Review

The Immune Response in Canine and Human Leishmaniasis and How This Influences the Diagnosis- a Review and Assessment of Recent Research

Larisa Maria Ivanescu , Bianca Lavinia Andronic * , Smaranda Grigore Hristodorescu , Gabriela Martinescu * , Raluca Mindru , Liviu Miron

Posted Date: 11 May 2023

doi: 10.20944/preprints202305.0830.v1

Keywords: leishmaniasis; immunological status; problem of diagnosis

Review

The Immune Response in Canine and Human Leishmaniasis and How this Influences the Diagnosis—A Review and Assessment of Recent Research

Larisa Ivanescu [1], Bianca Lavinia Andronic [1,*], Smaranda Grigore-Hristodorescu [2], Gabriela Martinescu [1,*], Raluca Mîndru [1] and Liviu Miron [1]

[1] Ion Ionescu de la Brad" University of Life Science, 8 Mihail Sadoveanu Alley, 700490, Iași, Romania; livmiron@yahoo.com, larisssa81@yahoo.com, raluca@mindru.com

[2] Grigore T. Popa" University of Medicine and Pharmacy, 16 Universității Street, 700115, Iași, Romania; marketing@praxismedica.ro

* Correspondence: biancalavinia7@gmail.com (B.L.A.); martinescugabi11@yahoo.co.uk (G.M.)

Abstract: Laboratory diagnosis of leishmaniasis remains a major challenge, demonstrating variable efficacy in detecting infected mammalian hosts. Thus, there is a great need for the accuracy of the identification of appropriate antigens to also improve diagnostic tests. This review brings to the fore the sensitivity of the balance in canine and human leishmaniasis, which greatly influences the progression of the disease and especially the diagnostic methods. The problem of diagnosis arises in asymptomatic leishmaniasis, since in these conditions the methods considered as reference in the diagnosis of leishmaniasis no longer show certainty, being influenced for the most part by the immune response of the host, which is different according to the presence of other associated diseases or even according to the breed, when we talk about dogs. Consequently, the diagnosis and surveillance of leishmaniasis cases remains an open topic, with the need for new methods adapted to the immunological status of the host.

Keywords: leishmaniasis ; immunological status ; problem of diagnosis.

1. Introduction

Leishmaniasis is a vector-borne, parasitic disease produced by more than 20 species of protozoa belonging to the genus *Leishmania*. The vectors are represented by approximately 90 species of sandflies. The parasite is either identified as a promastigote, which develops in the vector or as an amastigote which develops in the host targeting the reticulo-endothelial system in a multitude of tissues, mainly occuring in the bone marrow, the lymph nodes, the spleen and the liver [1,2].

Leishmaniasis can have three forms: 1. visceral leishmaniasis, reffered also as kala-azar, with approximately 50,000 to 90,000 new cases of leishmaniasis occurring annually globally, but unfortunately only 25-45% are reported to the WHO [3–5]; 2. cutaneous leishmaniasis (CL), considered to be the most frecquent form (approximately 95% of cases taking place in the Americas, the Middle East, the Mediterranean basin and Central Asia), with an annual estimate from 600,000 to 1 million new cases throughout, which are unfortunately underreported, as only approximately 200,000 are reported to the WHO [5,6]; 3. mucocutaneous leishmaniasis with over 90% of cases reported in Bolivia (plurinational state), Ethiopia, Brazil and Peru. Manifestations of the disease are different depending on the species of parasite and on the state of the host's immune system, ranging from fatal to self-limiting [7]. Immune control can be achieved through the activation of macrophages and an intact T-helper cell type 1 response (Th1); which underlines the major risk from visceral leishmaniasis, in case of immunosuppression [8].

 doi:10.20944/preprints202305.0830.v1

The dog represents the main domestic reservoir for *Leishmania infantum* mainly in the Americas [9,10]. Bihar State in India comprises over 90% of visceral leishmaniasis cases, making it the fourth most common tropical disease in morbidity and second only to malaria in mortality [11]. In the Mediterranean basin, China, the Middle East and South America the zoonotic form appears, produced by *L. infantum*, with the main reservoir being the dog. In East Africa, Nepal, Bangladesh and India, the anthroponotic form, produced by *L. donovani*, is predominantly found [2]. A study carried out in Brazil, using the CR technique, demonstrated a prevalence of asymptomatic leishmaniasis infection of up to 80% in the analysed dogs [12]. The processes of asymptomatic infection and the body's immunological reactions are not fully understood, which is why diagnostic methods must be adapted for each individual patient [13,14].

Domestic and wild canids are considered the main parasite reservoir, maintaining a continuous cycle of transmission of *L. chagasi* in the New World and of *L. infantum* in the Old World. In Europe, a particular interest (from the public health point of view and from the veterinary pharmaceutical field) is shown for canine visceral leishmaniasis (CVL), a serious disease manifested through a chronic evolution of viscerocutaneous signs. It is considered that no less than 2.5 million dogs manifest this form of disease, developing severe forms with a high degree of death [15]. Both symptomatic and asymptomatic dogs can be regarded as a natural reservoir of *L. infantum* for both dogs and humans [16], being a real danger in the endemic regions of Latin America and the Mediterranean [17].

2. Diagnosis in Humans Leishmaniasis

Firstly, after the sandfly bite, a small erythema appears, followed by an inflammatory reaction caused by the parasite, which can develop into an open ulcer or infiltrate in organs like liver or spleen [18]. Great importance must be given to the early diagnosis of leishmaniasis, since this can prevent both the evolution of severe clinical signs and mortality in humans with visceral leishmaniasis.

The diagnosis of leishmaniasis is challenging, based on history, clinical evaluation and laboratory data [19,20]. The parasitological diagnosis of leishmaniasis represents the gold standard, and it consists in the detection of parasites in tissue fragments or aspirated from an organ [21,22], or direct histology/microscopy in the case of cutaneous leishmaniasis [11,23]. However, this method is less and less used, as sample collection is invasive and requires skilled personnel trained in both protozoan identification and collection technique [24–26]. Thus, the lack of diagnostic certainty greatly limits disease control and contributes to the maintenance of a reservoir in nature. Immunological methods are most commonly used, although sensitivity and/or specificity have presented variability, as they are not invasive and do not require qualified personnel or special equipment [27–30]. Recently, leishmaniasis diagnosis has been based on the evaluation of specific proteins [31,32]. In symptomatic visceral leishmaniasis, there is an increased antibody response, both in dogs and in humans, and recombinant antigen-based assays can thus be used [33–35]. The challenge is asymptomatic leishmaniasis, in which we face a low serology against the protozoan, the tests showing a reduced sensitivity, as well as a cross-reactivity with other pathologies such as tuberculosis, Chagas disease, or malaria, which reduces the tests' specificity [36,37]. Recently, the rLiHyQ protein was identified in the antigens of the protozoan leishmania; this was cloned and used in ELISA experiments for determining the diagnosis in both human and canine leishmaniasis, showing sensitivity and specificity even in HIV-coinfected patients [19]. A study on an rK39 protein identified in a strain of *L. infantum* from Iran reported a sensitivity of 100% in humans and 97.6% in dogs in the ELISA test, as well as very good results in cross-assays, suggesting that it may represent a reliable diagnosis of canine and human visceral leishmaniasis in Iran [38]. The host's immune status presents great importance in leishmaniasis diagnosis, for this reason the protocol differs in HIV patients. In 1985, the first report of leishmaniasis associated with HIV infection appeared, with the number subsequently increasing in southern Europe and 35 other countries reporting cases of co-infection. Their number decreased after antiretroviral therapy was introduced [39].

 doi:10.20944/preprints202305.0830.v1

2.1. Parasitological Methods of Diagnosis

This technique still stands as the gold standard in leishmaniasis diagnosis, showing different sensitivity depending on the organ addressed (Table 1). In HIV-infected patients, microscopy of splenic aspirate shows the highest sensitivity; the one from the bone marrow presents a sensitivity of 81%, but with the specification that many cases came out positive after repeating the examination of the bone marrow preparation [40]. Culture has the advantage that it can increase the sensitivity more and also allows species identification, but has the disadvantage that it can take several weeks, that is why microculture is recommended [41]. Although it is a very specific technique, the sensitivity given by the microscopic detection of parasites is influenced by the smear quality, the experience of the specialist and the reagents used.

Table 1. The sensitivity and specificity rates acquired through the parasitological examination.

Direct parasitological examination (microscopy/culture)	Sensitivity(%)	Specificity(%)	References
Aspirated from the spleen (more common in East Africa and Indian subcontinent)	93%-99%; 81% in HIV patients;	>95% in patients with HIV coinfection;	[23,26,39]
Bone marrow aspiration/biopsy (more common in Europe, Brazil, and the United States)	53%–86%	67-94% in patients with HIV coinfection and up to 100% in other cases;	[1,8,22]
Lymph node aspiration	53%–65%	>95%	[21,42,43]
Peripheral blood from mononuclear cells (PBMCs)	52 %	91%	[44–46]

2.2. Serological Testing

For the detection of anti-leishmanial antibodies, serological tests include immunosorbent assay (ELISA), a direct agglutination test (DAT), a lateral flow immunochromatographic test (ICT) which uses the recombinant antigen rK39 and an indirect enzyme-linked immunofluorescence test [47,48].

In serological testing, the immunological status of the patients should be considered and in the immunocompetent patients, indirect tests with fluorescent antibodies are very useful in the diagnosis of visceral leishmaniasis, particularly in paediatric cases [49]. The most frequently used tests are the direct agglutination test and the rK39 rapid test, although the latter in Sri Lanka did not give the expected results, suggesting that local parasite antigenic variation may also have an influence [50]. In Brazil, India and Nepal, the ICT rK39 test has become the standard in the diagnosis of leishmaniasis [51]. The use of rK28 antigens in an RDT-type format gave superior results to rK39, with a sensitivity of 95.9% and specificity of 100% [28]. It has also been tested in patients with HIV co-infection, in whom serological test results are known to be poorer, but with a very good response between 92.3% and 100% [51].

The ELISA test showed satisfactory results in the diagnosis of symptomatic visceral leishmaniasis, being less expensive in comparison to in-vitro cultures and PCR tests from bone marrow or from samples belonging to other organs, but still showed low levels of values in immunocompromised individuals [51,52].

The direct agglutination test presents a very good sensitivity, but it has a number of drawbacks, as it demands for a laboratory equipped with a temperature-controlled incubator, overnight incubation, skilled personnel, which makes this test unable to be used at the point of care, and also, even if it can detect low levels of antibodies, because it uses multiple antigens it affects specificity [53,54].

