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Standardization of canine infections for the development of antileishmanial drugs

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Chemotherapy of leishmaniasis, both human and canine, is far from ideal and new antileishmanial drugs are urgently needed. Therapeutic arsenal against the disease is old, of low antileishmanial efficacy and hampered by toxicity issues in some cases. Furthermore, hospitalization required for some drugs, the price of the safer presentations is high and there are growing reports of leishmanial resistance to first line medicines. In addition to human visceral cases, canine infections by *Leishmania infantum* represent a first order veterinary pathology with dogs being the main reservoir for this zoonotic infection. Thus, dog infections by *L. infantum* are needed to develop veterinary drugs and are the best surrogate model for human leishmaniasis. Many contributions on the efficacy of new drugs or presentations in dogs have been published but the variety of experimental designs makes comparisons challenging thus reducing their value. Present study offers a comprehensive review of canine experimental infections with visceral *Leishmania*, with a particular focus on *L. infantum*. The review encompasses a range of topics, including animal housing, regulatory aspects, ethical considerations, infection methods, follow-up procedures, and outcomes. The final aim of our contribution is to promote the standardization of some experimental procedures to enhance the comparability of the studies performed. This, in turn, is expected to reduce the use of animals and to increase the efficiency of drug discovery and development.

KEYWORDS

3R principles, dog, drug development, drugs, experimental model, infection, infection follow-up, leishmaniasis

1 Introduction

The development of new drugs requires thorough evaluation in diverse animal models prior to testing in the target species. Several surrogate hosts, particularly rodents, can be experimentally infected with *Leishmania* species responsible for visceral leishmaniasis (VL), the most severe form of the disease (*Leishmania donovani* and *L. infantum*). Nonetheless, rodent models such as mice and hamsters present notable limitations in recapitulating the clinical course, pathological manifestations, and immune responses elicited during infection. In contrast, dogs represent a highly valuable model for investigating both the pathogenesis of VL and potential therapeutic approaches, as they are natural hosts of *L. infantum* (= *L. chagasi*), constitute an indispensable target for integrated disease control,

and closely mirror the human condition, given that the clinical presentation and lesions in both species are strikingly similar, with cutaneous lesions rarely reported in humans (Moreno and Alvar, 2002).

A standardized experimental model of canine leishmaniasis (CanL) should be capable of inducing active infection in all inoculated animals within a short period, displaying clear signs of infection and facilitating parasite detection. However, the unpredictable nature of the canine response to infection is the main stumbling block (e.g., Cacheiro-Llaguno et al., 2020). Some authors consider this to reflect the wide spectrum of clinical manifestations observed in natural infections in both this domestic animal species and humans (Garg and Dube, 2006). Additional factors, such as the virulence of the isolate, the parasite stage initiating the infection, the infective dose and route of inoculation, the age, sex, breed and physiological status of the dogs, can influence the progression and outcome of the infection. Although over 30 experiments involving dogs and *L. infantum*, as well as some research into canine infections by *L. mexicana* (Cruz-Chan et al., 2014) and *L. donovani* (Keenan et al., 1984; Konno et al., 2022), have been published, the small number of animals in most experiments, the variable designs and follow-up tools, and the differing purposes (vaccination, chemotherapy and pathology) make it difficult to obtain definitive conclusions.

The manuscript provides an overview of canine experimental infections with visceral *Leishmania*, with a particular focus on *L. infantum*. It covers the purpose of the infections, housing conditions, regulatory aspects, ethical considerations, follow-up procedures, and outcomes. The aim is to standardize some experimental procedures and make the studies performed comparable.

2 Canine infections

Preclinical studies using different animal species to test the prophylactic or therapeutic value of a prospective new drug before progressing to clinical trials are a critical step in drug development. In the case of leishmaniasis, infections in dogs are not only the most advanced surrogate model for human medicines but also a target indication given the extension and severity of canine leishmaniasis. Preclinical studies in dogs are among the most relevant steps in the process of antileishmanial drug discovery and development. Therefore, there are issues involved in animal experimentation that must be addressed: *ethical aspects and legislation*, *experimental design*, and *requirements from regulatory agencies*. Given the importance of these items, we considered that they should be included in our contribution.

2.1 Regulatory framework and ethics of animal experimentation

Animal experimentation is a controversial issue. While it is considered critical in the development of new drugs by some authors (https://japan-forward.com/beyond-the-propaganda-animal-testing-helps-save-lives/) there is a growing movement against animal trials. Although there are strong differences among countries and societies (Uchikoshi and Kasai, 2019; Su et al., 2022) and considered that animal experimentation is still needed by the scarce alternatives (Kiani et al., 2022) the opposition to the use of experimental animals in the drug discovery process is growing not only in developed countries (Blakemore and Peatfield, 2004; Pound et al., 2004; Akhtar, 2015; Parvatam et al., 2020; Bose, 2025). Many scientific and ethical advances have been, and the scientific community should incorporate ethical and scientific considerations in animal testing and research (Ferdowsian and Beck, 2011) including modifications in methodology leading to a significant reduction of animal use (Richter, 2017; 2024). New available technologies such as organoids, organ-on-chip systems along with *in silico* tools can reduce -and eventually replace- the animal experimentation in many areas. Anti-experimentalists claim that the actual benefits of animal experiments are overstated (e.g., Pound et al., 2004) given the scarce predictive value of the results obtained in animals in their translation to humans. This viewpoint, based on results obtained in poorly designed experiments, inadequate experimental models and follow-up, does not reflect the situation with antimicrobials, in a wide sense, and especially in the case of leishmaniasis. Dog infections with *Leishmania* sp., particularly the most virulent species, *L. donovani* and *L. infantum*, are not only the most advanced surrogate model for VL in humans but also a specific pathology of dogs very common in some areas, such as the Mediterranean Basin and South America (Berriatua et al., 2021; Dantas-Torres, 2024; Vilas-Boas et al., 2024). Moreover, the same currently marketed drugs are effective in human and canine leishmaniasis this supporting the value of the testing of potential drugs in infected dogs.

The Principles of Humane Experimental Technique, published by W. M. S. Russell and R. L. Burch in 1959 established the ethical framework known as the "3Rs" principles: Replacement, Reduction, and Refinement (Balls et al., 2009). These principles, despite their limitations, have been adopted by developed countries although the translation to national and international legislation is variable. In the European Union (EU) the first Directive for the protection of animals in science was adopted in 1986 and replaced by Directive 2010/63/EU (https://eur-lex.europa.eu/eli/dir/2010/63/oj/eng). Afterwards, Regulation (EU)2019/1010 (https://eur-lex.europa.eu/eli/reg/2019/1010/oj/eng) introduced new levels of transparency to help progress towards replacing animal use in science. This legislation has been translated into national laws by state members, and it is compulsory. In the United States of America (USA), the Animal Welfare Act (AWA) (https://www.aphis.usda.gov/sites/default/files/ac_bluebook_awa_508_comp_version.pdf) regulates the use of most warm-blooded vertebrates, including dogs, in research, teaching and testing. This primary regulation is complemented with a range of laws governing the treatment of animals by different regulatory bodies (USDA, Public Health Service) (https://olaw.nih.gov/resources/tutorial/relevant.htm#2b). The Japanese Law Concerning the Protection and Control of Animals enacted in 1973 was amended in 1999 and given the new title of the Law for the Humane Treatment and Management of Animals. This law protects all species of animals from cruelty. The regulatory framework, like that from the US relies on the law, national regulations and self-regulation by scientific societies (Kagiyama and Nomura, 2004).

