, reaching the highest density around day 10, following the pattern previously reported for *in vitro* cultured *L. infantum* promastigotes (Gossage et al., 2003).

On the other hand, the ability of *Leishmania* parasites to withstand the wide range of hostile and changeable conditions encountered throughout their life cycle has been explored as an indicator of virulence (Zilberstein and Shapira, 1994; Cortes et al., 2012; Mas et al., 2020). Several statistically significant differences were found between the strains analysed in the course of this study after exposure to different stress conditions (*i.e.*, thermal, nutritional and oxidative). However, none of them suggested any specific features associated with feline *L. infantum* parasites. In general, data from the analysis of thermal stress suggest that parasites from cats multiply well at higher temperatures, a feature that is known to be shared by viscerotropic species, including *L. infantum* (Callahan et al., 1996).

On the other hand, although well-tolerated, the exposure to lower temperatures resulted in slower parasite growth, a finding shared by most strains isolated from cats, dogs, and humans. Hlavacova et al. (2013) observed that the canine strain D-IMT373 developed well in specimens of *Lutzomyia longipalpis* and *P. perniciosus* (natural vectors) experimentally maintained at 20 ºC [optimal temperature range from 24 to 28 ºC; (Volf and Volfova, 2011)]. Thus, these results highlight the apparent ability of *L. infantum* strains from cats to potentially develop a heavy late-stage infection in natural vectors even at 20 ºC. The decreasing concentration of nutrients did not clearly affect growth of most strains analysed, but significant differences were mainly observed among feline strains, suggesting distinct metabolic needs that seem to be unrelated to the origin of the host from which they were isolated. In contrast, all strains were susceptible to $H_2O_2$ in a concentration-dependent manner, as previously described for *L. infantum* promastigotes of human origin (Cortes et al., 2018), suggesting their vulnerability to host-derived oxygen radicals.

Allopurinol, amphotericin B, miltefosine, and pentavalent antimonials are the antileishmanial drugs recommended for the treatment of canine leishmaniosis and/or human VL caused by *L. infantum* (WHO, 2010; Solano-Gallego et al., 2011). Although off-label, most of these drugs have also been used in FeL cases (Leal et al., 2018; Pereira et al., 2019). Drug resistance has been extensively described in human leishmaniosis and more scarcely in the canine disease (Ponte-Sucre et al., 2017; Campino and Maia, 2018). Nevertheless, no data are available on resistance to antileishmanial drugs in cats. The amastigote-macrophage model is currently the gold standard for *in vitro* drug resistance

detection (Baek et al., 2020). However, it presents several technical limitations compared to assays using promastigotes, which are easy to perform, fast, and robust (Maia et al., 2013; Baek et al., 2020). Therefore, in the current study, the susceptibility of *Leishmania* promastigotes of feline, canine and human origin to the drugs mentioned above was estimated from a dose-response curve, using and *in vitro* system.

Significant differences in allopurinol susceptibility were observed mainly between *Leishmania* strains of feline origin, which may be attributed to intrinsic differences in drug sensitivities without any host species-specific association. The allopurinol $IC_{50}$ values obtained for parasites isolated from cats (1.17 ± 0.36 mM) were similar to those from treated asymptomatic dogs (1.97 ± 1.3 mM; Yasur-Landau et al., 2016), indicating a high susceptibility of feline strains to this drug. Although allopurinol has been effective in several FeL cases (Pennisi et al., 2015), its use as monotherapy should be revised since *in vitro* experimental selection of allopurinol-resistant *L. infantum* promastigotes was easily achieved (Yasur-Landau et al., 2017).

Overall, strains isolated from cats, dogs, and humans presented an identical susceptibility to amphotericin B. The range of $IC_{50}$ values obtained (129.6-307.5 nM) appeared to be related to that previously reported for *L. infantum* strains isolated from both dogs and humans from Portugal (40,0-250,0 nM; Maia et al., 2013), as well as from dogs from an urban area of Alger (Algeria) (108.2-443.7 nM; Aït-Oudhia et al., 2012). In contrast, $IC_{50}$ of studied strains was considerably lower compared to that observed for an *L. infantum*/*L. major* hybrid (710.0 nM) isolated from an immunocompromised human patient following treatment with antileishmanial drugs, including amphotericin B (Maia et al., 2013). Although amphotericin B seems to be effective against feline strains of *Leishmania*, its use in the treatment of FeL cases should not be equated so as to avoid drug resistance selection to the first option for treatment of human VL due to *L. infantum* in the Old World (WHO, 2010).

Finally, and as far as the resistance to miltefosine was regarded, strain C-2147 presented the highest $IC_{50}$ (*i.e.*, 47.7 ± 5.1). Interestingly, values greater than 40 µM were recently reported for a naturally miltefosine-resistant strain (*i.e.*, MHOM/FR/2005/LEM5159) (Van Bockstal et al., 2020), and although the existence of a resistance profile among feline strains cannot be formally excluded, it should be carefully

considered. On the one hand, in the study cited above, promastigotes were exposed to miltefosine for an extended period (72 h *vs* 48 h), and the exact $IC_{50}$ was not provided. Instead, in this study, the $IC_{50}$ obtained for strain C-2147 was neither significantly different from that calculated for most other feline strains, nor from all strains isolated from dogs and humans. Thus, further studies using the amastigote-macrophage model are needed, as it better reflects the *in vivo* infection scenario (Hefnawy et al., 2017). Nevertheless, miltefosine seems to be a possible option for the medical management of FeL, as recently proposed by Leal et al. (2018).