An up-to-date commercial test is now available for the detection of antigens, namely the latex agglutination test (KATEX) which is used for leishmanial antigen detection in the urine of patients which present visceral leishmaniasis [55]. Although the sensitivity is not very good (60–80%), this

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 May 2023 doi:10.20944/preprints202305.0830.v1

antigen-based test can represent a real success in visceral leishmaniasis diagnosis in immunocompromised individuals who may have low antibody titres [56,57]. HIV/AIDS patients receiving antiretroviral treatment develop an asymptomatic leishmaniasis, with an increase in the parasite load, increasing also the risk of infection of sandfly vectors and therefore of disease transmission [29,58].

The use of serological tests is therefore less reliable in patients with HIV coinfection, in Europe it has been shown that more than 40% can give negative results [8,59]. However, studies have shown that serological test results are different in HIV patients, as opposed to other categories of immunocompromised patients like those who received organ transplants, in whom for example, the direct agglutination test had a high sensitivity of 80% in East Africa [60]. Also, the use of the indirect fluorescent antibody test showed a sensitivity of 48% in HIV patients and 93% in transplant patients [61].

Table 2. The sensitivity and specificity rates acquired through the serological tests.

	Sensitivity (%)	Specificity (%)	
Recombinant antigen rK39 (India and Nepal).	> 95.8	100	[51,52,62–64]
Direct agglutination test (DAT)	91.6 (84.1–96.3)	97.3 (92.3–99.4)	[51,65]
Recombinant antigen rK28	95.9	100	[28]
IFAT	87.5	95.8	[65–67]
Immunosorbent assay (ELISA)	75	95,8	[66,67]

In conclusion, the overall performance of rapid dipstick rk39 test had a score higher than ELISA and IFAT with a sensitivity and specificity of (95.8%) and (100%) respectively. This immunochromatographic test rk39 is non-invasive, rapid, easy to perform and low-cost diagnostic test, recommended to be used for the diagnosis of leishmaniasis, but a second method is still necessary to have a greater certainty of the result obtained, especially if we are talking about patients with co-infections, especially HIV, because both diseases target the same immune cells. Visceral leishmaniasis increases the rate of onset of AIDS and decreases the lifetime of HIV infected patients [68].

2.3. Molecular Techniques

The PCR techniques are recommended for samples that have a lower parasite load [69]. Moreira et al. (2007) [70] carried out a comparative study of different diagnostic methods,observing a sensitivity of 100%, 96% and respectively 95.65% for symptomatic, oligosymptomatic patients and asymptomatic ones and 100% specificity.

The PCR method has the disadvantage that it cannot differentiate between active visceral leishmaniasis and asymptomatic infections [71], as it presents a high risk of contamination.

Multiplex PCR

In this method, different DNA targets can be simultaneously amplified for the diagnosis of leishmaniasis using different types of markers, such as multicopy SL RNAs and minicircle kDNA, but it shows a lower sensitivity compared to the single PCR method [20].

Real-Time PCR (Quantitative PCR)

This method helps to monitor the parasite load in different tissues throughout the course of the disease and after treatment [72]. It presents the disadvantage that it is expensive and requires qualified personnel for interpretation. Using peripheral blood for the diagnosis of visceral

 doi:10.20944/preprints202305.0830.v1

leishmaniasis, a sensitivity and specificity of 91.3% and 29.6% respectively for real-time PCR and 97.78% and 61.82% for PCR have been demonstrated [73]. But the sensitivity for the classic PCR technique is dependent on the concentration of DNA in the sample. Thus, emphasis was placed on the RealTime PCR technique, succeeding in the identification of parasites without cross-amplification with another parasite subgenus [74].

3. The Immune Response in Human Leishmaniasis

In most individuals, leishmaniasis does not progress to a noticeable form of the disease, in regions with a high degree of endemicity, up to 30% of infected residents are asymptomatic [75]. Control of infection is based on activated, leishmanicidal macrophages and an intact specific T-helper type 1 (Th1) cell response. Cell-mediated immunity is indicated through a positive skin test result. The observable disease state is associated with a mixed Th1/T-helper type 2 (Th2) response. Increased levels of regulatory T cells contribute to the severe immunosuppression observed in visceral leishmaniasis [76]. Proinflammatory cytokines which mediate an effective Th1 response include interferon-c and TNF-a [77]. In the case of immunosuppression, a reactivation of the disease can occur, as the parasites remain viable after the primary infection [2,78]. In HIV infection, a depletion of CD4 T cells is observed, with a bias toward a Th2-type immune response, thus affecting both the innate and adaptive immune systems, along with macrophage functionality [79]. *Leishmania sp.* is located in the same host cells, in macrophages and dendritic cells, so that a co-infection with the HIV virus increases the pathogenicity [80].

In most cases of co-infection recorded in Europe, it presents a CD4 cell count <200 cells/ml at the diagnosis of visceral leishmaniasis [79]; but reasearch in Ethiopia, where *L. donovani* is predominant, showed higher CD4 cell counts [81]. The immunosuppression which favours visceral leishmaniasis, creating a predisposition to it, can also be induced by a series of immunosuppressive or immunomodulatory treatments, significantly affecting T-cell lymphocytes [82]. It should be taken into account that in humans co-infected with the HIV virus, its presence can be found in abnormal tissues like the intestine, the oral cavity, lung tissue or skin [8,83,84]. Post-kala azar dermal leishmaniasis was reported in higer numbers in immunosuppressed patients [85]. Post-kala azar dermal leishmaniasis in *L. infantum* infestation is not usually observed but it was reported in HIV patients [86,87].

In visceral leishmaniasis there is an overactivation of B cells, which leads to a marked polyclonal hypergammaglobulinemia, which may result in a positive indirect Coombs test and detectable levels of antinuclear antibodies, anti-cardiolipin antibodies, anti-dsDNA antibodies and rheumatoid factor IgM [88]. Circulating immune complexes, cryoglobulinemia and low complement levels could also be noticed [89], similar symptomatology being able to lead to a misdiagnosis of an autoimmune disease like rheumatoid arthritis or systemic lupus erythematosus and/or as an onset of the basic disease [90,91].

4. Immunological Response in Visceral Leishmaniasis in The Dog

Visceral leishmaniasis in dogs is very variable, being able to evolve from very severe symptomatic forms to asymptomatic or mild forms classified as oligosymptomatic. The clinical picture includes anaemia, lymphadenopathy, alopecia, diarrhoea, weight loss, onychogryphosis, locomotor problems, epistaxis, conjunctivitis, muscle atrophy, polyuria, polydipsia [92–94], the range of clinical symptoms being connected to the genetic makeup and immune response of individual animals, which can have a direct impact on their vulnerability or resistance to infection [95]. A big problem for public health, especially in Brazil, is represented by dogs without clinical manifestations, which represent a reservoir in nature, being an infestation source for vectors [96,97]. The non-specific symptomatology is related to the heterogeneity of immune responses, which occurs in leishmaniasis [98]. Thus, in leishmaniasis there can be a severe form, with the suppression of the cellular response and the formation of a high titre of antibodies, or there can be an asymptomatic form with the mounting of a protective immune response which leads to a positive or negative serology [17]. As a mandatory intracellular parasite with localisation in cells of the myeloid system (macrophages,

 doi:10.20944/preprints202305.0830.v1

monocytes, neutrophils, antigen-presenting cells), the protozoan has a distinctive and intricate effect on the immune system. In canine leishmaniasis, the first to interact with parasites are neutrophils, which can lead to parasite destruction by an oxidative burst or prolonged parasite survival [99]. During disease with clinical manifestations, *Leishmania* protozoa are thought to induce early apoptosis of neutrophils. Parasites manipulate the macrophage immune response within the parasitophorous vacuole by keeping neutrophils alive for an extended period. This enables the parasites to survive in the parasitophorous vacuole and be absorbed by macrophages without causing inflammatory reactions, ultimately resulting in parasite persistence [100].

In canine visceral leishmaniasis, immunological changes involving T cells are involved, such as the absence of delayed hypersensitivity (DTH) to Leishmania antigens [101], decreased numbers of T cells in the peripheral blood [102,103], absence of interleukin 2 (IL-2) production and absence of interferon gamma (IFN-γ) production [104]. The main mechanism involved in the protective immunity of dogs with *L. infantum* is the activation of macrophages by IFN-γ and TNF-α to clear intracellular amastigotes via nitric oxide–arginine. IL-12p40 and IL-2 and IFN-γ are also implicated in delaying disease onset in these animals, IL-12 being detected in lymph node cells from dogs protected against *L. infantum* after immunisation with LACK-expressing vaccine [105]. IL-10 production was correlated with active disease, and IL-2 and IFN-γ were predominantly reported in asymptomatic dogs. IL-17 released by Th17 cells increases IFN-ɣ production and reduces IL-10 [106]. In the clinical phase of the disease, excessive production of IL-17 results in the recruitment of an excessive number of neutrophils to sites of inflammation. This phenomenon causes tissue damage, which is particularly noticeable in the cutaneous and mucocutaneous forms of human leishmaniasis [107]. The expression of Toll-like receptors (TLRs) on the surface of monocytes, dendritic cells, and macrophages can trigger either activation or inhibition of cellular functions and the production of IL-12. This, in turn, can lead to a TH1 type response that limits or halts the intracellular survival of parasites, and which can be determined by measuring the proliferation of CD4+ T cells and the production of IFN-γ, which activates macrophages. [108].

The cytokine IL-4 was not observed in asymptomatic dogs, but was observed in symptomatic dogs, isolated from bone marrow aspirates of dogs which had more severe symptoms [109]. IgG1 and IgG2 subclasses were used more than total IgG as an indicator of canine visceral leishmaniasis status [110].

High levels of anti-Leishmania IgG1 antibodies and the appearance of clinical IgG2 antibodies were detected in symptomatic dogs, as well as IgE [111].