In December 2022, China implemented its first national standards document addressing laboratory animal welfare,

representing a major advancement in ethical research practices and international collaboration: https://std.samr.gov.cn/gb/search/gbDetailed?id=F159DFC2A79D47EFE05397BE0A0AF334. These regulations align with global best practices and establish guidelines for key aspects such as euthanasia, pain management, transportation, housing, breeding, personnel training, and ethical oversight. In addition to the original 3RS, some authors propose a fourth R: Responsibility, emphasizing the ethical and scientific accountability of researchers in designing and conducting animal experiments.

The final aim of a potentially useful molecule against leishmaniasis is to be used in clinical practice in humans, pets or both; therefore, preclinical studies are critical in the DDD process. However, evidence has shown that poor quality in the design and conduct of these studies has not only impeded clinical translation but also led to significant waste of valuable research resources (Huang et al., 2020). The bad design of the experiment not only directly affects the scientific value of the trial but also the efficient and humane use of animals and the failure of the experiment performed to fulfill the requirements posed the drug regulatory bodies to register the new therapeutic agent. Given the high cost (human, material) and the involved ethical dimensions of experimentation with animals, and particularly with dogs, scientists must be aware of the need to use an adequate design, including follow-up. Besides the ethical committees from the institutions there are several guides that should be consulted such as Norecopa (Smith, 2023; https://norecopa.no/) or the widely employed ARRIVE guidelines (Kilkenny et al., 2010; Percie du Sert et al., 2018; Percie du Sert et al., 2020a; Percie du Sert et al., 2020b).

The last step in the process will be the registration and eventual approval of the new drug by competent medicinal authorities mainly the larger ones such as EMA in Europe (https://www.ema.europa.eu/en/homepage); FDA in the USA (https://www.fda.gov/); PMDA from Japan (https://www.pmda.go.jp/english/); NMPA from China (https://english.nmpa.gov.cn/), and CDSCO from India (https://cdsco.gov.in/opencms/opencms/en/Home/). These agencies are, in fact, the institutional bodies to approve or reject the experimental work performed by scientists. Therefore, the advice of the regulatory agencies would improve the experimental design of the trials -this leading to an easier examination of the application for approval- and surely would help to refine the follow-up and perhaps to reduce the number of animals. On this ground we strongly support the close interaction between scientists going the experimental work and the professionals from the regulatory bodies.

2.2 The infective stage: promastigotes vs. amastigotes

Canine infections by *L. infantum* can be initiated with either promastigotes or amastigotes, although promastigotes are more easily cultured in a laboratory setting and are more similar to the parasitic stage inoculated by sandfly vectors, which is why they are widely used (Campino et al., 2000; Santos-Gomes et al., 2000; Moreno et al., 2007; Costa et al., 2013a; Martin et al., 2014; Gizzarelli et al., 2020). In general, inoculations based on promastigotes have been found to be less successful than those based on amastigotes (González et al., 1988; Carrera et al., 1996; Leandro et al., 2001; Maia et al., 2010). However, the efficacy of inoculation with both parasitic stages varies widely. While some experiments have shown 100% efficacy with promastigote inoculation, others have shown a null success rate with amastigote inoculation, depending on the inoculation route (0% with intradermal inoculation: Leandro et al., 2001) or the infection schedule (ca. 25%: Santos-Gomes et al., 2003).

The lower efficacy of promastigote inoculations is to be expected, since this multiplicative stage *in vitro* is not necessarily comparable to development in the vector's midgut. Their virulence decreases progressively after successive laboratory subcultures, and the metacyclic subpopulation—the only infective subpopulation—is not selected (PMID: 26768275; Martin et al., 2014), but rather late-log or stationary-phase promastigotes (e.g., Fernández-Cotrina et al., 2013; Martínez-Moreno et al., 1995; Alcolea et al., 2019). In some instances, experimental infections have been achieved through the natural intervention of sand flies feeding on dogs (e.g., Vlkova et al., 2011; Volfova, 2011; Aslan et al., 2016), or by using promastigotes supplemented with salivary gland extracts from the sand fly (Costa et al., 2013; Paranhos-Silva et al., 2003; Pinelli et al., 1994; Poot et al., 2005). In dogs, certain sand fly salivary proteins have been shown to act as immune chemo attractants, triggering an early innate response characterized by neutrophil recruitment at the bite site (Guimarães-Costa et al., 2021). These approaches, which more closely mimic the natural conditions of infection, nevertheless require the maintenance of a sand fly colony (Volf and Volfova, 2011; Vlkova et al., 2011) and a more labor-intensive methodology to deliver Sonicated Salivary Gland Homogenate (SGH) equivalent to five pairs of glands during infection. While this strategy may enhance immune responses in the context of vaccination studies (Abbehusen et al., 2018), the establishment of infection itself has proven to be no more efficient than that achieved with amastigotes or cultured promastigotes. For chemotherapeutic assays against established canine infections, it would therefore be preferable to use virulent promastigotes (stationary phase/metacyclic) or, better still, fresh amastigotes from a well-characterized *L. infantum* isolate.

To restore the virulence of *L. infantum* strains following prolonged expansion as promastigotes *in vitro*, infections are performed in mice and, more frequently, in hamsters. However, neither mice nor hamsters are natural hosts, so infectivity in these surrogate models does not necessarily imply recovery of virulence in natural hosts such as dogs and humans.

2.3 Infective dose and administration via

As there is no standardization of the infective dose administered, a wide variation is found in the experimental infections reported. Under natural conditions, dog infections are probably initiated by the inoculation of a few thousand metacyclic promastigotes by infected sandflies at most. With the exception of some experiments mimicking natural infections (e.g., Aslan et al., 2016: 20 infected sandflies per dog; Killick-Kendrick et al., 1994: 5,250–8,260 metacyclic promastigotes), the size of the inoculum administered with both promastigotes and amastigotes shows a wide range: 10^9 amastigotes/kg (Campino et al., 2000); 10^{10}–11 amastigotes/kg (Abranches et al., 1991); and much lower doses: from 10^8 (Olías-Molero et al., 2019) to 5.8×10^8 (Carrera et al.,

1996), or even 0.6×10^6 late-log phase promastigotes per dog (Fernández-Cotrina et al., 2013), with no substantial difference in outcome. Despite the heterogeneity of the follow-up methods used to assess the course of the infection, the virulence of the *Leishmania* isolate employed surely plays an important role, whereas the role of the other co-administered components (e.g., salivary glands of sandflies) is not essential (Costa D. J. et al., 2013; Paranhos-Silva et al., 2003). Overall, the infective dose does not seem to be a critical aspect of experimental inoculation of dogs with *L. infantum* for achieving an effective infection, although the course tends to be faster when a higher infective dose is administered. Thus, when infecting dogs with *L. infantum* to explore the chemotherapeutic value of a new drug, intravenous inoculation of freshly obtained amastigotes (10^8/animal) from a virulent isolate, or alternatively late-log (stationary phase) promastigotes (10^7/animal, ca. 10 kg), could be employed.