The feline strain C-6827 was significantly less susceptible to $Sb^V$ than most studied strains, with an $IC_{50}$ similar to that calculated for the *L. infantum* H-IMT369, a strain isolated from a human patient following treatment with meglumine antimoniate (a $Sb^V$ based drug; Glucantime) (Maia et al., 2013). However, when the parasites were exposed to the active trivalent form of this compound ($Sb^{III}$) no significant differences were revealed among the studied strains. For antileishmanial activity, $Sb^V$ must be reduced to $Sb^{III}$, which is more toxic to the parasites (Ponte-Sucre et al., 2017). This fact was also reflected in this study, where the $IC_{50}$ values calculated for $Sb^{III}$ were approximately 100 times lower than $Sb^V$, considering identical assay conditions. Thus, these results suggest the *a priori* absence of any resistance for this drug among feline strains. Clinical outcomes corroborate this hypothesis since the use of meglumine antimoniate in FeL cases has commonly resulted in clinical cure (Pennisi et al., 2015; Basso et al., 2016; Pereira et al., 2019).

Both immortalised cell lines and primary cells have long been used as *in vitro* models for studying the infectivity of *L. donovani* complex parasites (Maia et al., 2007; Vanaerschot et al., 2010; Araújo et al., 2020; Mas et al., 2020). Although cell lines appear to be more useful (i.e, easy to use, cost-effective, avoids many ethical objections, unlimited supply of material, and reproducibility results), it is known that biological changes resulting from serial passages may jeopardise their physiological importance (Kaur and Dufour, 2012). For this reason, monocyte-derived primary macrophages were used in the present study. Monocytes were isolated from a mix of peripheral blood collected from heterogeneous donors (*i.e.*, distinct breeds and/or sex and age) to promote a widely genotypic and phenotypic representativity of feline, canine, and human populations. Overall, when compared to those isolated from dogs and humans,

*Leishmania* strains isolated from cats showed a similar ability to infect feline, canine, and human macrophages. Focusing on feline strains, the percentage of infected cells and the number of amastigotes per infected cell were identical to those calculated for macrophages derived from human peripheral blood when infected with canine and human *L. infantum* (Maia et al., 2007; Mas et al., 2020). On the other hand, feline macrophages appeared to be permissive not only to strains isolated from cats but also from dogs and humans. These results were not altogether surprising since a previous study using similar assay conditions reported a comparable capacity of canine and human strains to infect distinct macrophage types (Maia et al., 2007). Nevertheless, it is important to highlight that the studied strains were subjected to successive periods of *in vitro* cultivation. This issue should be addressed in future studies since a high number of *in vitro* passages has been significantly correlated with loss of *L. infantum* virulence (Moreira et al., 2012). In addition, a divergence between *in vitro* and *in vivo* infectivity of *L. infantum* strains has been occasionally reported (Araújo et al., 2020; Mas et al., 2020). Therefore, further studies will be required to determine whether the reported findings are also validated *in vivo* in animal models of VL.

Multivariate cluster analysis has frequently been used to provide evidence for or against clustering structure in large data sets (Liao et al., 2016). In the present study, the formation of clusters seemed to be influenced by the over-representativity of strains isolated from cats. However, overall, it appears that feline strains are phenotypically identical to those isolated from dogs and humans.

## 5. Conclusions

To our knowledge, this is the first study assessing the phenotypic behaviour of feline *L. infantum* strains. A similar growth pattern, response to stress conditions, susceptibility to antileishmanial drugs, and infectivity was shared by strains isolated from cats, dogs, and humans. The findings herein reported provide a starting point for studying the virulence of *Leishmania* parasites of feline origin and contribute to the clarification of the hypothetical role of cats as reservoir hosts of the aetiological agent of zoonotic visceral leishmaniasis. Nevertheless, further epidemiological studies on transmission dynamics should be encouraged to fully establish the status of cats in the maintenance of *L. infantum* foci.

*Ethics statement*

This study was approved by the Ethical Committee of the IHMT (authorisation no. 10.20) as complying with Portuguese legislation for animal protection (Decree-Law no. 113/2013). Consent for blood collection was obtained from volunteers directly or, in the case of animals, from the legal detainer.