CD8+ lymphocytes were observed in asymptomatic dogs that were experimentally infected with *L. infantum*, as well as in immunized dogs, but not in symptomatic dogs . Genetic factors are also considered to play a significant role in the development of the disease, so polymorphisms of the canine NRAMP1 gene [112] and MHC class II alleles [113] have been shown to be important in the development of the disease, so that some breeds of dogs, such as the Ibizan Hound, do not develop signs of symptomatic leishmaniasis, which must be considered when working to control this disease. In canine patients, CD4+ Th1 lymphocytes activate macrophages to effector cells, leading to the production of *Leishmania*-specific Interferon-γ (IFN-γ). This process allows the host to combat the parasite and manage the advancement of the disease [107]. In general, dogs that experience clinical disease and poor clinical outcomes typically exhibit a reduction or absence of the cell-mediated immune response [114–116]. In "resistant" dogs, the Th1 immune response is dominant and is marked by Interferon-γ. However, some of these dogs may not produce antibodies or may have very low levels (sometimes below the cutoff), yet parasite DNA can still be detected in tissues such as bone marrow and lymph nodes in some of these individuals [117,118].

In order to achieve immunological control of *Leishmania infantum*, which is the main species causing canine leishmaniasis, a state of equilibrium between inflammatory and regulatory responses is necessary. This balance usually occurs between pro-inflammatory Th1 CD4+ T cells that are responsible for managing parasite multiplication and regulatory T cells 1 which produce immunosuppression, necessary to alleviate exaggerated inflammation, but which if predominates, leads to in the progression of canine leishmaniasis. Multiple factors are involved in the development

 doi:10.20944/preprints202305.0830.v1

of the disease, for example hunting dogs are more susceptible to developing the disease due to frequent co-infections acquired during hunting and poor constitution. Those in whom the disease does not progress remain in a subclinical state. The presence of parasites in peripheral blood can be identified through qPCR and/or detection of humoral immune responses. Although enzyme-linked immunosorbent assay (ELISA) may reveal low antibody titers, a robust cellular immune response, including CD4+ T cell proliferation in response to parasite antigen, is observed.

CD4+ T cells have a major role in intracellular control of pathogens by generating IFN-γ-activating macrophages. TH1 cells also contribute to the production of IL-3 , CXCL2 and TNF-α, which play an important role in the recruitment, maintenance and differentiation of macrophages at the location of the infection, as well as IL-2 cells, which lead to the formation of more T cells, creating a favorable environment inside cells for the destruction of Leishmania. In the course of subclinical disease, canine patients have proliferative cytotoxic CD8+ T cells, which help control *L. infantum* by eliminating infected macrophages [119]. Studies showed by qPCR that even in subclinical form dogs had increased levels of IL-18 and IL-6 in peripheral blood mononuclear cells, which aided IL-12 in macrophage activation [120]. With the progression of leishmaniasis, the immune system is no longer able to maintain a balance in the inflammation process without producing pathology [121].

5. Diagnosis of Canine Leishmaniasis

The diagnosis of canine leishmaniasis is intricate and multiple strategies are required to be used. In endemic zones, it is recommended to employ the clinical diagnosis in combination with specific techniques according to the clinical signs and laboratory results, such as quantitative serology (IFAT- immunofluorescence antibody test, or ELISA- the enzyme-linked immunosorbent assay). This is to examine the humoral response, beacause in clinical leishmaniasis the antibody levels are elevated**.** In addition, along with parasitological diagnosis, molecular diagnosis is used to detect specific DNA through biopsy and/or cytology (conventional PCR, qPCR, nested PCR) [115].

As in human leishmaniasis, in canine leishmaniasis (CVL) diagnosis, the gold standard remains the parasitological diagnosis that includes the microscopic examination of smears and aspirates from the spleen, liver, bone marrow, lymphatic nodes, and of biopsy material from damaged or intact skin [111,122]. But this method also has a number of drawbacks, the result depending on the experience of the observer, the parasite load, as well as the immune response developed by the host [101]. The most used are serological tests, but which present a series of disadvantages, especially those based on parasitic antigens, as they cross-react with other *Leishmania* species, and also with *Trypanosoma* species, with which they are co-endemic [103,111]. Several recombinant proteins, including rLb6H from *Leishmania braziliensis*, have been tested and found to be effective in diagnosing human leishmaniasis. The use of this protein has produced positive outcomes in diagnosing visceral leishmaniasis caused by *Leishmania infantum* and American cutaneous leishmaniasis resulting from infection with species of *Leishmania* such as *L. braziliensis*, *L. amazonensis*, *L. shawi*, and *L. guyanensis*. However, these proteins still have drawbacks due to their tendency to produce cross-reactivity with sera from patients with toxoplasmosis, malaria, Chagas disease, paracoccidioidomycosis, tuberculosis, and histoplasmosis [102]. rKLO8, a kinesin protein derived from *Leishmania donovani* found in Sudan, has demonstrated strong specificity and sensitivity, particularly in patients with VL in India and East Africa [123]. Furthermore, the utilization of both rKLO8 and rK26 proteins in combination has enhanced the specificity and sensitivity of serodiagnosis for CVL, surpassing that of monospecific ELISA [104]. The most used are the fusion proteins rK39 and rK28, also used successfully in human medicine; rK28 (obtained from the fusion of rK9, rK26 and rK39 from *L. donovani*), being recommended by the Brazilian Ministry of Health since 2011, in the form of a rapid immunochromatographic test. In the diagnosis of leishmaniasis in dogs, it is extremely important to differentiate vaccinated dogs from those infected with *Leishmania*.

Patients who presented a series of clinical signs came out positive in the ELISA and IFAT tests, making them safe in the diagnosis of clinical leishmaniasis [124].

The use of the Real Time PCR technique for the detection of *L. infantum* proved clearly superior to the conventional PCR technique, which failed to detect *Leishmania* DNA in samples with very low

 doi:10.20944/preprints202305.0830.v1

or very high numbers of parasites [125,126]. Consequently, studies utilizing polymerase chain reaction (PCR) to detect DNA in different canine tissues have reported increased rates of positivity [124]. A study carried out by Vito Priolo in 2022 shows that there are no differences according to PCR positivity among a series of studied variables such as the age, gender and race of the patients taken into account. Nested and semi-nested PCR techniques are crucial for distinguishing between different species. This method involves the use of two sets of primers in two consecutive cycles, wherein the second set amplifies the secondary target in the first product. Despite being highly sensitive, this approach has limitations due to the possibility of diagnostic contamination [127].

6. Discussion

Immunoassays were the most personalised, being appropriate for endemic regions, but still unable to distinguish active infections. In addition, molecular methods have high specificity and sensitivity but do not indicate whether the infection is active. The parasitological techniques, which are considered the gold standard in the diagnosis of leishmaniasis, have a number of drawbacks as they require time, qualified personnel, and in the case of an asymptomatic infection where the parasite load is low, the identification is limited, with the risk of a false positive response. In asymptomatic cases, antibody titres are also low, and serological tests are limited as in HIV co-infections. Therefore, the diagnosis and surveillance of leishmaniasis cases remains an open topic, with the need for new methods adapted to the immunological status of the host.

In countries where sandfly species exist, it is believed that both humans and dogs who have a subclinical form of leishmaniasis play a significant role in spreading and transmitting the disease [128]. In order to reduce the progression and transmission of the disease, it is very important to maintain the functionality of the T cells, trying through vaccines and specific therapies to eliminate the disease. Chronic exposure to *Leishmania* protozoan antigen can lead to T-cell exhaustion, which can be reactivated by using a vaccine antigen and TLR agonists.

Recent studies have proposed the potential use of vaccines in combination with chemotherapy as immunotherapies for infected dogs. There are 3 canine leishmaniasis vaccines available and used on the market, namely: CaniLeish® (Virbac Santé Animale, France), Leish-Tec® (Ceva Animal Health, Brazil), and Letifend® (Laboratorios Leti, Spain) [129], with the specification that the manufacturers only recommend vaccinating seronegative dogs. The challenge is the difficulty of identifying completely healthy dogs due to the existence of gaps in current diagnosis. A study conducted by Toepp et al. in 2018 [130] showed that adverse effects were low, approximately 3%, when vaccinating healthy subclinical dogs with LeishTec, a study which should be considered and used in the vaccination/immunotherapy protocol of infected healthy animals. But it must be taken into account that during the sandfly season, other vectors such as mosquitoes and ticks are also active, which can transmit a number of pathogens such as *Dirofilaria spp., Babesia spp., Ehrilichia spp., Anaplasma spp.*; all these pathogens can evolve as co-infections with *Leishmania*, a fact which affects the immune system and can lead to complications of leishmaniasis [131].

7. Conclusions

Canine leishmaniasis remains a challenge, as the balance of the immune system is very delicate in trying to prevent the replication and growth of *Leishmania* parasites. Thus, in the attempt to establish a disease control protocol, combinations of vaccination and immunotherapy should also be taken into account, as well as diagnostic protocols, since no diagnostic method has proven to be 100% safe in asymptomatic leishmaniasis. Consequently, for the diagnosis of the disease in endemic areas, combinations of diagnostic methods should be established, adapted to the region where the disease evolves.

This problem is also encountered in human medicine, especially if we talk about patients with HIV, or other immunosuppressive diseases, which affect the immune system differently in leishmaniasis.

In conclusion, leishmaniasis remains a disease which requires detailed research regarding the response of the immune system in various conditions, as well as the establishment of safe diagnostic methods.

Consequently, it is necessary to introduce this disease to the list of diseases of great interest also in the context of global warming and the movement of the human and animal population, which increase the risk of global dissemination.