2.4 Dog breed, age and sex

Observational studies of dogs in endemic areas of canine leishmaniasis (CanL) have revealed variations in disease prevalence and severity in naturally infected dogs. Relative variations in breed resistance and/or resilience have been observed (Burnham et al., 2020; Edo et al., 2021), and a correlation between susceptibility to *L. infantum* and MHC class II polymorphism has been described (Quinnell et al., 2003b). Insofar, no conclusive evidence of breed-related increased susceptibility or resistance is available, and all breeds studied can become infected. Establishing a genetic basis for susceptibility or resistance is difficult at both the individual and breed levels. Several published reports do not specify the breed of dog used (Martin et al., 2014), while others have used mixed-breed animals (Abranches et al., 1991; Oliveira et al., 1993; Martínez-Moreno et al., 1995; Campino et al., 2000; Santos-Gomes et al., 2000; 2003; Moreno et al., 2007). Using mixed-breed animals could be beneficial when large groups are included in the experimental design. However, due to obvious logistical, ethical and economic constraints, the number of animals is generally low, so the use of genetically close dogs of manageable body size and behavior would be advantageous. The vast majority of experiments have been performed with beagles, a standard breed of dog in animal experimentation. The use of other breeds of dog has been anecdotal, such as German Shepherd Dogs (Santos-Gomes et al., 2003). No correlation has been found between infection outcome and dog breed in the published results. The use of experimental animals is regulated by common law across the European Union (Directive 2010/63/EU) and by national legal transpositions. Under such legislation, animals used must be specifically bred for experimentation, and beagles can be easily obtained from authorized breeders (see above).

While most researchers agree on the use of beagle dogs, the number, age and sex of the experimental animals used in different experiments varies widely. The number of animals used is an important issue, particularly when mixed-breed dogs are used, as more individual variations can be expected. Several published reports include 5–7 animals, sometimes including both male and female dogs, without matched uninfected control animals. These reports use different inoculation routes within each experiment and sometimes use different *Leishmania* species (e.g., Keenan et al., 1984, 3 *L. donovani* + 3 *L. chagasi*; Abranches et al., 1991, 7; Carrera et al., 1996, 5 (F + M); Santos-Gomes et al., 2000; Konno et al., 2022, 3). Experiments involving larger numbers of animals (e.g., >15) are abundant (Killick-Kendrick et al., 1994, 26; Paranhos-Silva et al., 2003, 16; Ramiro et al., 2003, 15; Poot et al., 2005, 21; Poot et al., 2006, 15; Moreno et al., 2007, 48; Costa D. J. et al., 2013, 35; Fernández-Cotrina et al., 2013, 25; Martin et al., 2014, 20; Hosein et al., 2015, 31; Olías-Molero et al., 2019; 2021a; Gizzarelli et al., 2020, 20; Alonso et al., 2023, 30) for different purposes (e.g., vaccination trials, biopathological studies and drug efficacy). In the published reports, the efficacy of inoculation using various assessment techniques (e.g., biopsies, nPCR, qPCR, cell culture and microscopy) is generally high (>80% as a rule, with most achieving 100% efficacy when all diagnostic techniques are combined). Variations are mainly due to the inoculation route and the infective stage employed.

In principle, experiments involving larger numbers of animals are of superior value. In general, these experiments had a more accurate follow-up and produced a full spectrum of conditions (e.g., the absence of specific antibodies despite a *Leishmania* burden in the lymph nodes, even after 7 months post-inoculation, or the absence of clinical signs in over 75% of inoculated animals) (Costa et al., 2013a; Gizzarelli et al., 2020). The efficacy also depended on the organ examined (Rodríguez-Cortés et al., 2007). Considering the published records, it would be recommended to use a well-characterized dog breed, not necessarily of the same sex and younger than 1 year old, regardless of the experimental purpose. There is no clear relationship between dog breed and susceptibility to *L. infantum*. Thus, beagle dogs produced by authorized breeders following national and international ethical regulations must be used, with emphasis placed on the importance of the 3Rs (and better 4Rs: Reduce, Refine, Replace and Responsibility). Well-designed experiments with careful inoculation procedures and adequate follow-up do not necessarily require large sample sizes.

2.5 Efficacy criteria

Studies investigating experimental leishmaniosis in dogs have employed various sample types and procedures to determine the animals' infection status, disease progression, and parasite load in target organs. Monitoring *Leishmania* infection in dogs requires a combination of molecular, serological and parasitological techniques that balance diagnostic accuracy with animal welfare. The most appropriate method depends on the study's purpose, required precision, and invasiveness of the procedure. Therefore, it is crucial to strike a balance between minimizing the impact on the animal model and obtaining reliable data to accurately evaluate the efficacy of vaccines or treatments for canine leishmaniosis.

2.6 Follow-up: clinico-pathological parameters

When studying the natural history of an infection or evaluating chemotherapeutic agents or vaccination procedures, it is necessary to reproduce the clinical course of a chronic disease such as canine

leishmaniosis (CanL). Manifestations include a variety of clinical signs, lesions, biopathological alterations, and immune responses, both specific and non-specific. Dog leishmaniosis can be considered an immunopathology, the outcome of which depends on an imbalance in the immune system of infected animals. Experimentally infected dogs with *L. infantum* exhibit a variety of clinical signs and lesions that become increasingly frequent and severe over time. Their appearance is assessed over a period ranging from several weeks (>4–10 weeks post inoculation (pi)) (Carrera et al., 1996; Olías-Molero et al., 2019) to months (Poot et al., 2005; Rodríguez-Cortés et al., 2007; Fernández-Cotrina et al., 2018), and this timing is related to the experimental design, including the inoculated parasite stage and inoculation via, the virulence of the used parasite isolate, and the sampling calendar. Lymphadenomegaly, which is frequent in CanL, is followed by splenomegaly and is sometimes observed very early on (2 months post inoculation).

Cutaneous lesions are also characteristic, particularly rapidly evolving exfoliative dermatitis, which may or may not be accompanied by alopecia, as well as ulcerative dermatitis. In addition, other clinical manifestations have been described, including cutaneous alterations (erythema, hyperkeratosis, papular, nodular, or pustular dermatitis, onychogryphosis) and systemic signs (hyperthermia, keratoconjunctivitis, blepharitis, uveitis, epistaxis, vascular disorders, pallor of mucous membranes, and muscular atrophy). Decreased appetite and consequent weight loss are also commonly observed. Not all these alterations are observed in all infected and diseased dogs in the different experiments, thus mimicking the course of the infection under natural conditions, and their severity depends on how long ago the infection occurred and the individual dog's resistance/resilience. There have been many attempts to quantify these clinical signs in the form of 'scoring systems', and various models have been proposed (e.g., Ciaramella et al., 1997; Koutinas et al., 1999; Paltrinieri et al., 2010; Foglia Manzillo et al., 2013; Manna et al., 2009; Roura et al., 2013; Silva et al., 2017; Olías-Molero et al., 2019; Galán-Relaño et al., 2022).