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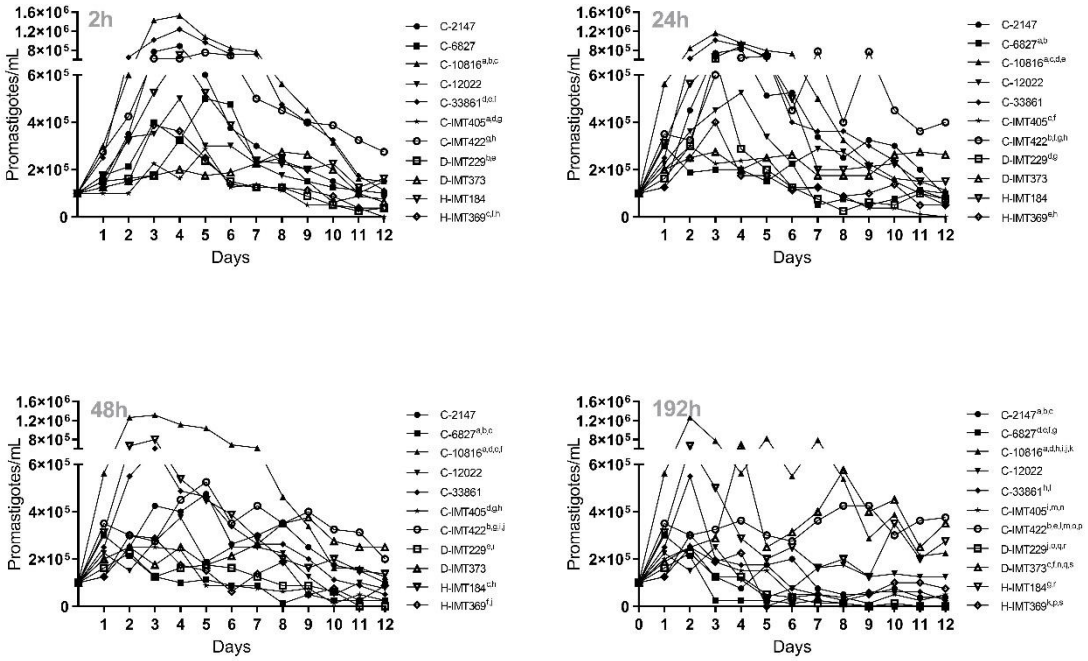
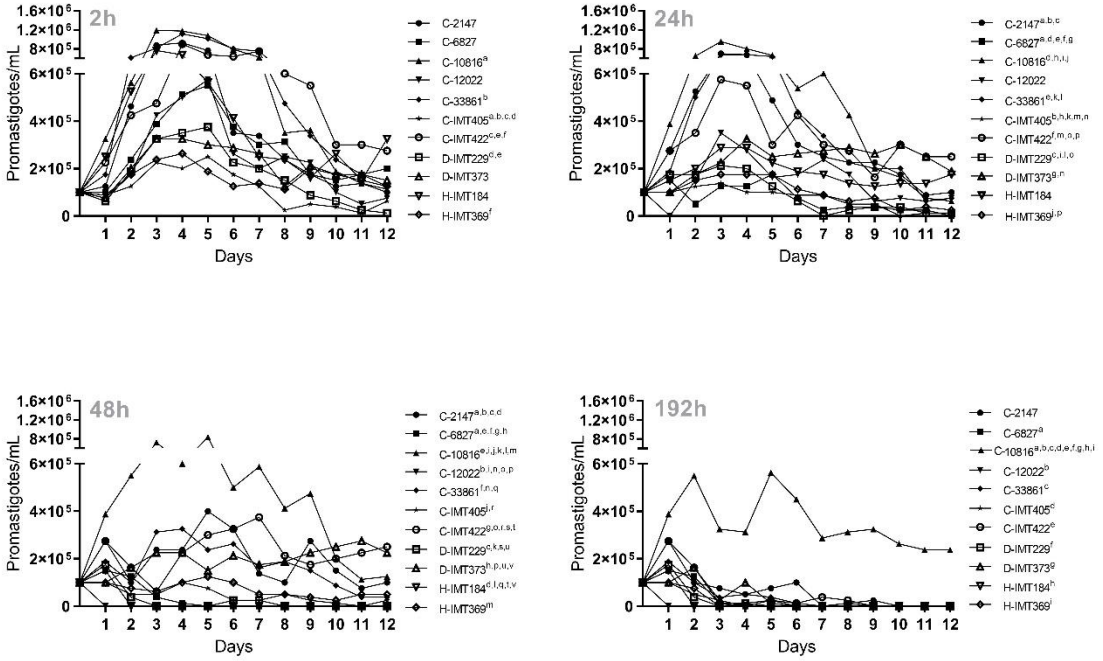
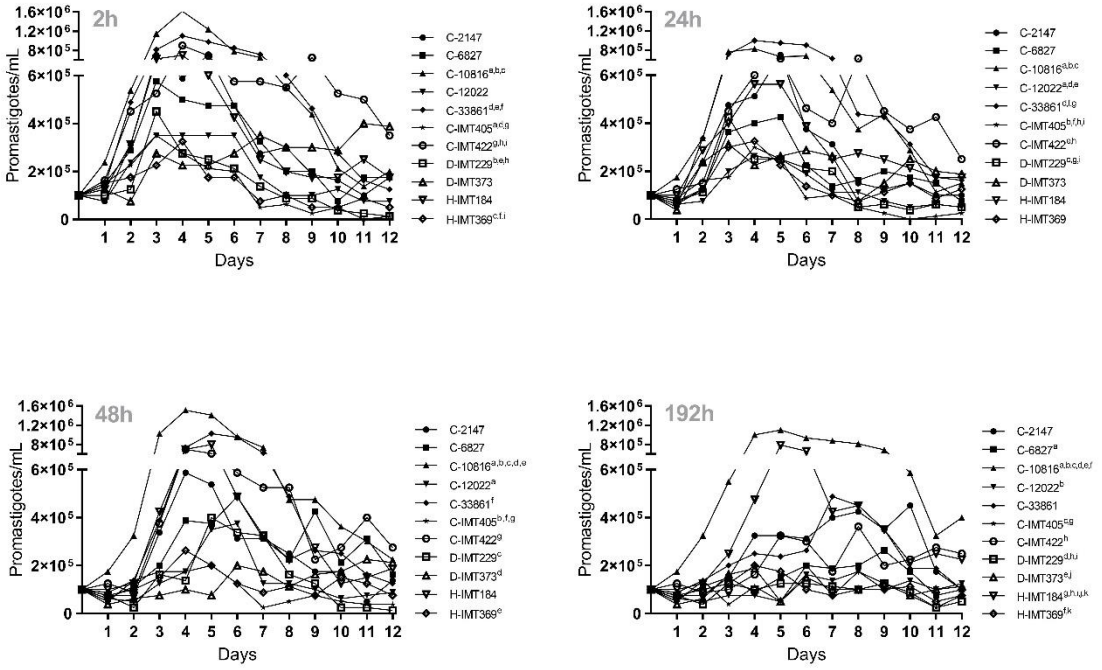
# 8. Supplementary data