Author Contributions: Conceptualization, L.M. and L.I.,B.L.A.; methodology, G.M., L.M. and L.I.; investigation, S.G., L.I., B.L.A., and R.L; resources, G.M., S.G., R.L.; data curation, L.I., L.M.; writing—original draft preparation, L.M. B.L.A. and L.I.; writing—review and editing,L.M., L.I., G.M., B.L.A.; visualization, L.M. and R.M.; supervision, L.M., S.G. and L.I.; project administration, L.M.; funding acquisition, S.G., R.M., B.L.A. and G.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable here.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. van Griensven, J.; Diro, E. Visceral Leishmaniasis. *Infectious Disease Clinics of North America* **2012**, *26* (2), 309–322. https://doi.org/10.1016/j.idc.2012.03.005.
2. van Griensven, J.; Diro, E. Visceral Leishmaniasis: Recent Advances in Diagnostics and Treatment Regimens. *Infectious Disease Clinics of North America* **2019**, *33* (1), 79–99. https://doi.org/10.1016/j.idc.2018.10.005.
3. WHO. Leishmaniasis. 2016. Available online: http://www.who.int/topics/leishman iasis/en/ (accessed on 23 March 2023)
4. Pace, D. Leishmaniasis. *Journal of Infection* **2014**, *69*, S10–S18. https://doi.org/10.1016/j.jinf.2014.07.016
5. Alvar, J.; Vélez, I. D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; Boer, M. D.; the WHO Leishmaniasis Control Team. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS ONE* **2012**, *7* (5), e35671. https://doi.org/10.1371/journal.pone.0035671
6. Reithinger, R.; Dujardin, J.-C.; Louzir, H.; Pirmez, C.; Alexander, B.; Brooker, S. Cutaneous Leishmaniasis. *Lancet Infect Dis* **2007**, *7* (9), 581–596. https://doi.org/10.1016/S1473-3099(07)70209-8.
7. Torres-Guerrero, E.; Quintanilla-Cedillo, M. R.; Ruiz-Esmenjaud, J.; Arenas, R. Leishmaniasis: A Review. *F1000Res* **2017**, *6*, 750. https://doi.org/10.12688/f1000research.11120.1.
8. van Griensven, J.; Carrillo, E.; López-Vélez, R.; Lynen, L.; Moreno, J. Leishmaniasis in Immunosuppressed Individuals. *Clinical Microbiology and Infection* **2014**, *20* (4), 286–299. https://doi.org/10.1111/1469-0691.12556.
9. Marcondes, M.; Day, M. J. Current Status and Management of Canine Leishmaniasis in Latin America. *Res Vet Sci* **2019**, *123*, 261–272. https://doi.org/10.1016/j.rvsc.2019.01.022.
10. Travi, B. L.; Cordeiro-da-Silva, A.; Dantas-Torres, F.; Miró, G. Canine Visceral Leishmaniasis: Diagnosis and Management of the Reservoir Living among Us. *PLoS Negl Trop Dis* **2018**, *12* (1), e0006082. https://doi.org/10.1371/journal.pntd.0006082.
11. Altamura, F.; Rajesh, R.; Catta-Preta, C. M. C.; Moretti, N. S.; Cestari, I. The Current Drug Discovery Landscape for Trypanosomiasis and Leishmaniasis: Challenges and Strategies to Identify Drug Targets. *Drug Development Research* **2022**, *83* (2), 225–252. https://doi.org/10.1002/ddr.21664.
12. Pederiva, M. M. C.; Santos, S. M. dos; Rivarola, L. G. S.; Guerreiro, V. J.; Lopes, K. S.; Lima Junior, M. S. da C.; Neitzke-Abreu, H. C. Asymptomatic Leishmania Infection in Humans: A Systematic Review. *Journal of Infection and Public Health* **2023**, *16* (2), 286–294. https://doi.org/10.1016/j.jiph.2022.12.021.
13. Mody, R. M.; Lakhal-Naouar, I.; Sherwood, J. E.; Koles, N. L.; Shaw, D.; Bigley, D. P.; Co, E.-M. A.; Copeland, N. K.; Jagodzinski, L. L.; Mukbel, R. M.; Smiley, R. A.; Duncan, R. C.; Kamhawi, S.; Jeronimo, S. M. B.; DeFraites, R. F.; Aronson, N. E. Asymptomatic Visceral Leishmania Infantum Infection in US Soldiers Deployed to Iraq. *Clinical Infectious Diseases* **2019**, *68* (12), 2036–2044. https://doi.org/10.1093/cid/ciy811.

14. Burza, S.; Croft, S. L.; Boelaert, M. Leishmaniasis. *The Lancet* **2018**, *392* (10151), 951–970. https://doi.org/10.1016/S0140-6736(18)31204-2.
15. Athanasiou, L. V.; Kontos, V. I.; Saridomichelakis, M. N.; Rallis, T. S.; Diakou, A. A Cross-Sectional Sero-Epidemiological Study of Canine Leishmaniasis in Greek Mainland. *Acta Tropica* **2012**, *122* (3), 291–295. https://doi.org/10.1016/j.actatropica.2012.02.003.
16. Otranto, D.; Dantas-Torres, F. The Prevention of Canine Leishmaniasis and Its Impact on Public Health. *Trends in Parasitology* **2013**, *29* (7), 339–345. https://doi.org/10.1016/j.pt.2013.05.003.
17. Jr, G. G.; Teva, A.; dos-Santos, C. B.; Santos, F. N.; Pinto, I. de-Souza; Fux, B.; Leite, G. R.; Falqueto, A. Field Trial of Efficacy of the Leish-Tec® Vaccine against Canine Leishmaniasis Caused by Leishmania Infantum in an Endemic Area with High Transmission Rates. *PLOS ONE* **2017**, *12* (9), e0185438. https://doi.org/10.1371/journal.pone.0185438.
18. Reithinger, R.; Dujardin, J.-C. Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. *Journal of Clinical Microbiology* **2007**, *45* (1), 21–25. https://doi.org/10.1128/JCM.02029-06.
19. Santos, T. T. O.; Ramos, F. F.; Gonçalves, I. A. P.; Tavares, G. S. V.; Ludolf, F.; Bandeira, R. S.; Silva, A. M.; Oliveira-da-Silva, J. A.; Reis, T. A. R.; Machado, A. S.; Lage, D. P.; Freitas, C. S.; Vale, D. L.; Martins, V. T.; Alves, L. A.; Guimarães, N. S.; Chaves, A. T.; Chávez-Fumagalli, M. A.; Cota, G. F.; Silveira, J. A. G.; Tupinambás, U.; Gonçalves, D. U.; Christodoulides, M.; Coelho, E. A. F. Potential of Recombinant LiHyQ, a Novel Leishmania Infantum Protein, for the Diagnosis of Canine Visceral Leishmaniasis and as a Diagnostic and Prognostic Marker for Human Leishmaniasis and Human Immunodeficiency Virus Co-Infection: A Preliminary Study. *Acta Trop* **2021**, *224*, 106126. https://doi.org/10.1016/j.actatropica.2021.106126.
20. Thakur, S.; Joshi, J.; Kaur, S. Leishmaniasis Diagnosis: An Update on the Use of Parasitological, Immunological and Molecular Methods. *J Parasit Dis* **2020**, *44* (2), 253–272. https://doi.org/10.1007/s12639-020-01212-w.
21. Shirian, S.; Oryan, A.; Hatam, G.-R.; Panahi, S.; Daneshbod, Y. Comparison of Conventional, Molecular, and Immunohistochemical Methods in Diagnosis of Typical and Atypical Cutaneous Leishmaniasis. *Archives of Pathology & Laboratory Medicine* **2014**, *138* (2), 235–240. https://doi.org/10.5858/arpa.2013-0098-OA.
22. Elmahallawy, E. K.; Sampedro Martinez, A.; Rodriguez-Granger, J.; Hoyos-Mallecot, Y.; Agil, A.; Navarro Mari, J. M.; Gutierrez Fernandez, J. Diagnosis of Leishmaniasis. *J Infect Dev Ctries* **2014**, *8* (8), 961–972. https://doi.org/10.3855/jidc.4310.
23. Srivastava, P.; Dayama, A.; Mehrotra, S.; Sundar, S. Diagnosis of Visceral Leishmaniasis. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **2011**, *105* (1), 1–6. https://doi.org/10.1016/j.trstmh.2010.09.006.
24. Srividya, G.; Kulshrestha, A.; Singh, R.; Salotra, P. Diagnosis of Visceral Leishmaniasis: Developments over the Last Decade. *Parasitol Res* **2012**, *110* (3), 1065–1078. https://doi.org/10.1007/s00436-011-2680-1.
25. Pessoa-E-Silva, R.; Vaitkevicius-Antão, V.; de Andrade, T. A. S.; de Oliveira Silva, A. C.; de Oliveira, G. A.; Trajano-Silva, L. A. M.; Nakasone, E. K. N.; de Paiva-Cavalcanti, M. The Diagnosis of Canine Visceral Leishmaniasis in Brazil: Confronting Old Problems. *Exp Parasitol* **2019**, *199*, 9–16. https://doi.org/10.1016/j.exppara.2019.02.012.
26. Barrett, M. P.; Croft, S. L. Management of Trypanosomiasis and Leishmaniasis. *British Medical Bulletin* **2012**, *104* (1), 175–196. https://doi.org/10.1093/bmb/lds031.
27. Mettler, M.; Grimm, F.; Capelli, G.; Camp, H.; Deplazes, P. Evaluation of Enzyme-Linked Immunosorbent Assays, an Immunofluorescent-Antibody Test, and Two Rapid Tests (Immunochromatographic-Dipstick and Gel Tests) for Serological Diagnosis of Symptomatic and Asymptomatic Leishmania Infections in Dogs. *Journal of Clinical Microbiology* **2005**, *43* (11), 5515–5519. https://doi.org/10.1128/JCM.43.11.5515-5519.2005.
28. Pattabhi, S.; Whittle, J.; Mohamath, R.; El-Safi, S.; Moulton, G. G.; Guderian, J. A.; Colombara, D.; Abdoon, A. O.; Mukhtar, M. M.; Mondal, D.; Esfandiari, J.; Kumar, S.; Chun, P.; Reed, S. G.; Bhatia, A. Design, Development and Evaluation of RK28-Based Point-of-Care Tests for Improving Rapid Diagnosis of Visceral Leishmaniasis. *PLoS Negl Trop Dis* **2010**, *4* (9), e822. https://doi.org/10.1371/journal.pntd.0000822.
29. Das, V. N. R.; Siddiqui, N. A.; Verma, R. B.; Topno, R. K.; Singh, D.; Das, S.; Ranjan, A.; Pandey, K.; Kumar, N.; Das, P. Asymptomatic Infection of Visceral Leishmaniasis in Hyperendemic Areas of Vaishali District, Bihar, India: A Challenge to Kala-Azar Elimination Programmes. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **2011**, *105* (11), 661–666. https://doi.org/10.1016/j.trstmh.2011.08.005.