Clinical scoring is complemented by both haematological and biochemical parameters. Haematological evaluation includes red blood cell count, packed cell volume, haemoglobin and indices, white blood cell count, and platelet count. The most common finding is non-regenerative anaemia (Ciaramella et al., 1997; Meléndez-Lazo et al., 2018), as well as leukopenia associated with neutropenia, mild eosinopenia, and decreased lymphocytes. Thrombocytopenia frequently occurs in CanL and is related to different pathogenic mechanisms (Cortese et al., 2009; Foglia Manzillo et al., 2013), indicating a high parasite burden in the bone marrow.

These blood determinations are complemented by several clinical biochemistry parameters, primarily hepatic enzymes (ASL and ALT) and markers of renal function (urea and creatinine). Alterations in plasma proteins are an early marker of disease (Foglia Manzillo et al., 2013; Olías-Molero et al., 2019), characterized by increased total protein, hyperglobulinemia and hypoalbuminemia. Decreased plasma albumin is considered a poor prognosis indicator of CanL (Geisweid et al., 2012) as it is a negative acute phase protein (APP) whose synthesis is regulated by proinflammatory cytokines (Cerón et al., 2018). It also correlates to the clinical picture of infected animals. Elevated globulins, particularly the γ fraction, are indicative of polyclonal lymphocyte activation and are closely correlated with the clinical status of the animals, confirming their value in diagnosing and monitoring infection (Paradies et al., 2010). Elevation of the α2 fraction, to which most alpha-fetoprotein (AFP) migrate in the early stages of infection, has been identified as an early marker of infection (Paltrinieri et al., 2010). Due to hypoalbuminemia and hyperglobulinemia, the A/G ratio is inverted, which is strongly correlated with the severity of the disease, making this index one of the most sensitive markers of CanL.

Renal alterations are common in leishmaniosis, and chronic kidney disease (CKD) is a sign of poor prognosis and one of the main causes of death in CanL (Koutinas et al., 1999; Solano-Gallego et al., 2011; Costa D. J. et al., 2013). In advanced stages under natural conditions, CKD is characterized by azotemia (Meléndez-Lazo et al., 2018). In experimental infections, the duration of the studies is generally shorter, so these renal alterations are not to be expected. However, an early marker that is more specific than creatinine has been incorporated into the IRIS guideline on the diagnosis and monitoring of CKD: symmetric dimethylarginine (SDMA) (http://www.iris-kidney.com/pdf/2_IRIS_Staging_of_CKD_2023.pdf).

In chronic liver disease, an elevated AST/ALT ratio is predictive of long-term complications such as fibrosis and cirrhosis. In the case of leishmaniosis, a mild to moderate increase in transaminases has been observed from 10 weeks post-infection (pi) onwards (Olías-Molero et al., 2019), and this could be correlated with the clinical course of the disease in animals. Alteration of pancreatic enzymes (amylase and lipase) in infected animals reflects systemic involvement of the disease. The relevance of hyponatremia, which is associated with a poor prognosis in human patients (Daher et al., 2017), has not yet been evaluated in CanL.

Regardless of the scoring scales proposed by the different systems (see above), they are useful tools for diagnosing and monitoring the clinical evolution and staging of animals after therapy. However, the rationale behind using a final numerical value (clinico-pathological score), which is different for each scoring system and composed of entirely different alterations (e.g., dermal vs. visceral), is debatable. Moreover, the majority are based on clinical observations of naturally infected animals and therefore lack, in general, time-course validation of the alterations alongside the infection. Thus, while they can be considered useful for clinical management in practice, they are less valuable for experimental infections.

2.7 Monitoring parasite load in the experimental model of canine leishmaniosis

The most used methods for the parasitological follow-up of *L. infantum* infection include direct parasitological analysis (with or without culture of the biological sample obtained) and indirect molecular methods.

2.7.1 Cytology

Light microscopic examination (40x, 100x) of fine needle biopsies of lymph nodes (e.g., popliteal lymph nodes and other easily accessible lymph nodes), bone marrow aspirate smears, skin

biopsies or liver biopsies allow visualization of the parasite in these samples. Biological samples can be stained with a metachromatic stain, such as Giemsa or May–Grünwald Giemsa. This method is widely used in clinical practice for diagnosing *Leishmania* infection in dogs. One obvious advantage of this method is its 100% specificity. However, sensitivity may be affected by uneven parasite distribution in tissues and the observer's experience. Saridomichelakis et al. (2005) evaluated the efficacy of lymph node and bone marrow cytology in diagnosing canine leishmaniosis. Cytology of these samples was found to have 100% specificity. However, although the technique was highly specific, it had a lower ability to detect infection in asymptomatic dogs. Additionally, bone marrow samples can be obtained from dogs via sternal aspiration (Paparcone et al., 2013), although this requires sedation or anesthesia, which poses additional risks to the animal.

2.7.2 Parasite culture

Undoubtedly, parasitological culture of bone marrow, spleen, lymph node, skin and other tissue samples is the most reliable way to confirm the presence of viable parasites. However, limiting dilution assays are time-consuming and may have low sensitivity in chronic infections. In addition, depending on the site chosen to obtain the sample, they can also be invasive procedures. Costa et al. (2013b) investigated the experimental infection of dogs with *Leishmania* and phlebotomine sandfly saliva. They monitored disease progression through clinical, serological, and parasitological assessments, including parasite detection in bone marrow and lymph node aspirates. While these techniques provide comprehensive information on infection, aspirate collection can be painful and requires technical expertise. In another study of canine leishmaniasis, Cruz-Chan et al. (2014) developed a dog model of experimental infection with *L. mexicana*. They evaluated infection by observing clinical signs and performing parasitological cultures and molecular techniques, such as PCR, on skin and lymph node samples. Although cultures confirmed parasite viability, they were laborious and required specific laboratory conditions.