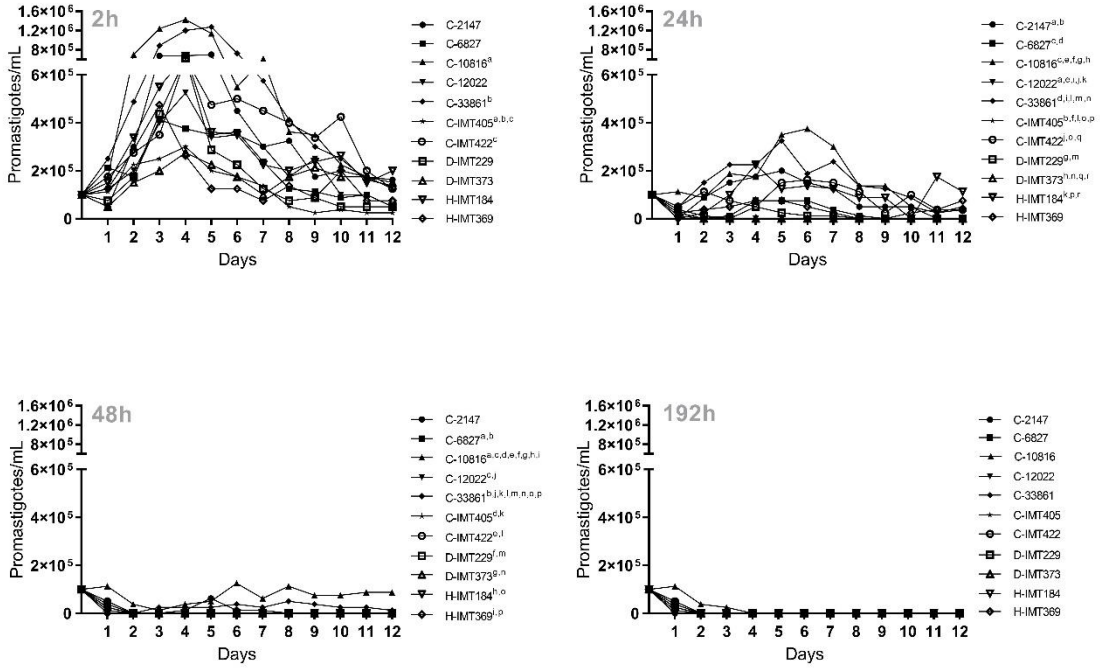

**Supplementary Figure 1.** Promastigotes growth behaviour at 28ºC for 2, 24, 48 and 192 h. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparisons. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.



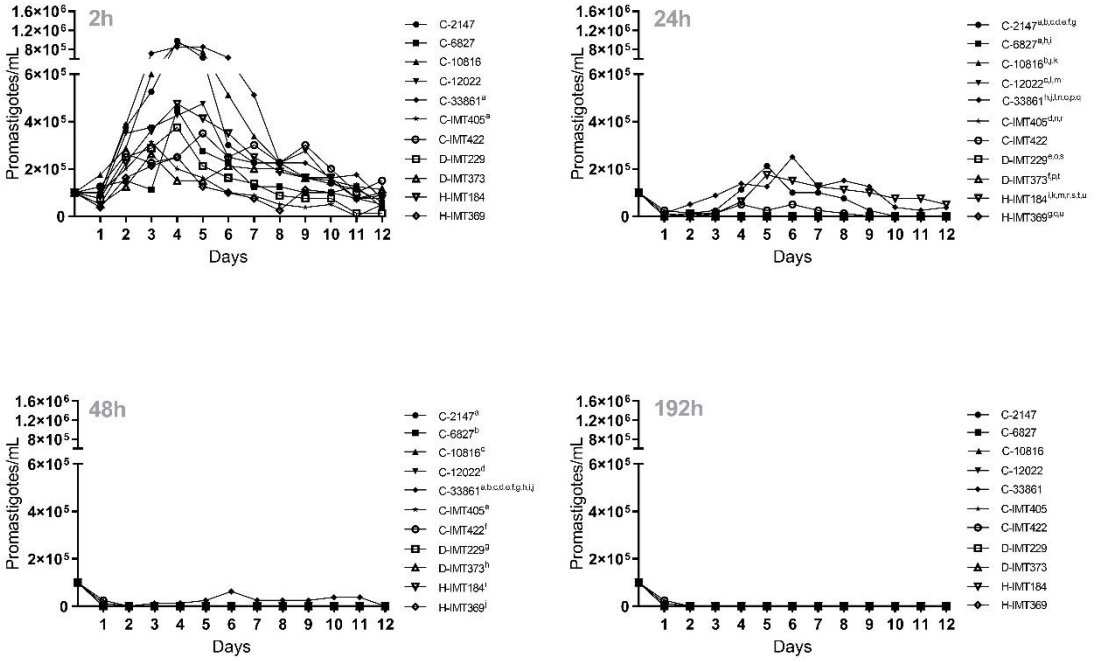

**Supplementary Figure 2.** Promastigotes growth behaviour at 33ºC for 2, 24, 48 and 192 h. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparisons. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.



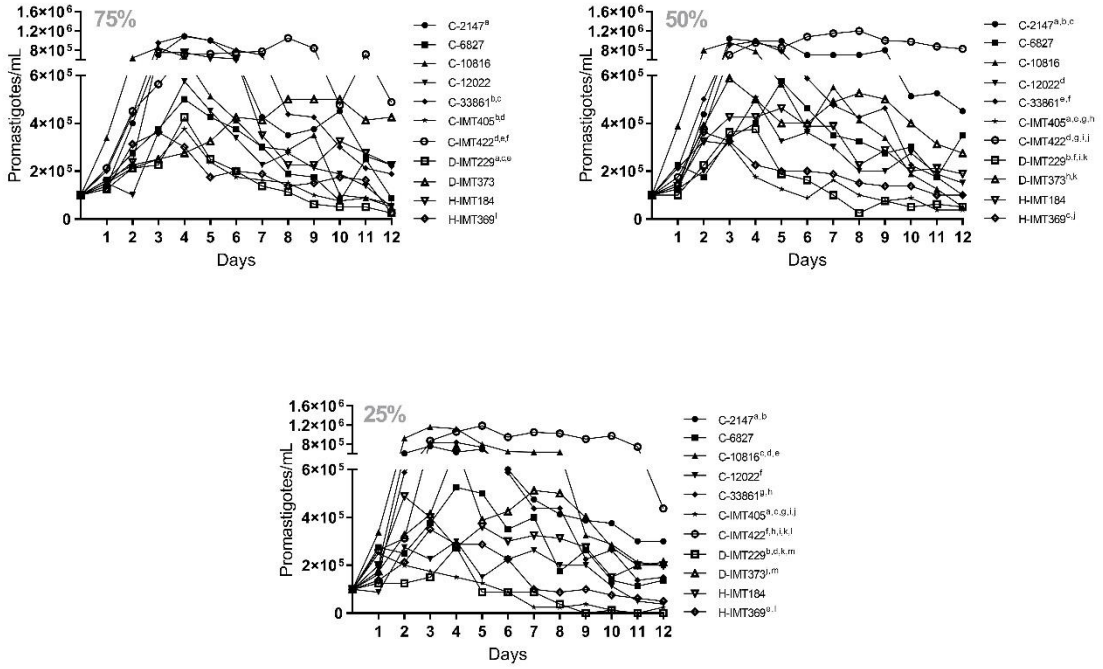

**Supplementary Figure 3.** Promastigotes growth behaviour at 20ºC for 2, 24, 48 and 192 h. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparisons. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.