30. Reimão, J. Q.; Coser, E. M.; Lee, M. R.; Coelho, A. C. Laboratory Diagnosis of Cutaneous and Visceral Leishmaniasis: Current and Future Methods. *Microorganisms* **2020**, *8* (11), 1632. https://doi.org/10.3390/microorganisms8111632.
31. Faria, A. R.; Veloso, L. de C.; Coura-Vital, W.; Reis, A. B.; Damasceno, L. M.; Gazzinelli, R. T.; Andrade, H. M. Novel Recombinant Multiepitope Proteins for the Diagnosis of Asymptomatic Leishmania Infantum-Infected Dogs. *PLOS Neglected Tropical Diseases* **2015**, *9* (1), e3429. https://doi.org/10.1371/journal.pntd.0003429.
32. Farahmand, M.; Nahrevanian, H. Application of Recombinant Proteins for Serodiagnosis of Visceral Leishmaniasis in Humans and Dogs. *Iran Biomed J* **2016**, *20* (3), 128–134. https://doi.org/10.7508/ibj.2016.03.001.
33. Dhom-Lemos, L.; Viana, A. G.; Cunha, J. L. R.; Cardoso, M. S.; Mendes, T. A. O.; Pinheiro, G. R. G.; Siqueira, W. F.; Lobo, F. P.; Teles, L. F.; Bueno, L. L.; Guimarães-Carvalho, S. F.; Bartholomeu, D. C.; Fujiwara, R. T. Leishmania Infantum Recombinant Kinesin Degenerated Derived Repeat (RKDDR): A Novel Potential Antigen for Serodiagnosis of Visceral Leishmaniasis. *PLOS ONE* **2019**, *14* (1), e0211719. https://doi.org/10.1371/journal.pone.0211719.
34. Pereira, I. E.; Silva, K. P.; Menegati, L. M.; Pinheiro, A. C.; Assunção, E. A. O.; Araújo, M. D. L. P.; Abass, E.; Duthie, M. S.; Steinhoff, U.; Teixeira, H. C. Performance of Recombinant Proteins in Diagnosis and Differentiation of Canine Visceral Leishmaniasis Infected and Vaccinated Dogs. *European Journal of Microbiology and Immunology* **2020**, *10* (3), 165–171. https://doi.org/10.1556/1886.2020.00018.
35. Lévêque, M. F.; Battery, E.; Delaunay, P.; Lmimouni, B. E.; Aoun, K.; L'Ollivier, C.; Bastien, P.; Mary, C.; Pomares, C.; Fillaux, J.; Lachaud, L. Evaluation of Six Commercial Kits for the Serological Diagnosis of Mediterranean Visceral Leishmaniasis. *PLOS Neglected Tropical Diseases* **2020**, *14* (3), e0008139. https://doi.org/10.1371/journal.pntd.0008139.
36. Rodrigues, M. R.; Santos, L. M. O.; Miyazaki, C. K.; Martins, V. T.; Ludolf, F. R.; Kursancew, A. C.; Ramos, F. F.; Dias, D. S.; Oliveira, J. S.; Vieira, P. M. A.; Roatt, B. M.; Machado de Ávila, R. A.; Gonçalves, D. U.; Menezes-Souza, D.; Coelho, E. A. F.; Duarte, M. C. Immunodiagnosis of Human and Canine Visceral Leishmaniasis Using Recombinant Leishmania Infantum Prohibitin Protein and a Synthetic Peptide Containing Its Conformational B-Cell Epitope. *J Immunol Methods* **2019**, *474*, 112641. https://doi.org/10.1016/j.jim.2019.112641.
37. Daltro, R. T.; Leony, L. M.; Freitas, N. E. M.; Silva, Â. A. O.; Santos, E. F.; Del-Rei, R. P.; Brito, M. E. F.; Brandão-Filho, S. P.; Gomes, Y. M.; Silva, M. S.; Donato, S. T.; Jeronimo, S. M. B.; Monteiro, G. R. de G.; Carvalho, L. P.; Magalhães, A. S.; Zanchin, N. I. T.; Celedon, P. A. F.; Santos, F. L. N. Cross-Reactivity Using Chimeric Trypanosoma Cruzi Antigens: Diagnostic Performance in Settings Where Chagas Disease and American Cutaneous or Visceral Leishmaniasis Are Coendemic. *Journal of Clinical Microbiology* **2019**, *57* (8), e00762-19. https://doi.org/10.1128/JCM.00762-19.
38. Hosseini Farash, B. R.; Mohebali, M.; Kazemi, B.; Fata, A.; Raoofian, R.; AKHOUNDI, B.; MOJARRAD, M.; Mastroeni, P.; Sharifi-Yazdi, M. kazem; Tanipour, M. H. The RK39 Antigen from an Iranian Strain of Leishmania Infantum: Detection of Anti-Leishmania Antibodies in Humans and Dogs. *Iranian Journal of Parasitology* **2020**, *15*. https://doi.org/10.18502/ijpa.v15i1.2526.
39. Alvar, J.; Aparicio, P.; Aseffa, A.; Den Boer, M.; Cañavate, C.; Dedet, J.-P.; Gradoni, L.; Ter Horst, R.; López-Vélez, R.; Moreno, J. The Relationship between Leishmaniasis and AIDS: The Second 10 Years. *Clin Microbiol Rev* **2008**, *21* (2), 334–359. https://doi.org/10.1128/CMR.00061-07.
40. Besada, E.; Njålla, R. J.; Nossent, J. C. Imported Case of Visceral Leishmaniasis Presenting as Pancytopenia in a Norwegian Patient Treated with Methotrexate and Etanercept for Psoriasis Arthritis. *Rheumatol Int* **2013**, *33* (10), 2687–2689. https://doi.org/10.1007/s00296-012-2483-4.
41. Ates, S. C.; Bagirova, M.; Allahverdiyev, A. M.; Kocazeybek, B.; Kosan, E. Utility of the Microculture Method for Leishmania Detection in Non-Invasive Samples Obtained from a Blood Bank. *Acta Tropica* **2013**, *128* (1), 54–60. https://doi.org/10.1016/j.actatropica.2013.06.009.
42. Boelaert, M.; Bhattacharya, S.; Chappuis, F.; El Safi, S. H.; Hailu, A.; Mondal, D.; Rijal, S.; Sundar, S.; Wasunna, M.; Peeling, R. W. Evaluation of Rapid Diagnostic Tests: Visceral Leishmaniasis. *Nat Rev Microbiol* **2007**, *5* (11), S31–S39. https://doi.org/10.1038/nrmicro1766.
43. Guerra, J. M.; Fernandes, N. C. C. A.; Ressio, R. A.; Magno, J. A.; Kimura, L. M.; Barbosa, J. E. de R.; Bertollo, D. M. B.; Taniguchi, H. H.; Hiramoto, R. M.; Motoie, G.; Tolezano, J. E.; Cogliati, B. Evaluation of

cytopathological techniques for the diagnosis of canine visceral leishmaniosis with lymph node samples. *Journal of Comparative Pathology* **2019**, *172*, 62–71.

44. Hide, M.; Singh, R.; Kumar, B.; Bañuls, A. L.; Sundar, S. A Microculture Technique for Isolating Live Leishmania Parasites from Peripheral Blood of Visceral Leishmaniasis Patients. *Acta Tropica* **2007**, *102* (3), 197–200. https://doi.org/10.1016/j.actatropica.2007.04.015.
45. Maurya, R.; Mehrotra, S.; Prajapati, V. K.; Nylén, S.; Sacks, D.; Sundar, S. Evaluation of Blood Agar Microtiter Plates for Culturing Leishmania Parasites To Titrate Parasite Burden in Spleen and Peripheral Blood of Patients with Visceral Leishmaniasis. *Journal of Clinical Microbiology* **2010**, *48* (5), 1932–1934. https://doi.org/10.1128/JCM.01733-09.
46. Galdino, H.; Saar Gomes, R.; dos Santos, J. C.; Pessoni, L. L.; Maldaner, A. E.; Marques, S. M.; Gomes, C. M.; Dorta, M. L.; de Oliveira, M. A. P.; Joosten, L. A. B.; Ribeiro-Dias, F. Leishmania (Viannia) Braziliensis Amastigotes Induces the Expression of TNFα and IL-10 by Human Peripheral Blood Mononuclear Cells in Vitro in a TLR4-Dependent Manner. *Cytokine* **2016**, *88*, 184–192. https://doi.org/10.1016/j.cyto.2016.09.009.
47. Schallig, H.; Schoone, G.; Kroon, C.; Hailu, A.; Chappuis, F.; Veeken, H. Development and Application of "simple" Diagnostic Tools for Visceral Leishmaniasis. *Med Microbiol Immunol* **2001**, *190* (1), 69–71. https://doi.org/10.1007/s004300100083.
48. Singh, S.; Gilman-Sachs, A.; Chang, K.-P.; Reed, S. G. Diagnostic and Prognostic Value of K39 Recombinant Antigen in Indian Leishmaniasis. *The Journal of Parasitology* **1995**, *81* (6), 1000–1003. https://doi.org/10.2307/3284056.
49. Cruz, I.; Chicharro, C.; Nieto, J.; Bailo, B.; Cañavate, C.; Figueras, M.-C.; Alvar, J. Comparison of New Diagnostic Tools for Management of Pediatric Mediterranean Visceral Leishmaniasis. *Journal of Clinical Microbiology* **2006**, *44* (7), 2343–2347. https://doi.org/10.1128/JCM.02297-05.
50. Siriwardana, H. V. Y. D.; Karunanayake, P.; Goonerathne, L.; Karunaweera, N. D. Emergence of Visceral Leishmaniasis in Sri Lanka: A Newly Established Health Threat. *Pathog Glob Health* 2017, *111* (6), 317–326. https://doi.org/10.1080/20477724.2017.1361564.
51. Bezuneh, A.; Mukhtar, M.; Abdoun, A.; Teferi, T.; Takele, Y.; Diro, E.; Jemaneh, A.; Shiferaw, W.; Wondimu, H.; Bhatia, A.; Howard, R. F.; Ghalib, H.; Ireton, G. C.; Hailu, A.; Reed, S. G. Comparison of Point-of-Care Tests for the Rapid Diagnosis of Visceral Leishmaniasis in East African Patients. *Am J Trop Med Hyg* **2014**, *91* (6), 1109–1115. https://doi.org/10.4269/ajtmh.13-0759.
52. Deepachandi, B.; Weerasinghe, S.; Soysa, P.; Chatterjee, M.; Siriwardana, Y. Serological Studies on RK39 Negative Visceral Leishmaniasis in an Endemic Focus of Leishmania Donovani Induced Cutaneous Leishmaniasis. *Diagnostic Microbiology and Infectious Disease* **2023**, *105* (3), 115873. https://doi.org/10.1016/j.diagmicrobio.2022.115873.
53. Gidwani, K.; Picado, A.; Ostyn, B.; Singh, S. P.; Kumar, R.; Khanal, B.; Lejon, V.; Chappuis, F.; Boelaert, M.; Sundar, S. Persistence of Leishmania Donovani Antibodies in Past Visceral Leishmaniasis Cases in India. *Clin Vaccine Immunol* **2011**, *18* (2), 346–348. https://doi.org/10.1128/CVI.00473-10.
54. Ostyn, B.; Gidwani, K.; Khanal, B.; Picado, A.; Chappuis, F.; Singh, S. P.; Rijal, S.; Sundar, S.; Boelaert, M. Incidence of Symptomatic and Asymptomatic Leishmania Donovani Infections in High-Endemic Foci in India and Nepal: A Prospective Study. *PLOS Neglected Tropical Diseases* **2011**, *5* (10), e1284. https://doi.org/10.1371/journal.pntd.0001284.
55. Attar, Z. J.; Chance, M. L.; el-Safi, S.; Carney, J.; Azazy, A.; El-Hadi, M.; Dourado, C.; Hommel, M. Latex Agglutination Test for the Detection of Urinary Antigens in Visceral Leishmaniasis. *Acta Tropica* **2001**, *78* (1), 11–16. https://doi.org/10.1016/S0001-706X(00)00155-8.
56. Riera, C.; Fisa, R.; Lopez, P.; Ribera, E.; Carrió, J.; Falcó, V.; Molina, I.; Gállego, M.; Portús, M. Evaluation of a Latex Agglutination Test (KAtex) for Detection of Leishmania Antigen in Urine of Patients with HIV-Leishmania Coinfection: Value in Diagnosis and Post-Treatment Follow-Up. *Eur J Clin Microbiol Infect Dis* **2004**, *23* (12), 899–904. https://doi.org/10.1007/s10096-004-1249-7.
57. Hatam, G. R.; Ghatee, M. A.; Hossini, S. M. H.; Sarkari, B. Improvement of the Newly Developed Latex Agglutination Test (Katex) for Diagnosis of Visceral Lieshmaniasis. *Journal of Clinical Laboratory Analysis* **2009**, *23* (4), 202–205. https://doi.org/10.1002/jcla.20312.
58. Molina, R.; Jiménez, M.; García-Martínez, J.; Martín, J. V. S.; Carrillo, E.; Sánchez, C.; Moreno, J.; Alves, F.; Alvar, J. Role of Asymptomatic and Symptomatic Humans as Reservoirs of Visceral Leishmaniasis in a Mediterranean Context. *PLOS Neglected Tropical Diseases* **2020**, *14* (4), e0008253. https://doi.org/10.1371/journal.pntd.0008253.