2.7.3 Quantitative PCR (qPCR)

In this case, samples are taken from the following tissues: peripheral blood, bone marrow, lymph nodes, the spleen, the liver, the skin and, more recently, non-invasive samples such as hair, urine, oral and vulvar swabs (Belinchón-Lorenzo et al., 2013; Hernández et al., 2015; Karakuş et al., 2015; Aschar et al., 2016; Cavalera et al., 2022). The advantages include high sensitivity and specificity. Konno et al. (2022) developed an experimental model of *L. donovani* infection in beagle dogs. During the eight-month observation period, liver biopsies were performed to assess the parasite load in the liver using real-time PCR and immunohistochemistry. Although these techniques were sensitive and allowed accurate quantification of the parasite, obtaining liver biopsies is an invasive procedure that poses risks to the animal. In another comprehensive study, Fernández-Cotrina et al. (2013) reproduced canine visceral leishmaniosis through experimental infection with *L. infantum*. They used PCR to detect parasite DNA in bone marrow, lymph node and spleen samples. While these techniques were highly sensitive and enabled the early detection of infection, they required invasive procedures. However, PCR analysis of hair is a reliable, non-invasive method of diagnosing canine leishmaniosis. Its sensitivity may vary depending on the degree of infection and the body region sampled. Suitable body regions for hair collection include the periocular and peribuccal areas, which have a higher parasite load in infected dogs, and the ears, which are a common site of *Leishmania* parasite colonization. The dorsal and lumbar regions are also used in some studies, but they have lower sensitivity. Belinchón-Lorenzo et al. (2013) detected *L. infantum* kinetoplast minicircle DNA in the hair of dogs with leishmaniosis using real-time PCR. Blood, lymph node and ear hair samples were analyzed from 28 dogs, which were divided into two groups: 13 infected dogs and 15 healthy dogs. Parasitic DNA was detected in all the lymph node samples from infected dogs. PCR sensitivity in ear hair was like that in blood, with nine out of 13 hair samples and eight out of 13 blood samples being positive. Furthermore, *L. infantum* DNA was found in hair samples taken from all analyzed body areas and hair sections, as well as in epidermal keratinocytes.

Regardless of the method used to monitor parasite load, adequate clinical monitoring of the following parameters is required: weight loss, skin lesions, splenomegaly and lymphadenopathy. Moreover, when samples are taken from different tissues, it is strongly recommended to use stereological techniques to avoid biases in the determination of the parasite burden (Warille et al., 2023).

2.8 Immune response

In leishmaniosis, clinical signs and lesions are a consequence of immune-mediated processes that occur because of the infection (Day, 2011). When dogs are infected with *L. infantum*, they mount an immune response that is not necessarily protective. This response includes two main effector mechanisms: a cellular response and the production of both specific and non-specific antibodies (Abs). Most studies of CanL have focused on the humoral response, not only because it is more accessible, but also because determining specific Ab levels is routinely used to diagnose and monitor animals with leishmaniosis. However, other studies have examined solid tissues (e.g., spleen, liver and lymph nodes), peripheral lymphocyte proliferation (specific or non-specific responses to SLA or ConA), cell types (e.g., $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$ and FoxP3+) and indexes (e.g., CD4+/CD8+) (Abranches et al., 1991; Costa et al., 2013a; Killick-Kendrick et al., 1994; Konno et al., 2022; Leandro et al., 2001; Martin et al., 2014; Martínez-Moreno et al., 1995; Moreno et al., 2007; Paranhos-Silva et al., 2003; Pinelli et al., 1994; Poot et al., 2005; 2006; Rodriguez-Cortés et al., 2007; Santos-Gomes et al., 2003; Ramiro et al., 2003; Ramos et al., 2008; Alcolea et al., 2019; Alonso et al., 2023) or cytokines, mainly IL10, IFN-γ and less frequently IL2, UIL6, IL12, IL17A, TLRs and others (Aslan et al., 2016; Campino et al., 2000; Costa D. J. et al., 2013; Cruz-Chan et al., 2014; Hosein et al., 2015; Konno et al., 2022; Martin et al., 2014; Pinelli et al., 1994; Poot et al., 2005; 2006; Ramiro et al., 2003; Ramos et al., 2008; Alcolea et al., 2019) have been performed. As correctly pointed out, the correlation between levels of γIFN and other cytokines and the clinical course in naturally or experimentally infected dogs with *L. infantum* is far from clear (Maia and Campino, 2018). Frequently, contradictory results are obtained, reflecting the individual variability observed even in genetically similar animals (e.g., beagles). Different patterns possibly relate to experimental designs and methodologies, among other factors. Thus, while it is

important to characterize the individual responses and tissue-specificities of the immune response in CanL, this information is probably less useful when evaluating the cell-based response in experimentally infected dogs (infection and post-treatment monitoring).

2.8.1 Peripheral antibody response

Contrary to that reported in the cytokine profile and timing in natural and experimental *L. infantum* infection in dogs, a constant finding in the course of canine leishmaniosis is that the infected animals develop a remarkable specific anti-*Leishmania* IgG response (Abranches et al., 1991; Martínez-Moreno et al., 1995; Carrera et al., 1996; Campino et al., 2000; Costa et al., 2013a; Fernández-Cotrina et al., 2013 Olías-Molero et al., 2019) although timing of onset is variable and can be delayed (Rodríguez-Cortés et al., 2007; Fernández-Cotrina et al., 2018) probably related to the experimental design (e.g., infective dose, infective stage, route of inoculation), and also the sensitivity and specificity of the immunological techniques employed. Most used techniques are IFAT, different types of ELISA, immunochromatographic techniques (e.g., lateral flow self-contained devices), Western blot and other techniques. IFAT is still considered the "gold standard" for the diagnosis of CanL in clinical practice. Its sensitivity and specificity have been studied, in comparison with ELISA, for diagnostic and follow-up purposes (Mancianti et al., 1995; Martínez-Moreno et al., 1995; Maia et al., 2010) and it is widely accepted despite being laborious and requiring qualified personnel and expensive equipment. IFAT titration is frequently used in veterinary clinical practice to monitor the clinical evolution of dogs infected with *L. infantum*, including their response after chemotherapy, but in longitudinal studies no clear correlation between IFAT titers and clinical status of the animals has been found (Olías-Molero et al., 2019). For their part, different ELISA formats have been employed - including recombinant antigens (i.e., rK39, rK28) (Maia et al., 2010), synthetic peptides, soluble *Leishmania* extracts (ELISAsla)- both in cross-sectional and longitudinal studies. In general, ELISA presents higher sensitivity than IFAT although in more advanced stages of experimental infection (>10 weeks pi) there is concordance between IFAT and ELISA results. In-house ELISA with fixed promastigotes (pELISA) allows an earlier diagnosis than those reported with slaELISA and with the rK39 immunochromatographic test in experimentally infected dogs (90–120 days pi) (Poot et al., 2005; Maia et al., 2010). WB is a highly sensitive technique proposed for diagnosis and follow-up in CanL (Gottstein et al., 1988; Rachamim et al., 1991; Aisa et al., 1998; Fernández-Pérez et al., 1999). There is no global consensus on the pattern of antigenic recognition in dogs with leishmaniosis. A relatively small number of recognized Ags is the rule in experimental infections (Abranches et al., 1991; Carrera et al., 1996; Moreno et al., 1999) and the wide variety found in naturally infected dogs (Fernández-Pérez et al., 1999; Vargas-Duarte et al., 2009) may be related to both the variety of clinical presentations in naturally infected dogs and to the presence of co-infections by other pathogens with which they share antigens (e.g., *Ehrlichia*, *Trypanosoma*, *Toxoplasma*, *Neospora*, *Babesia*) (Zanette et al., 2014). These aspects must be considered when efficacy trials are performed with naturally infected animals, in endemic regions.