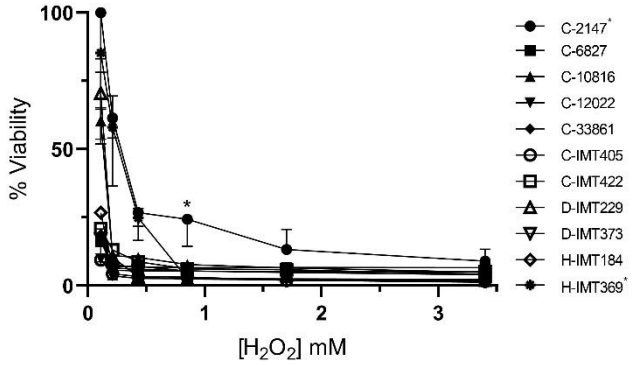

**Supplementary Figure 4.** Promastigotes growth behaviour at 37ºC for 2, 24, 48 and 192 h. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparison. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.



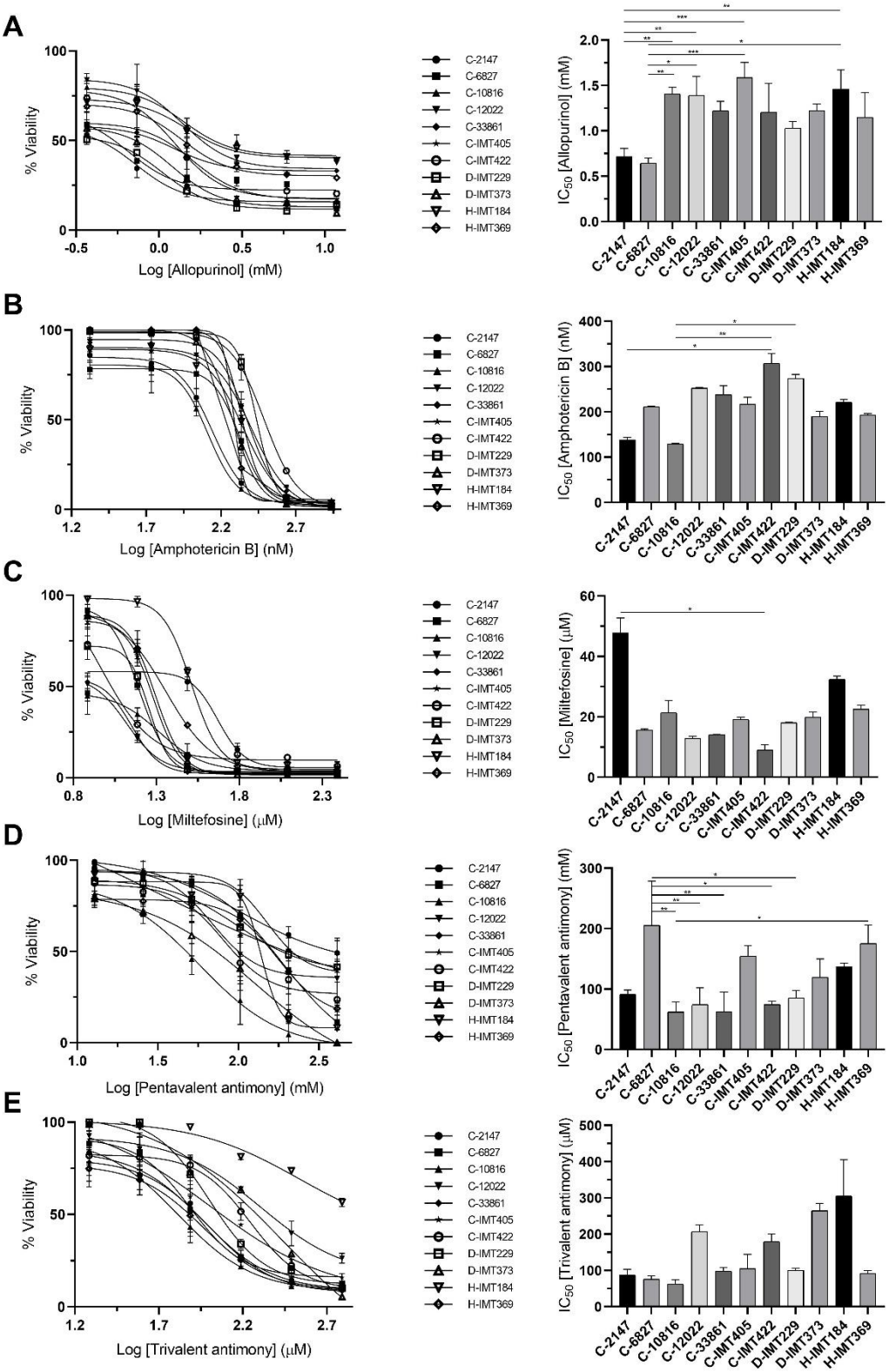

**Supplementary Figure 5.** Promastigotes growth behaviour at 40ºC for 2, 24, 48 and 192 h. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparison. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.