59. Medrano, F. J.; Cañavate, C.; Leal, M.; Rey, C.; Lissen, E.; Alvar, J. The Role of Serology in the Diagnosis and Prognosis of Visceral Leishmaniasis in Patients Coinfected with Human Immunodeficiency Virus Type-1. *Am J Trop Med Hyg* **1998**, *59* (1), 155–162. https://doi.org/10.4269/ajtmh.1998.59.155.
60. Cota, G. F.; Sousa, M. R. de; Demarqui, F. N.; Rabello, A. The Diagnostic Accuracy of Serologic and Molecular Methods for Detecting Visceral Leishmaniasis in HIV Infected Patients: Meta-Analysis. *PLOS Neglected Tropical Diseases* **2012**, *6* (5), e1665. https://doi.org/10.1371/journal.pntd.0001665.
61. Antinori, S.; Cascio, A.; Parravicini, C.; Bianchi, R.; Corbellino, M. Leishmaniasis among Organ Transplant Recipients. *The Lancet Infectious Diseases* **2008**, *8* (3), 191–199. https://doi.org/10.1016/S1473-3099(08)70043-4.
62. Maia, Z.; Lírio, M.; Mistro, S.; Mendes, C. M. C.; Mehta, S. R.; Badaro, R. Comparative Study of RK39 Leishmania Antigen for Serodiagnosis of Visceral Leishmaniasis: Systematic Review with Meta-Analysis. *PLOS Neglected Tropical Diseases* **2012**, *6* (1), e1484. https://doi.org/10.1371/journal.pntd.0001484.
63. Bern, C.; Jha, S. N.; Joshi, A. B.; Thakur, G. D.; Bista, M. B. Use of the Recombinant K39 Dipstick Test and the Direct Agglutination Test in a Setting Endemic for Visceral Leishmaniasis in Nepal. *The American Journal of Tropical Medicine and Hygiene* **2000**, *63* (3), 153–157. https://doi.org/10.4269/ajtmh.2000.63.153.
64. Gao, C.; Yang, Y.; Shi, F.; Wang, J.; Steverding, D.; Wang, X. Development of an Immunochromatographic Test for Diagnosis of Visceral Leishmaniasis Based on Detection of a Circulating Antigen. *PLOS Neglected Tropical Diseases* **2015**, *9* (6), e0003902. https://doi.org/10.1371/journal.pntd.0003902.
65. Mikaeili, F.; Fakhar, M.; Sarkari, B.; Motazedian, M. H.; Hatam, G. Comparison of Serological Methods (ELISA, DAT and IFA) for Diagnosis of Visceral Leishmaniasis Utilizing an Endemic Strain. *Iran J Immunol* **2007**, *4* (2), 116–121.
66. Mniouil, M.; Fellah, H.; Amarir, F.; Sadak, A.; Et-touys, A.; Bakri, Y.; Moustachi, A.; Tassou, F. Z.; Hida, M.; Lyagoubi, M.; Adlaoui, E. B.; Rhajaoui, M.; Sebti, F. Comparative Evaluation of Immunochromatographic Dipstick Test (ICT) Rk39, Soluble Antigen ELISA and IFAT for the Sero-Diagnosis of Visceral Leishmaniasis in Morocco. *Acta Tropica* **2018**, *182*, 185–189. https://doi.org/10.1016/j.actatropica.2018.03.007.
67. Alam, M. J.; Rahman, K. M.; Asna, S. M.; Muazzam, N.; Ahmed, I.; Chowdhury, M. Z. Comparative Studies on IFAT, ELISA & DAT for Serodiagnosis of Visceral Leishmaniasis in Bangladesh. *Bangladesh Med Res Counc Bull* **1996**, *22* (1), 27–32.
68. Pintado, V.; Martín-Rabadán, P.; Rivera, M. L.; Moreno, S.; Bouza, E. Visceral Leishmaniasis in Human Immunodeficiency Virus (HIV)-Infected and Non-HIV-Infected Patients: A Comparative Study. *Medicine* **2001**, *80* (1), 54–73. https://doi.org/10.1097/00005792-200101000-00006.
69. Antinori, S.; Calattini, S.; Longhi, E.; Bestetti, G.; Piolini, R.; Magni, C.; Orlando, G.; Gramiccia, M.; Acquaviva, V.; Foschi, A.; Corvasce, S.; Colomba, C.; Titone, L.; Parravicini, C.; Cascio, A.; Corbellino, M. Clinical Use of Polymerase Chain Reaction Performed on Peripheral Blood and Bone Marrow Samples for the Diagnosis and Monitoring of Visceral Leishmaniasis in HIV-Infected and HIV-Uninfected Patients: A Single-Center, 8-Year Experience in Italy and Review of the Literature. *Clinical Infectious Diseases* **2007**, *44* (12), 1602–1610. https://doi.org/10.1086/518167.
70. Moreira, M. A. B.; Luvizotto, M. C. R.; Garcia, J. F.; Corbett, C. E. P.; Laurenti, M. D. Comparison of Parasitological, Immunological and Molecular Methods for the Diagnosis of Leishmaniasis in Dogs with Different Clinical Signs. *Veterinary Parasitology* **2007**, *145* (3), 245–252. https://doi.org/10.1016/j.vetpar.2006.12.012.
71. Sudarshan, M.; Sundar, S. Parasite Load Estimation by QPCR Differentiates between Asymptomatic and Symptomatic Infection in Indian Visceral Leishmaniasis. *Diagnostic Microbiology and Infectious Disease* **2014**, *80* (1), 40–42. https://doi.org/10.1016/j.diagmicrobio.2014.01.031.
72. Manna, L.; Reale, S.; Vitale, F.; Picillo, E.; Pavone, L. M.; Gravino, A. E. Real-Time PCR Assay in Leishmania-Infected Dogs Treated with Meglumine Antimoniate and Allopurinol. *The Veterinary Journal* **2008**, *177* (2), 279–282. https://doi.org/10.1016/j.tvjl.2007.04.013.
73. da Costa Lima, M. S.; Zorzenon, D. C. R.; Dorval, M. E. C.; Pontes, E. R. J. C.; Oshiro, E. T.; Cunha, R.; Andreotti, R.; de Fatima Cepa Matos, M. Sensitivity of PCR and Real-Time PCR for the Diagnosis of Human Visceral Leishmaniasis Using Peripheral Blood. *Asian Pacific Journal of Tropical Disease* **2013**, *3* (1), 10–15. https://doi.org/10.1016/S2222-1808(13)60003-1.
74. Colombo, F. A.; Pereira-Chioccola, V. L.; Meira, C. da S.; Motoie, G.; Gava, R.; Hiramoto, R. M.; de Almeida, M. E.; da Silva, A. J.; Cutolo, A. A.; Menz, I. Performance of a Real Time PCR for Leishmaniasis Diagnosis Using a L. (L.) Infantum Hypothetical Protein as Target in Canine Samples. *Experimental Parasitology* **2015**, *157*, 156–162. https://doi.org/10.1016/j.exppara.2015.08.014.