Three of the immunodominant Ags, heat shock proteins (HSP83, HSP70 and chaperonin HSP60) (Requena et al., 2015), are characteristic immunogens of *Leishmania* infections, and the simultaneous reactivity of HSP83 and HSP70 is considered a marker of LV (Kaur and Kaur, 2013), including CanL, although HSP70 apparently cross-reacts with *T. cruzi* (Angel et al., 1996). The marker character of chaperonin HSP60 is less well known, although it has been described to react with sera from dogs with subclinical natural infection (Agallou et al., 2016). Combination of chimeric proteins (PQ10, PQ20) did not yield satisfactory results in terms of precocity but apparently is more sensitive than conventional PCR (Faria et al., 2015; 2017). The combination of early markers (HSP83 + HSP70 +HSP60 + 32 kDa +30 kDa) in a dot-ELISA format has been suggested (Olías-Molero et al., 2019) given the current availability of *Leishmania* HSP, their early recognition and the correlation between reactivity and the clinical status of the dogs.

2.8.2 IgG subclasses in CanL: dynamics and significance. *Caveat*

The polarization of the immune response found in *Leishmania* infections in murine models (Th1 vs. Th2), and its relationship with Abs subclasses (IgG_{2a} vs. IgG_1) and disease evolution (resistance vs. susceptibility) have fostered interest in examining the role of IgG subclasses in CanL. However, no clear pattern about their relative importance has been found neither in studies performed with experimental canine infections nor in samplings of naturally infected dogs: in some cases the presence of clinical signs and lesions has been associated with elevated levels of anti-*L. infantum* IgG_1 (e.g., Lima et al., 2017; Iniesta et al., 2007); the opposite has also been described, with high levels of specific IgG_2 (e.g., Reis et al., 2006; Reis et al., 2009; Teixeira Neto et al., 2010). Reasons that may explain this heterogeneity include the absence of quantitative determinations of IgG and total IgG subclasses, the variability among experimental designs, differential immunogenicity and virulence of *Leishmania* infecting inoculum, age of the dogs, and the methodologies used, among others. It is evident that these factors play a relevant role. Moreover, there is scarce information about the actual role played by IgG subclasses in dogs (Mazza et al., 1994) and their relationship to that found in surrogate rodent species: mice (e.g., Heinzel et al., 1989) or hamsters (Kushawaha et al., 2012; Jiménez-Antón et al., 2019). The second concern is linked to the lack of specificity of immunological determinations which, in turn, are dependent on the specificity of the reagents. Marketed anti-IgG_1 and anti-IgG_2 are not specific to dog IgG subclasses (Day, 2007; Marcondes et al., 2011) and it is very likely that variable and inconsistent results obtained with them (e.g., Solano-Gallego et al., 2001; Lima et al., 2017; Reis et al., 2008) could be biased and need to be reconsidered. In fact, in the few cases that subclasses have been determined with specific MAb, *L. infantum* infection elicits an elevation of all canine IgG subclasses (Quinnell et al., 2003a; Strauss-Ayali et al., 2007; Marcondes et al., 2011; Olías-Molero et al., 2020) although the predominant subclass, IgG_1, shows an earlier elevation.

2.9 Post-mortem assessment of *Leishmania* burden

CanL is a systemic disease and, therefore, *L. infantum*-infected cells can be found virtually in every organ and tissue of infected

animals. Moreover, although less frequently in short-term experimental infections, general lesions are to be expected (e.g., emaciation, pallor of mucosa) along specific ones in the most parasitized organs (spleen, liver, lymph nodes and bone marrow). Gross pathology main lesions observed include mild hepatomegaly, lymph node enlargement and variable splenomegaly. Gross pathology could be complemented with histology and histochemistry of liver, spleen and kidneys (Nieto et al., 1992; Abbehusen et al., 2017; Alves et al., 2019).

In experimental infections with *L. infantum* a variety of techniques have been used to estimate the leishmanial burden in target organs. Different formats of PCR (nested-PCR, RT-PCR, q-PCR) have been employed in infected dogs (Campino et al., 2000; Leandro et al., 2001; Paranhos-Silva et al., 2003; Moreno et al., 2007; Rodríguez-Cortés et al., 2007; Ramos et al., 2008; Maia et al., 2010; Aslan et al., 2016; Fernández-Cotrina et al., 2013; Hosein et al., 2015; Alcolea et al., 2019; Gizzarelli et al., 2020; Alonso et al., 2023) and the actual determination of viable parasites by back-transformation of amastigotes and culture (Keenan et al., 1984; Killick-Kendrick et al., 1994; Martínez-Moreno et al., 1995; Campino et al., 2000; Santos-Gomes et al., 2000; 2003; Leandro et al., 2001; Ramiro et al., 2003; Poot et al., 2005; 2006; Moreno et al., 2007; Maia et al., 2010; Fernández-Cotrina et al., 2013; Costa D. J. et al., 2013). It should be recommended to perform quantification of the parasite burden by using limiting dilution assays (Buffet et al., 1995) with the samples taken from experimental animals. PCR is more sensitive than culture, but the latter gives a picture of the viable parasites, and the combination of both types of determinations could be an advantage. In a few cases (e.g., Costa et al., 2013) infectivity of the experimental dogs has been assessed by xenodiagnosis (feeding sandflies on infected dogs) but facilities to maintain phlebotomine colonies are out of reach for most laboratories.

2.10 Dosing of antileishmanial drugs in canine infections

The final aim of the discovery of new drugs is the use in the treatment of the infection in target species. In the case of leishmaniosis, dogs are not only the best surrogate animal species to test potential drugs against human VL but also the natural host of several *Leishmania* species, particularly *L. infantum* (=*L.chagasi*). Prevalence of zoonotic VL in dogs (CanL) in endemic areas, severity and bad prognosis of canine infections along the epidemiological role of the species and the generalized use of most antileishmanial drugs intended for humans to treat the dog infections, give to the test in dogs a prominent position.

Naturally infected dogs are considered the main reservoir for *L. infantum* (Moreno and Alvar, 2002). This role is also found in areas where several other human *Leishmania* sp affect dogs (Dantas-Torres, 2024). Thus, dog culling has been a suggested practice to reduce the prevalence of the human disease in some endemic regions such as South America (Nunes et al., 2010; Sevá et al., 2016; Bermudi et al., 2020; Giunchetti et al., 2023). However, its real impact is controversial (Vieira and Coehlo, 1998; Costa, 2011; Costa D. N. C. C. et al., 2013; Ribeiro et al., 2018; Sousa-Paula et al., 2019) and ethical considerations make this measure currently unacceptable. By large, the main control system of CanL in endemic areas is chemotherapy, accompanied with insect repellents.

Over 20 drugs have been used in leishmaniasis (Olías-Molero et al., 2021a; 2021b) this suggesting that the available chemotherapy is inadequate. Antileishmanial therapeutics relies on a reduced chemical arsenal, old and of limited efficacy, and eliciting significant side effects (*vide infra*). Exploration and testing in the adequate surrogate models of new chemical entities and combinations against leishmanial infections is an urgent need (Cordeiro da Silva et al., 2024). To make matters worse there is no universal availability of the antileishmanial chemotherapeutical agents. In the European Union (EU), antimonial compounds -particularly in the form of meglumine antimoniate (MeSb)-, miltefosine (Mil), and allopurinol (AL) are approved for veterinary use; treatment of dogs with amphotericin B (AmB) is not indicated and the drug is restricted for human use. Thus, despite their limited efficacy and side effects elicited most antileishmanial drugs are available to treat CanL. Contrary to this situation, in the Americas there is not a common regulatory framework and the veterinary practitioners from different countries face distinct challenges.