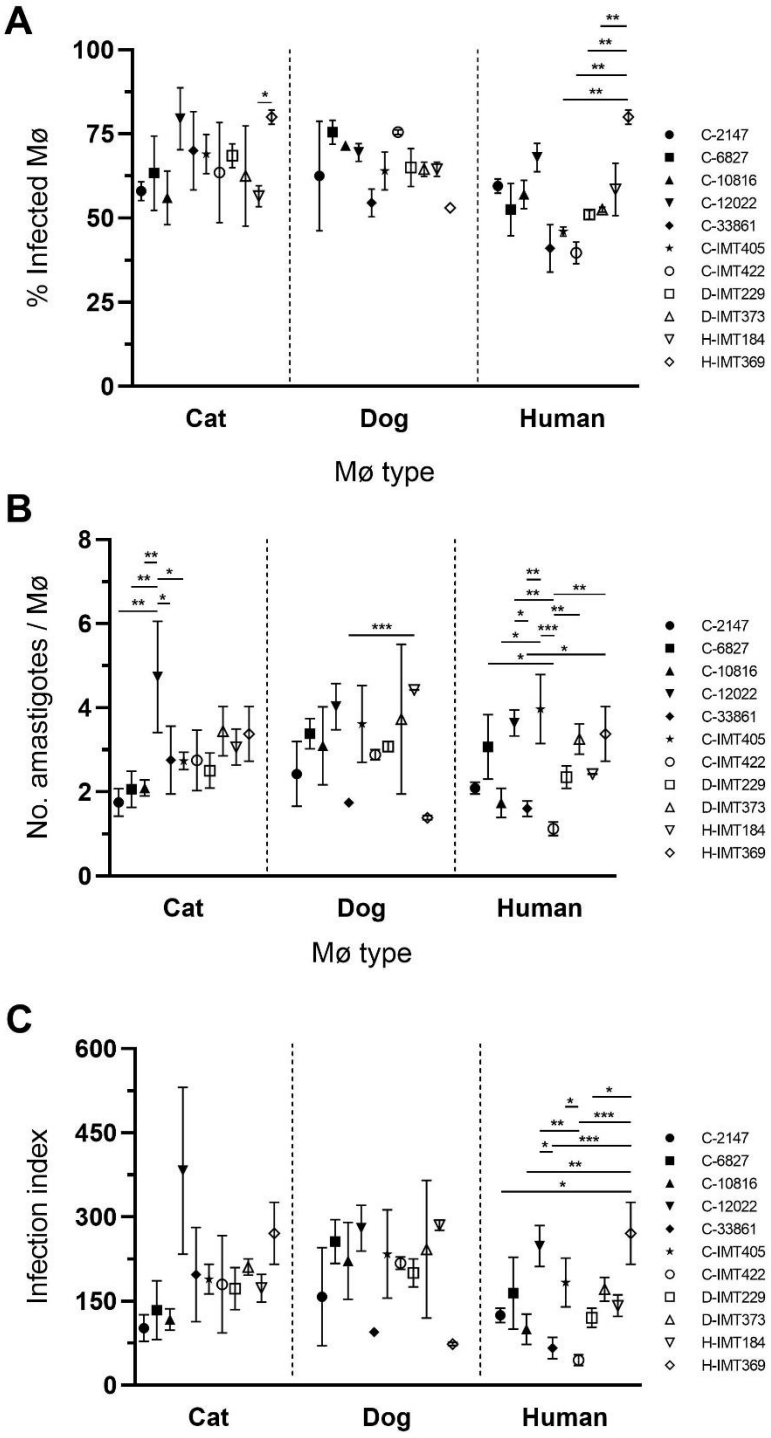

**Supplementary Figure 6**. Effect of nutrient depletion on promastigotes growth behaviour. The percentage of complete RPMI-1640 medium used (v/v) is indicated in each graph's upper left corner. Results are expressed as median values of four replicates. Superscripted letters represent significant differences ($p < 0.05$) from the pairwise comparison. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.




**Supplementary Figure 7.** Parasite viability (%) of the feline, canine, and human *L infantum* strains exposed to hydrogen peroxide ($H_2O_2$). Results are expressed as median values and interquartile range (Q1-Q3) of four replicates and three independent assays. * Statistically significant difference ($p < 0.05$) revealed after pairwise comparison. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.




**Supplementary Figure 8.** Parasite viability (%) and $IC_{50}$ values for antileishmanial drugs in susceptibility assays with the feline, canine, and human *L. infantum* strains. Results are expressed as mean values and standard deviation of four replicates and three independent assays. A, allopurinol; B, amphotericin B; C, miltefosine; D, pentavalent antimony; E, trivalent antimony. $^{*}$ $p < 0.05$; $^{**}$ $p < 0.01$; $^{***}$ $p < 0.001$. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.




**Supplementary Figure 9.** *Ex vivo* infectivity of the feline, canine, and human *L. infantum* strains assessed using distinct monocyte-derived primary macrophages. The percentage of infected cells (A) and the number of parasites per infected cell (B) were shown. The infection index was determined by multiplying the individual data from (A) by (B). Results are expressed as mean values and standard deviation of two replicates and two independent assays. $^{*}$ $p < 0.05$; $^{**}$ $p < 0.01$; $^{***}$ $p < 0.001$. Prefixes C, D and H, refer to strains isolated from cats, dogs, and humans, respectively.

# CHAPTER 6

## General discussion and conclusions

# 1. General discussion and conclusions

This doctoral project was developed around a focal question: What is the role of domestic cats in the epidemiology of zoonotic leishmaniosis?

Zoonotic leishmaniosis caused by *L. infantum* is a severe vector-borne disease affecting humans and other mammals worldwide. Disease control requires an integrated "One Health" approach in which identifying key reservoir hosts is crucial. In this context, dogs have attracted most research interests for years due to their *status* as primary domestic reservoir hosts for human infection by *L. infantum*. Nevertheless, there is accumulating evidence that other mammals than dogs may also have a relevant role in maintaining this zoonotic species in domestic and peridomestic environments (Cardoso *et al.*, 2021). Among domestic mammals, cats are considered the most probable additional reservoir hosts of *L. infantum* (Asfaram, Fakhar & Teshnizi, 2019). Despite the increasing trend to regard cats as more than an occasional source of infection, there are several criteria that, according to the WHO (2010), must be fulfilled to incriminate a mammal species as a reservoir host of *Leishmania* spp.:

***"A reservoir host is likely to be sufficiently abundant and long-lived to provide a significant food source for sand flies."***

Following dogs, cats are the second most popular animals owned as pets worldwide (GfK, 2016) and are often present in areas where the peridomestic and domestic transmission cycles of the parasite occur (Maia *et al.*, 2010; Iatta *et al.*, 2019; Rocha *et al.*, 2019). Also, these felids are among the blood-feeding preferences of phlebotomine sand flies in endemic areas of zoonotic leishmaniosis caused by *L. infantum* (Abbate *et al.*, 2020).