75. Michel, G.; Pomares, C.; Ferrua, B.; Marty, P. Importance of Worldwide Asymptomatic Carriers of Leishmania Infantum (L. Chagasi) in Human. *Acta Trop* **2011**, *119* (2–3), 69–75. https://doi.org/10.1016/j.actatropica.2011.05.012.
76. Murray, H. W.; Berman, J. D.; Davies, C. R.; Saravia, N. G. Advances in Leishmaniasis. *The Lancet* **2005**, *366* (9496), 1561–1577. https://doi.org/10.1016/S0140-6736(05)67629-5.
77. Kushwaha, V.; Kaur, S. Lymphatic Filariasis and Visceral Leishmaniasis Coinfection: A Review on Their Epidemiology, Therapeutic, and Immune Responses. *Acta Tropica* **2021**, *224*, 106117. https://doi.org/10.1016/j.actatropica.2021.106117.
78. Bogdan, C. Mechanisms and Consequences of Persistence of Intracellular Pathogens: Leishmaniasis as an Example. *Cellular Microbiology* **2008**, *10* (6), 1221–1234. https://doi.org/10.1111/j.1462-5822.2008.01146.x.
79. Ortiz, A. M.; Silvestri, G. Immunopathogenesis of AIDS. *Curr Infect Dis Rep* **2009**, *11* (3), 239–245. https://doi.org/10.1007/s11908-009-0035-1.
80. Andreani, G.; Lodge, R.; Richard, D.; Tremblay, M. J. Mechanisms of Interaction between Protozoan Parasites and HIV. *Current Opinion in HIV and AIDS* **2012**, *7* (3), 275. https://doi.org/10.1097/COH.0b013e32835211e9.
81. Santos-Oliveira, J. R.; Giacoia-Gripp, C. B.; Alexandrino de Oliveira, P.; Amato, V. S.; Lindoso, J. Â. L.; Goto, H.; Oliveira-Neto, M. P.; Mattos, M. S.; Grinsztejn, B.; Morgado, M. G.; Da-Cruz, A. M. High Levels of T Lymphocyte Activation in Leishmania-HIV-1 Co-Infected Individuals despite Low HIV Viral Load. *BMC Infect Dis* **2010**, *10*, 358. https://doi.org/10.1186/1471-2334-10-358.
82. Chinen, J.; Buckley, R. H. Transplantation Immunology: Solid Organ and Bone Marrow. *Journal of Allergy and Clinical Immunology* **2010**, *125* (2), S324–S335. https://doi.org/10.1016/j.jaci.2009.11.014.
83. Puig, L.; Pradinaud, R. Leishmania and HIV Co-Infection: Dermatological Manifestations. *Annals of Tropical Medicine & Parasitology* **2003**, *97* (sup1), 107–114. https://doi.org/10.1179/000349803225002589.
84. Jeziorski, E.; Blanchet, C.; Ludwig, C.; Lalande, M.; Coste, V.; Dereure, J.; Rodière, M. Récidive sous forme pseudotumorale d'une leishmaniose viscérale. *Archives de Pédiatrie* **2009**, *16*, S129–S131. https://doi.org/10.1016/S0929-693X(09)75316-3.
85. Ritmeijer, K.; Veeken, H.; Melaku, Y.; Leal, G.; Amsalu, R.; Seaman, J.; Davidson, R. N. Ethiopian Visceral Leishmaniasis: Generic and Proprietary Sodium Stibogluconate Are Equivalent; HIV Co-Infected Patients Have a Poor Outcome. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **2001**, *95* (6), 668–672. https://doi.org/10.1016/S0035-9203(01)90110-5.
86. Rihl, M.; Stoll, M.; Ulbricht, K.; Bange, F.-C.; Schmidt, R.-E. Successful Treatment of Post-Kala-Azar Dermal Leishmaniasis (PKDL) in a HIV Infected Patient with Multiple Relapsing Leishmaniasis from Western Europe. *Journal of Infection* **2006**, *53* (1), e25–e27. https://doi.org/10.1016/j.jinf.2005.09.015.
87. Stark, D.; Pett, S.; Marriott, D.; Harkness, J. Post-Kala-Azar Dermal Leishmaniasis Due to Leishmania Infantum in a Human Immunodeficiency Virus Type 1-Infected Patient. *J Clin Microbiol* **2006**, *44* (3), 1178–1180. https://doi.org/10.1128/JCM.44.3.1178-1180.2006.
88. Sakkas, L. I.; Boulbou, M.; Kyriakou, D.; Makri, I.; Sinani, C.; Germenis, A.; Stathakis, N. Immunological Features of Visceral Leishmaniasis May Mimic Systemic Lupus Erythematosus. *Clinical Biochemistry* **2008**, *41* (1), 65–68. https://doi.org/10.1016/j.clinbiochem.2007.10.008.
89. Atta, A. M.; Carvalho, E. M.; Jerônimo, S. M. B.; Sousa Atta, M. L. B. Serum Markers of Rheumatoid Arthritis in Visceral Leishmaniasis: Rheumatoid Factor and Anti-Cyclic Citrullinated Peptide Antibody. *Journal of Autoimmunity* **2007**, *28* (1), 55–58. https://doi.org/10.1016/j.jaut.2006.12.001.
90. Voulgari, P.; Pappas, G.; Liberopoulos, E.; Elisaf, M.; Skopouli, F.; Drosos, A. Visceral Leishmaniasis Resembling Systemic Lupus Erythematosus. *Ann Rheum Dis* **2004**, *63* (10), 1348–1349. https://doi.org/10.1136/ard.2003.014480.
91. Venizelos, I.; Tatsiou, Z.; Papathomas, T. G.; Orazi, A. Visceral Leishmaniasis in a Rheumatoid Arthritis Patient Treated with Methotrexate. *International Journal of Infectious Diseases* **2009**, *13* (4), e169–e172. https://doi.org/10.1016/j.ijid.2008.09.012.
92. Ciaramella, P.; Oliva, G.; De Luna, R.; Ambrosio, R.; Cortese, L.; Persechino, A.; Gradoni, L.; Scalone, A. A Retrospective Clinical Study of Canine Leishmaniasis in 150 Dogs Naturally Infected by Leishmania Infantum. *Veterinary Record* **1997**, *141* (21), 539–543. https://doi.org/10.1136/vr.141.21.539.
93. Chagas, Ú. M. R.; de Avelar, D. M.; Marcelino, A. P.; Paz, G. F.; Gontijo, C. M. F. Correlations between Tissue Parasite Load and Common Clinical Signs in Dogs Naturally Infected by Leishmania Infantum. *Veterinary Parasitology* **2021**, *291*, 109368. https://doi.org/10.1016/j.vetpar.2021.109368.

94. Baneth, G.; Koutinas, A. F.; Solano-Gallego, L.; Bourdeau, P.; Ferrer, L. Canine Leishmaniosis – New Concepts and Insights on an Expanding Zoonosis: Part One. *Trends in Parasitology* **2008**, *24* (7), 324–330. https://doi.org/10.1016/j.pt.2008.04.001.
95. Baneth, G. Leishmaniasis. Greene: Infectious diseases of the dog and cat, 3rd Ed. Saunders Elsevier, Saint Louis, **2006**, pp. 685–698.
96. Laurenti, M. D.; Rossi, C. N.; Matta, V. L. R. da; Tomokane, T. Y.; Corbett, C. E. P.; Secundino, N. F. C.; Pimenta, P. F. P.; Marcondes, M. Asymptomatic Dogs Are Highly Competent to Transmit Leishmania (Leishmania) Infantum Chagasi to the Natural Vector. *Veterinary Parasitology* **2013**, *196* (3), 296–300. https://doi.org/10.1016/j.vetpar.2013.03.017.
97. Silva, F. M. de F.; Santos, E. M. de S.; Torres, S. M.; Yamasak, E. M.; Ramos, R. A. N.; Alves, L. C. Parasite Load in Intact and Ulcerative Skin of Dogs with Leishmaniais. *Rev. Bras. Parasitol. Vet.* **2016**, *25*, 127–130. https://doi.org/10.1590/S1984-29612016014.
98. HOSEIN, S.; BLAKE, D. P.; SOLANO-GALLEGO, L. Insights on Adaptive and Innate Immunity in Canine Leishmaniosis. *Parasitology* **2017**, *144* (1), 95–115. https://doi.org/10.1017/S003118201600055X.
99. Almeida, B. F. M.; Narciso, L. G.; Bosco, A. M.; Pereira, P. P.; Braga, E. T.; Avanço, S. V.; Marcondes, M.; Ciarlini, P. C. Neutrophil Dysfunction Varies with the Stage of Canine Visceral Leishmaniosis. *Veterinary Parasitology* **2013**, *196* (1), 6–12. https://doi.org/10.1016/j.vetpar.2013.02.016.
100. Regli, I. B.; Passelli, K.; Hurrell, B. P.; Tacchini-Cottier, F. Survival Mechanisms Used by Some Leishmania Species to Escape Neutrophil Killing. *Frontiers in Immunology* **2017**, *8*.
101. Solano-Gallego, L.; Llull, J.; Ramos, G.; Riera, C.; Arboix, M.; Alberola, J.; Ferrer, L. The Ibizian Hound Presents a Predominantly Cellular Immune Response against Natural Leishmania Infection. *Veterinary Parasitology* **2000**, *90* (1), 37–45. https://doi.org/10.1016/S0304-4017(00)00223-5.
102. Martínez-Moreno, A.; Moreno, T.; Martínez-Moreno, F. J.; Acosta, I.; Hernández, S. Humoral and Cell-Mediated Immunity in Natural and Experimental Canine Leishmaniasis. *Veterinary Immunology and Immunopathology* **1995**, *48* (3), 209–220. https://doi.org/10.1016/0165-2427(95)05434-8.
103. De Luna, R.; Vuotto, M. L.; Ielpo, M. T. L.; Ambrosio, R.; Piantedosi, D.; Moscatiello, V.; Ciaramella, P.; Scalone, A.; Gradoni, L.; Mancino, D. Early Suppression of Lymphoproliferative Response in Dogs with Natural Infection by Leishmania Infantum. *Veterinary Immunology and Immunopathology* **1999**, *70* (1), 95–103. https://doi.org/10.1016/S0165-2427(99)00073-2.
104. Santos-Gomes, G. M.; Rosa, R.; Leandro, C.; Cortes, S.; Romão, P.; Silveira, H. Cytokine Expression during the Outcome of Canine Experimental Infection by Leishmania Infantum. *Veterinary Immunology and Immunopathology* **2002**, *88* (1), 21–30. https://doi.org/10.1016/S0165-2427(02)00134-4.
105. Ramiro, M. J.; Zárate, J. J.; Hanke, T.; Rodriguez, D.; Rodriguez, J. R.; Esteban, M.; Lucientes, J.; Castillo, J. A.; Larraga, V. Protection in Dogs against Visceral Leishmaniasis Caused by Leishmania Infantum Is Achieved by Immunization with a Heterologous Prime-Boost Regime Using DNA and Vaccinia Recombinant Vectors Expressing LACK. *Vaccine* **2003**, *21* (19), 2474–2484. https://doi.org/10.1016/S0264-410X(03)00032-X.
106. Rodriguez-Cortes, A.; Martori, C.; Martinez-Florez, A.; Clop, A.; Amills, M.; Kubejko, J.; Llull, J.; Nadal, J. M.; Alberola, J. Canine Leishmaniasis Progression Is Associated with Vitamin D Deficiency. *Sci Rep* **2017**, *7* (1), 3346. https://doi.org/10.1038/s41598-017-03662-4.
107. Toepp, A. J.; Petersen, C. A. The Balancing Act: Immunology of Leishmaniosis. *Research in Veterinary Science* **2020**, *130*, 19–25. https://doi.org/10.1016/j.rvsc.2020.02.004.
108. Strauss-Ayali, D.; Baneth, G.; Shor, S.; Okano, F.; Jaffe, C. L. Interleukin-12 Augments a Th1-Type Immune Response Manifested as Lymphocyte Proliferation and Interferon Gamma Production in Leishmania Infantum-Infected Dogs. *International Journal for Parasitology* **2005**, *35* (1), 63–73. https://doi.org/10.1016/j.ijpara.2004.10.015.
109. Quinnell, R. J.; Courtenay, O.; Shaw, M.-A.; Day, M. J.; Garcez, L. M.; Dye, C.; Kaye, P. M. Tissue Cytokine Responses in Canine Visceral Leishmaniasis. *The Journal of Infectious Diseases* **2001**, *183* (9), 1421–1424. https://doi.org/10.1086/319869.
110. Deplazes, P.; Smith, N. C.; Arnold, P.; Lutz, H.; Eckert, J. Specific LgG1 and LgG2 Antibody Responses of Dogs to Leishmania Infantum and Other Parasites. *Parasite Immunology* **1995**, *17* (9), 451–458. https://doi.org/10.1111/j.1365-3024.1995.tb00914.x.
111. Barbiéri, C. L. Immunology of Canine Leishmaniasis. *Parasite Immunology* **2006**, *28* (7), 329–337. https://doi.org/10.1111/j.1365-3024.2006.00840.x.