There are currently no Food and Drug Administration (FDA)–approved drugs for the treatment of leishmaniasis in any non-human species- in the US. Mil (Impavido, for human use) and Allopurinol can be used off-label, and MeSb must be obtained according to FDA requirements for importing veterinary drugs (Petersen and Barr, 2009; Levitan and Finnegan, 2023). In Brazil, probably the country with the highest number of dogs infected with zoonotic VL, the only medication regulated by the Ministry of Agriculture, Livestock and Supply (MAPA) for the treatment of the disease in animals is Mil (Krolow et al., 2022). This drug was authorized in Brazil in 2017, more than a decade after its introduction in Europe (Ribeiro et al., 2018). However, Mil has lower efficacy than MeSb and the latter is not authorized. Possibly, international efforts in South America on antileishmanial drugs regulate their use are needed to harmonize regulations and recommendations.

First-in-dogs (FID) assays, for veterinary purposes, or to be used afterwards in humans (First-in-humans, FIH), benefit from the research carried out previously *in vitro* and *in vivo* in rodent models. However, appropriate translation from models to target species is not a straightforward process and unexpected side effects can be present. No specific information is available on the dosage during development of antileishmanial agents. Therefore, the general approach followed in the development of other drugs must be followed.

Dosage translation based exclusively on the body weight of the species to be treated is not appropriate and the body surface area (BSA) was proposed as a normalization standard (Reagan-Shaw et al., 2008) and this allometric method was recommended by the Food and Drug Administration (FDA). However, this method is considered inadequate, and several other parameters must be considered, including physiological, pharmacokinetic and toxicological data (Blanchard and Smoliga, 2015). Dosing includes not only amounts of drugs but also time schedule of administration. In this line several approaches for the allometric scaling based on the dose-to-body surface area have been examined besides other approaches (e.g., pharmacokinetically guided, minimal

anticipated biological level, pharmacokinetic-pharmacodynamic modeling, similar drug approach and others) (Nair et al., 2018).

Pharmacokinetics depends on both characteristics of active molecules related to absorption, distribution, metabolization and elimination but also particularities of the animal to be treated. Some practical approaches have been published and could be used in the dose scaling (Nair and Jacob, 2016) with very useful information on the interspecies dose adaptation that we strongly recommend consulting for the first trial in dogs. Briefly, doses administered to dogs should be ca. ¼ of the dose administered to hamster and 1/6.7 of the dose administered to mice (Jacob et al., 2022).

Efficacy of the potential antileishmanial molecule must be assessed in naturally or, better, experimentally infected dogs (*vide supra*) using the appropriate experimental design, including infected + untreated animals, uninfected and untreated, besides the animal group medicated with the investigational drug (dose, schedule), and the animal group treated with any of the available drugs against canine leishmaniasis.

There is a growing societal concern, mainly in wealthy regions, for the use of experimental animals. As a result, national and international institutions have tightened the regulations and the number of animals used has been greatly reduced in the EU (10% decrease between 2018 and 2022) (https://www.eara.eu/post/2022-eu-figures-on-the-number-of-animals-used-in-research-are-welcomed-by-the-biomedical-community), UK (https://www.understandinganimalresearch.org.uk/news/eu-wide-animal-research-statistics-2022; https://www.gov.uk/government/news/animal-testing-to-be-phased-out-faster-as-uk-unveils-roadmap-for-alternative-methods.2025), and a strategic road map has been launched by FDA to reduce the animals employed and their replacement by validated alternative methodologies (e.g., such as organ-on-a-chip systems, computational modeling) (https://www.fda.gov/media/186092/download?attachment). This objective should be welcome as well as the development of scientific approaches such as the extensive use of historical controls reducing the current size of control groups (Kramer and Font, 2017), systematic heterogenization (Richter, 2017) or the design of mini experiments combined with Bayesian updating (Richter, 2024). Scientific community has been eager to adopt the inter nationally recognized principles (3R + Responsibility) but there still is necessity of animals for research and regulatory purposes. As recently pointed out by the National Association for Biomedical Research "As scientific knowledge evolves, so too should scientific practices—but decisions that impact public health must remain grounded in science, not politics "(https://www.nabr.org/about-nabr/news/nabr-statement-fda-animal-testing.2025).

In the case of VL, the use of experimental infections in dogs is entirely justified not only on regulatory grounds when developing drugs for human leishmaniasis but also because dogs besides their role as the main reservoir for humans are also a natural host for *L. infantum*. It can be argued by Ethical Committees that there is an extensive knowledge on the effect of *L. infantum* infection in dogs and, therefore, the inclusion of infected and untreated animals would not be justified in the testing of new antileishmanial drugs. However, as previously presented, experimental designs of published essays are highly variable, making comparisons hardly valid. Contrary to experiments not involving infectious agents, the virulence of *Leishmania* is strongly related to the isolate/strain employed and an infected and untreated control must be included to guarantee the validity of the experiment.

Therapeutical arsenal is reduced and only a few drugs are used: allopurinol (AL), miltefosine (Mil), meglumine antimoniate (MeSb) and amphotericin B (AmB). AL and Mil are orally administered and they have fewer adverse effects than the parenteral drugs MeSb and AmB. Parenteral drugs are considered as most effective in acute stage of leishmaniosis although not appropriate for weak animals. Combinations of these drugs is the usual practice to increase efficacy and decrease toxicity (Bastos et al., 2022). AmB is available in different parenteral commercial forms. The cheapest one is the deoxycholate form which is the most toxic one and usually administered once a day at the dose of 1 mg/mg for 15 or 21 days with a cure rate around 80% (Ibiapina et al., 2022). Liposomal formulations such as AmBisome® are more expensive AmB forms but with higher cure rate and less toxicity than the conventional deoxycholate form. However, it is strictly recommended to restrict AmB for humans to minimize cross resistance between humans and canine isolates (Olías-Molero et al., 2021b).