***"Intense host–sand fly contact is necessary."***

In this thesis (**Chapter 2)**, the cats' exposure to phlebotomine sand flies was assessed for the first time by quantifying specific IgG antibodies against *P. perniciosus* saliva. This approach has been proven to be a reliable way to monitor vector exposure in natural dog populations in endemic areas (Velez *et al.*, 2018; Maia *et al.*, 2020). About half of sampled cats showed antibodies to *P. perniciosus* saliva, which strongly suggests that cats in endemic foci have intense contact with the principal vector species of *L.*

*infantum* in the Western Mediterranean region. Moreover, the detection of antibodies to *P. perniciosus* saliva was significantly higher in adult cats suggesting a recurrent and accumulative exposure to *L. infantum* vectors. Nevertheless, feline antibodies to saliva seem to be relatively short-living. This conclusion is based on the association between IgG positive and sampling period, with cats during phlebotomine sand fly activity showing significantly higher levels of antibodies to *P. perniciosus* saliva than during winter months. Similar observations have been reported in dogs from endemic areas (Kostalova *et al.*, 2015; Velez *et al.*, 2018; Maia *et al.*, 2020). However, further longitudinal studies in cat populations are needed to provide solid evidence about the seasonal dynamics of feline antibody response to vectors and correlation type between antibody production and the number of phlebotomine sand fly bites. Also, the ability of repellents licenced for use in cats to reduces the frequency of *P. perniciosus* bites and subsequently *L. infantum* infection should be addressed in future works. In the present study, it was concluded that infected cats are frequently exposed to phlebotomine sand fly bites. Recent research has demonstrated that the treatment of dogs with isoxazolines might be a powerful strategy to control zoonotic vector-borne diseases such as leishmaniosis (Miglianico *et al.*, 2018; Bongiorno *et al.*, 2020; Queiroga *et al.*, 2020). Considering that, cats may be a source of infection to proven vectors of *L. infantum* (Maroli *et al.*, 2007; da Silva *et al.*, 2010; Mendonça *et al.*, 2020), it is relevant to assess if the use of isoxazolines in these felids reduces pool-infected vectors in endemic areas of zoonotic leishmaniosis.

***"The proportion of individuals that become infected during their lifetime is usually considerable."***

Still, in **Chapter 2**, the propotion of infected cats in endemic foci was estimated using serological and molecular tests. Based on the combined results, approximately seven out of every 100 cats sampled were infected by, or had been exposed to, *Leishmania* parasites. This proportion was similar to those obtained in the most extensive epidemiological survey performed in cats in Portugal so far (Maia *et al.*, 2014). However, the proportion of feline *Leishmania* infection in endemic countries, including Portugal, is widely variable [from 0 to >70 %; (Baneth *et al.*, 2020; Neves *et al.*, 2020)] and appears to be influenced by heterogeneity/performance of diagnostic methodologies (mainly cut-off, target gene and sample used for testing) and sampling bias (Cardoso *et al.*, 2021).

Sampling bias was, undoubtfully, a limitation of the current study as feline samples were obtained by convenience, which compromised the extrapolation of results to the target population (*i.e.*, which means that results cannot extrapolated to cats in endemic foci). Therefore, large-scale longitudinal studies using sampling methods based on the probability theory and different diagnostic tests (*i.e.*, molecular and serological) should be used to determine the true prevalence and incidence of feline *Leishmania* infection in endemic foci.

***"Parasites should be available in the skin or the blood in sufficient numbers to be taken up by a sand fly."***

The results herein reported (**Chapter 2**) suggest that *Leishmania* parasites are relatively common in the peripheral blood of cats (*i.e.*, an conclusion made by the detection of *Leishmania* DNA using PCR tests), including those without clinical signs compatible with leishmaniosis. Indeed, *L. infantum* DNA has been frequently found in the blood and skin of cats in endemic areas (Maia *et al.*, 2010; Chatzis *et al.*, 2014; Marcondes *et al.*, 2018). Nevertheless, xenodiagnosis and parasite culture are the most reliable mehtods to confirm whether mammals harbour parasites and that the protozoa are viable (Paltrinieri *et al.*, 2016). Unfortunately, none of the blood samples incubated on Novy-MacNeal-Nicolle medium led to the isolation of viable parasites, here which were only identified in cultures of lymph node biopsies from a cat with clinical leishmaniosis. However, there seems to be no doubt that domestic cats harbour parasites in the skin and blood in sufficient numbers to infect vectors due to the following reasons: *(i) Leishmania infantum* has been isolated from different biological samples of feline origin, including blood (Pocholle *et al.*, 2012) and skin (Poli *et al.*, 2002; Basso *et al.*, 2016); and *(ii)* it has been demonstrated that naturally infected cats are infectious to proven vectors of *L. infantum* in both the New and Old Worlds (Maroli *et al.*, 2007; da Silva *et al.*, 2010; Mendonça *et al.*, 2020).

***"The course of infection in a reservoir host should be long enough and the infection should be sufficiently non-pathogenic to allow the parasites to survive any non-transmission season."***

In this thesis (**Chapter 3)**, the clinical and parasitological follow-up of a naturally infected cat was described. Unfortunately, most screened animals were strays, which

severely compromised the objective of monitoring the course of *Leishmania* infection in cats. In the remaining cases (*i.e.*, non-stray cats which tested positive for *Leishmania* DNA or antibodies during the screening phase), there was a lack of compliance by owners or the animals died and were excluded. The exception was an adult cat with clinical leishmaniosis and concurrent regressive FeLV infection, which was continuously followed over a year. Only a small percentage of infected cats develop clinical disease, which is frequently associated with impaired immunocompetence due to concomitant infectious, debilitating diseases or immunosuppressive therapies (Pennisi & Persichetti, 2018). In the followed case, a chronic and insidious pattern of infection was identified, and for the first time, the presence of parasites in cats' inflammatory breast fluid was reported. Allopurinol monotherapy had an unsuccessful outcome, but when combined with meglumine antimoniate resulted in clinical remission. Also, the cat remained seropositive, but no *Leishmania* DNA was found in blood samples six months after the end of the combined treatment. Nevertheless, it has been demonstrated in previous studies that *Leishmania* parasites might persist in treated cats (Pocholle *et al.*, 2012; Pimenta *et al.*, 2015; Attipa *et al.*, 2017), suggesting that treatment may lead to clinical cure but may not eliminate the infection, as seen in dogs. Moreover, the development of acute fatal systemic disease in cats has been reported in rare cases, which may indicate that even in sick cats, the infection is sufficiently non-pathogenic to ensure parasite survival during long periods in the host.