112. Altet, L.; Francino, O.; Solano-Gallego, L.; Renier, C.; Sánchez, A. Mapping and Sequencing of the Canine NRAMP1 Gene and Identification of Mutations in Leishmaniasis-Susceptible Dogs. *Infect Immun* **2002**, *70* (6), 2763–2771. https://doi.org/10.1128/IAI.70.6.2763-2771.2002.
113. Quinnell, R. J.; Kennedy, L. J.; Barnes, A.; Courtenay, O.; Dye, C.; Garcez, L. M.; Shaw, M.-A.; Carter, S. D.; Thomson, W.; Ollier, W. E. R. Susceptibility to Visceral Leishmaniasis in the Domestic Dog Is Associated with MHC Class II Polymorphism. *Immunogenetics* **2003**, *55* (1), 23–28. https://doi.org/10.1007/s00251-003-0545-1.
114. Martínez-Orellana, P.; Marí-Martorell, D.; Montserrat-Sangrà, S.; Ordeix, L.; Baneth, G.; Solano-Gallego, L. Leishmania Infantum-Specific IFN-γ Production in Stimulated Blood from Dogs with Clinical Leishmaniosis at Diagnosis and during Treatment. *Veterinary Parasitology* **2017**, *248*, 39–47. https://doi.org/10.1016/j.vetpar.2017.10.018.
115. Solano-Gallego, L.; Montserrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. Leishmania Infantum-Specific Production of IFN-γ and IL-10 in Stimulated Blood from Dogs with Clinical Leishmaniosis. *Parasites Vectors* **2016**, *9* (1), 317. https://doi.org/10.1186/s13071-016-1598-y.
116. Zribi, L.; El-Goulli, A. F.; Ben-Abid, M.; Gharbi, M.; Ben-Sghaier, I.; Boufaden, I.; Aoun, K.; Bouratbine, A. Use of an Interferon Gamma Release Assay (IGRA) to Test T-Cell Responsiveness to Soluble Leishmania Infantum Antigen in Whole Blood of Dogs from Endemic Areas. *Veterinary Parasitology* **2017**, *246*, 88–92. https://doi.org/10.1016/j.vetpar.2017.08.029.
117. Lombardo, G.; Pennisi, M. G.; Lupo, T.; Migliazzo, A.; Caprì, A.; Solano-Gallego, L. Detection of Leishmania Infantum DNA by Real-Time PCR in Canine Oral and Conjunctival Swabs and Comparison with Other Diagnostic Techniques. *Veterinary Parasitology* **2012**, *184* (1), 10–17. https://doi.org/10.1016/j.vetpar.2011.08.010.
118. Oliva, G.; Scalone, A.; Foglia Manzillo, V.; Gramiccia, M.; Pagano, A.; Di Muccio, T.; Gradoni, L. Incidence and Time Course of Leishmania Infantum Infections Examined by Parasitological, Serologic, and Nested-PCR Techniques in a Cohort of Naïve Dogs Exposed to Three Consecutive Transmission Seasons. *Journal of Clinical Microbiology* **2006**, *44* (4), 1318–1322. https://doi.org/10.1128/JCM.44.4.1318-1322.2006.
119. Alexandre-Pires, G.; de Brito, M. T. V.; Algueró, C.; Martins, C.; Rodrigues, O. R.; da Fonseca, I. P.; Santos-Gomes, G. Canine Leishmaniosis. Immunophenotypic Profile of Leukocytes in Different Compartments of Symptomatic, Asymptomatic and Treated Dogs. *Veterinary Immunology and Immunopathology* **2010**, *137* (3), 275–283. https://doi.org/10.1016/j.vetimm.2010.06.007.
120. Solcà, M. S.; Andrade, B. B.; Abbehusen, M. M. C.; Teixeira, C. R.; Khouri, R.; Valenzuela, J. G.; Kamhawi, S.; Bozza, P. T.; Fraga, D. B. M.; Borges, V. M.; Veras, P. S. T.; Brodskyn, C. I. Circulating Biomarkers of Immune Activation, Oxidative Stress and Inflammation Characterize Severe Canine Visceral Leishmaniasis. *Sci Rep* **2016**, *6* (1), 32619. https://doi.org/10.1038/srep32619.
121. Boggiatto, P. M.; Ramer-Tait, A. E.; Metz, K.; Kramer, E. E.; Gibson-Corley, K.; Mullin, K.; Hostetter, J. M.; Gallup, J. M.; Jones, D. E.; Petersen, C. A. Immunologic Indicators of Clinical Progression during Canine Leishmania Infantum Infection. *Clin Vaccine Immunol* **2010**, *17* (2), 267–273. https://doi.org/10.1128/CVI.00456-09.
122. Pinelli, E.; Killick-Kendrick, R.; Wagenaar, J.; Bernadina, W.; del Real, G.; Ruitenberg, J. Cellular and Humoral Immune Responses in Dogs Experimentally and Naturally Infected with Leishmania Infantum. *Infection and Immunity* **1994**, *62* (1), 229–235. https://doi.org/10.1128/iai.62.1.229-235.1994.
123. Pinelli, E.; Gonzalo, R. M.; Boog, C. J. P.; Rutten, V. P. M. G.; Gebhard, D.; Del Real, G.; Ruitenberg, E. J. Leishmania Infantum-Specific T Cell Lines Derived from Asymptomatic Dogs That Lyse Infected Macrophages in a Major Histocompatibility Complex-Restricted Manner. *European Journal of Immunology* **1995**, *25* (6), 1594–1600. https://doi.org/10.1002/eji.1830250619.
124. Priolo, V.; Martínez-Orellana, P.; Pennisi, M. G.; Raya-Bermúdez, A. I.; Jurado-Tarifa, E.; Masucci, M.; Donato, G.; Bruno, F.; Castelli, G.; Solano-Gallego, L. Leishmania Infantum Specific Humoral and Cellular Immune Responses in Cats and Dogs: A Comparative Cross-Sectional Study. *Veterinary Sciences* **2022**, *9* (9), 482. https://doi.org/10.3390/vetsci9090482.
125. Mary, C.; Faraut, F.; Lascombe, L.; Dumon, H. Quantification of Leishmania Infantum DNA by a Real-Time PCR Assay with High Sensitivity. *Journal of Clinical Microbiology* **2004**, *42* (11), 5249–5255. https://doi.org/10.1128/JCM.42.11.5249-5255.2004.
126. Rolão, N.; Cortes, S.; Rodrigues, O. R.; Campino, L. QUANTIFICATION OF LEISHMANIA INFANTUM PARASITES IN TISSUE BIOPSIES BY REAL-TIME POLYMERASE CHAIN REACTION AND

POLYMERASE CHAIN REACTION–ENZYME-LINKED IMMUNOSORBENT ASSAY. *para* **2004**, *90* (5), 1150–1154. https://doi.org/10.1645/GE-264R1.

127. Akhoundi, M.; Downing, T.; Votýpka, J.; Kuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; Granouillac, B.; Gradoni, L.; Sereno, D. Leishmania Infections: Molecular Targets and Diagnosis. *Molecular Aspects of Medicine* **2017**, *57*, 1–29. https://doi.org/10.1016/j.mam.2016.11.012.
128. Nylén, S.; Sacks, D. Interleukin-10 and the Pathogenesis of Human Visceral Leishmaniasis. *Trends in Immunology* **2007**, *28* (9), 378–384. https://doi.org/10.1016/j.it.2007.07.004.
129. Solano-Gallego, L.; Cardoso, L.; Pennisi, M. G.; Petersen, C.; Bourdeau, P.; Oliva, G.; Miró, G.; Ferrer, L.; Baneth, G. Diagnostic Challenges in the Era of Canine Leishmania Infantum Vaccines. *Trends in Parasitology* **2017**, *33* (9), 706–717. https://doi.org/10.1016/j.pt.2017.06.004.
130. Toepp, A.; Larson, M.; Grinnage-Pulley, T.; Bennett, C.; Anderson, M.; Parrish, M.; Fowler, H.; Wilson, G.; Gibson-Corely, K.; Gharpure, R.; Cotter, C.; Petersen, C. Safety Analysis of Leishmania Vaccine Used in a Randomized Canine Vaccine/Immunotherapy Trial. *The American Journal of Tropical Medicine and Hygiene* **2018**, *98* (5), 1332–1338. https://doi.org/10.4269/ajtmh.17-0888.
131. Toepp, A. J.; Monteiro, G. R. G.; Coutinho, J. F. V.; Lima, A. L.; Larson, M.; Wilson, G.; Grinnage-Pulley, T.; Bennett, C.; Mahachi, K.; Anderson, B.; Ozanne, M. V.; Anderson, M.; Fowler, H.; Parrish, M.; Willardson, K.; Saucier, J.; Tyrell, P.; Palmer, Z.; Buch, J.; Chandrashekar, R.; Brown, G. D.; Oleson, J. J.; Jeronimo, S. M. B.; Petersen, C. A. Comorbid Infections Induce Progression of Visceral Leishmaniasis. *Parasites & Vectors* **2019**, *12* (1), 54. https://doi.org/10.1186/s13071-019-3312-3.