The recommended dose is 100 mg of meglumine antimoniate per kilogram of live weight (lw) per day (Medkour et al., 2020). If it is possible to administer several injections per day, it is recommended to administer the daily dose divided into two injections of 50 mg of meglumine antimoniate/kg lw separated by an interval of 12 h. The duration of the initial treatment is 3 weeks. This can be extended for 1 more week (https://cimavet.aemps.es/cimavet/pdfs/es/ft/191+ESP/FT_191+ESP.pdf). MeSb has therapeutic success in approximately between 65% and 100% of cases and it is usually combined with AL. One example is the combination of MeSb at 100 mg/kg SC, once daily, for 28 days and AL between 20 or 30 mg/kg, per os, daily, for 6 months. This combination results in 42.1% cured animals (Kasabalis et al., 2020). AL treatment requires around 20 mg/kg, usually divided in two oral daily doses of 10 mg/kg for long time treatment, sometimes for life (Nascimento et al., 2019). AL administration improves clinical signs although combination with another drug is required to improve efficacy. Mil is usually administered at the dose of 2 mg/kg for 28 days. The efficacy is around 90% after several months of treatment (Gizzarelli et al., 2023) and can be improved by combination with AL. Mil + AL combination is usually considered as convenient and very popular due to the easy oral administration of Mil. Dosing may be 2 mg/kg Mil for 28 days and 10 mg/kg AL, BID, for a period between 2 and 12 months. Although it is not strictly necessary to use -as positive therapeutic control- a drug of similar administration via to the one used for the investigational molecule is an advantage. In standard clinical practice, treatment of a disease includes medication against the etiological agent and supportive therapy to recover the physiological normality of the diseased animal. This possibility should be considered when testing a potential new drug. Even using the same supportive therapy for all treated animals, results could be biased by the different mechanisms of action involved depending on the drug employed. This aspect, including the ethical considerations involved, should be considered.

Current chemotherapy for leishmaniasis is inadequate in terms of efficacy, side effects elicited and affordability. Moreover, emergence of resistance to antimonials and Mil has been reported. Under such circumstances, there is an urgent need for new drugs against leishmaniasis (Alunda, 2023). The crisis involving the "translation" of basic scientific findings into human applications and potential treatments is recognized both in academia and industry in general (Seyhan, 2019) and in the case of

antileishmanial drugs (Olías-Molero et al., 2021b). Lack of experimental models has been considered among the causes of the high attrition rate in drug discovery. Information on the experimental canine infection with *L. infantum* for drug discovery and development is highly variable and the establishment of a standard procedure is not an easy task. We present, based on our experience, some key points on the canine experimental model with the aim of reducing the number of dogs used without compromising the validity of the results obtained (Box 1).

BOX 1 Testing new drugs and/or combinations in dogs against experimental infections with *Leishmania infantum*. TIPS.

Regulatory framework

- ✓ Check for advice from national Regulatory bodies. Legislation can vary depending on the country where the experiment is performed. Early advice can help to refine the experimental design, reducing animals and increasing the value of the experiment even for registration purposes.
- ✓ Availability of animal facilities with adequate containment measures

Ethical issues

- ✓ Strict compliance with the international and national ethical regulations for animal experimentation, in particular dogs.
- ✓ Strong commitment to apply the 3Rs + Responsibility by using a well-designed experiment providing the maximum information output with the minimum number or animals and duration of the experiment avoiding unnecessary suffering of the animals.
- ✓ Animal facilities for long-term stays of animals including adequate kennels, walking and socialization area under supervision, and enriched environment.

Parasites, Hosts and Experimental design

- ✓ *Leishmania infantum* strain: When possible, a well characterized fresh isolate from dogs. Ideally, they should be used without subpassages to avoid selection of lines/clones adapted to culture media. This is restricted to endemic areas where fresh isolates can be obtained. Preserved isolates and sub passaged strains in surrogate hosts (mice, hamsters) cannot necessarily keep the same virulence of the wild strains.
- ✓ Dog breed: No clear correlation has been found between infection and outcome among dog breeds. To minimize individual variation and to comply with regulations on animal experimentation the use of animals specifically bred for experimentation, from a well characterized breed (such as Beagle) is preferred.
- ✓ It is recommended to purchase the dogs from well recognized breeders and the animals with complete documentation and vaccination and antiparasitic treatments administered before reaching the facilities.
- ✓ Dog age: Animals around 1 year (young adult) are convenient. In most endemic areas natural infections start at this age.
- ✓ Dog sex: Females are preferred to avoid aggressive behavior more common in males.
- ✓ Experimental design: Use the most adequate design, considering advice from national and international agencies, the purpose of the experiment -including the comparison with the standard chemo therapeutic approach, minimum duration of the experiment and parasite dose and burden. Canonical experiments, with all control groups included, are preferred given the variability of *L.infantum*virulence and the individual response of inoculated animals.
- ✓ Dog number: Use the minimum required number to get significant results. Genetic similarity of pure-bred dogs helps to reduce the number of animals required.
- ✓ Complete record of all events and procedures during the experimental infection (e.g. number of animals inoculated vs animals infected). This record must be disclosed if required.

(Continued in next column)

BOX 1 (*Continued*)

- ✓ *L. infantum* infective stage: Infective promastigotes (metacyclic, or late-log promastigotes) and, better, freshly obtained amastigotes from a naturally infected animal.
- ✓ *L. infantum* infective dose: Ranging from 10^7 infective promastigotes/10 kg – 10^8 amastigotes/inoculated dog. A Beagle female of 1 year age weights from 9-10 kg.
- ✓ *L. infantum* inoculation route: IV via, administered in sterile physiological saline, is preferred. Differences have been found using other via (SC, IM) and with adjuvants (e.g. sandfly saliva) but for the purpose of getting a reproducible infection IV is adequate. Volume should be reduced as much as possible (could be administered in less than 2 mL/animal)
- ✓ Investigational molecule: Use single-batch synthetic or natural product and use the appropriate administration route (IV, oral) based on pharmacological and toxicological results from surrogate rodent models and pilot assay in dog.
- ✓ Reference drug: Use, as a rule, a marketed drug with the approved treatment schedule (dosage, route). Route of administration should be the same as the one used for the investigational molecule(s).

Follow-up and efficacy assessment

- ✓ Clinical follow-up is a must to determine clinical signs and lesions. Therefore, involvement of trained veterinarians is critical. Lesional scoring is of help to follow-up the infection and the possible recovery after treatment. Numerical scores can be used if justified.
- ✓ Complete record of all events and procedures along the experimental infection and treatment (e.g. supportive therapy administered). This record must be disclosed if required.
- ✓ Biochemical and hematological markers are abundant (liver functionality markers, hematological parameters -particularly non-regenerative anemia-)
- ✓ Immunological follow-up. Antibodies (Ab) (specific IgG response determined by ELISA or IFAT) and cytokines are useful.*Caveat*: Check actual specificity of marketed Abs against IgG subclasses from dogs
- ✓ Avoid euthanasia if possible: Parasite burden can be determined in vivo by using invasive (lymph nodes, biopsies-exeresis) or non-invasive procedures (e.g. hair); direct (parasite observation and semi quantitation) or indirect (e.g. PCR-based methods).

Author contributions

AO-M: Data curation, Writing – review and editing, Investigation, Methodology, Writing – original draft. MC-C: Writing – original draft, Investigation, Data curation, Writing – review and editing. MJ-A: Data curation, Writing – review and editing, Writing – original draft, Investigation. MM-B: Writing – original draft, Investigation, Writing – review and editing, Data curation. JC: Writing – review and editing, Funding acquisition, Writing – original draft. JJT: Writing – original draft, Writing – review and editing, Methodology. JA: Writing – review and editing, Supervision, Writing – original draft.

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The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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