***"The parasites in reservoir hosts must be the same as those in humans."***

In this thesis (**Chapter 4)**, it was demonstrated (using a MLST-based approach) that strains isolated from cats were genetically similar to those of *L. infantum* isolated from humans (accidental hosts), dogs (proven primary reservoirs), and *P. ariasi* and *P. perniciosus* (proven vectors). However, the monophyly of *L. donovani* and *L. infantum* was not consistently revealed, and thereby feline strains could only be identified as *L. donovani* sensu lato. Indeed, this species complex has been the subject of controversy in recent decades due to several taxonomic inconsistencies, that included the description of *L. archibaldi* and *L. chagasi* as separate species from *L. donovani* and *L. infantum*, respectively (Maurício, 2018). Moreover, there is evidence confirming the occurrence of genetic recombination within the *L. donovani* complex (Rogozin *et al.*, 2020). Consequently, no markers have been found that can provide unambiguous identification

of any species within the *L. donovani* complex. Thus, for diagnosis purposes and epidemiological studies, Maurício (2018) suggested that it may be more helpful to recognise this complex as a single species, *L. donovani*. However, Franssen *et al.* (2020) analysed the whole-genome of 151 *L. donovani* complex strains recently and demonstrated that *L. infantum* and *L. donovani* are not, in fact, synonymous species. However, the same authors confirmed the existence of highly divergent *L. donovani* complex strains whose identity at the species level is debatable (Franssen *et al.*, 2020). Therefore, a high-resolution view of the entire genome of *Leishmania* strains isolated from cats needs to be delivered by future works.

The detection of DNA of *L. major* and putative *L. major*/*L. donovani* complex hybrid parasites among screened cats was an unexpected finding which reinforces their possible circulation in Southern Europe (Ravel *et al.*, 2006; Campino *et al.*, 2013), bringing new challenges for futures epidemiological studies.

Since ecological interactions shape parasite traits (Mideo & Reece, 2012), the phenotypic profile of genetically defined *L. donovani* s.l. strains isolated from cats was also characterised (**Chapter 5**). Overall, feline strains showed similar behaviour to *L. infantum* strains isolated from humans and dogs under optimal and stressed growth conditions as well as exposure to drugs. Moreover, it was also demonstrated that parasite strains isolated from cats can infect different types of macrophages (*i.e.*, of canine, feline, and human origin) in an identical way to those isolated from dogs and humans. Additionally, feline macrophages were found to be permissive not only to strains isolated from cats but also to strains isolated from dogs and humans. Some statistically significant differences were observed between the studied strains using different assays, but none of them suggested any specific features associated with parasites isolated from cats. As a result, it can be deduced that *L. infantum* strains isolated from cats, dogs and humans share similar phenotypes.

The absence of *in vivo* studies should be regarded as a limitation of the current study. This type of studies is useful to provide deeper knowledge about infectivity of *Leishmania* strains (Cortes *et al.*, 2018; Araújo *et al.*, 2020; Mas *et al.*, 2020). Furthermore, epigenetic changes are also associated with *Leishmania* phenotypic variation (Kamhawi & Serafim, 2020). Therefore, future studies should be performed to

elucidate *in vivo* behaviour of *L. infantum* strains isolated from cats as well as to uncovered possible epigenetic mechanisms underlying infection by these parasites.

By integrating knowledge at different levels (*i.e.*, epidemiological, molecular, experimental, and clinical data) it can be concluded that cats fulfilled all criteria to be recognised as reservoir hosts.

In addition, new insights into the diagnosis and prevention of feline *Leishmania* infection are provided in this doctoral thesis, through the development of a pioneering diagnostic algorithm for clinical decision support and establishment of a set of prevention guidelines.

Although crucial issues concerning the role of cats in the epidemiology of zoonotic leishmaniosis were settled in this thesis, there are still some outstanding questions that will need to be addressed in the future:

- How often are cats in endemic foci bitten by phlebotomine sand flies?
- What is the true proportion of cats infected with *L. infantum* in endemic foci?
- Can cats ensure parasite transmission in the absence of dogs in endemic areas where both hosts occur sympatrically?
- Can the proportion of new human and animal cases of *L. infantum* in endemic foci owed to transmission from cats be determined?
- Can cats coinfected with *L. infantum* and FIV be more infectious to proven vectors and contribute to transmission as "super-spreaders" in endemic foci?
- Do cats treated with anti-leishmanial drugs remain infectious to phlebotomine sand flies?
- In cats, can isoxazolines reduce pool-infected vectors in endemic foci?
- What is the impact of stray populations of cats on the spread of zoonotic leishmaniosis?
- Can *Leishmania* infection in cats be transmitted by non-vectorial ways?
- Can markers be developed to assess resistance and susceptibility of cats to leishmaniosis?
- Do *L. infantum* strains isolated from cats display the same genetic composition of those isolated from humans, dogs, and phlebotomine sand flies?
- How does the immune system of cats respond to *Leishmania* infection?
- What is the *in vivo* infectivity and virulence of *L. infantum* strains isolated from cats?
- Which factors are associated with feline leishmaniosis susceptibility, resistance, and severity?
- Can a vaccine be developed to avoid feline leishmaniosis and block transmission from cats?

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