Chapter 11

Biomarkers in Leishmaniasis: From Basic Research to Clinical Application

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Abstract

Leishmania is an intracellular protozoan parasite and the etiological agent of a vector-borne disease known as leishmaniasis. This neglected tropical disease exhibits high morbidity and mortality putting at risk people from multiple countries worldwide. It is endemic in 97 countries and 700,000–1 million new cases are estimated to occur each year. Leishmaniasis management is very challenging, the symptoms are non-pathognomonic (in both human and canine populations) and the treatments are associated with significant toxicity. Therefore, the need for detection in symptomatic and asymptomatic hosts is important to tackle the dissemination of infection, increasing the need for highly specific biomarkers. In this complex the available disease biomarkers will be addressed in a retrospective manner, focusing on their development from laboratory to their direct use in clinical settings.

Keywords: leishmaniasis, biomarkers, diagnosis techniques

1. Introduction

1.1. Leishmaniasis as a neglected tropical disease

Neglected tropical diseases are a diverse group of diseases that prevail in tropical and subtropical countries. The geographical distribution of these diseases associated with lack of sanitation, close contact with domestic animals, livestock and infectious vectors contributes to disease dissemination, affecting more than 1 billion people and costs billions each year in developing economies.

Leishmaniasis is a devastating and significantly under-recognized vector-borne disease that causes serious global health burden. After malaria, it is the second parasitic disease with the highest mortality rate [1]. It mainly affects populations in Africa, Asia and Latin America, and is associated with malnutrition, population displacement, poor housing, weak immune system and lack of resources [1].

Although this disease was initially limited to the tropics and subtropics, several factors lead to the spread of leishmaniasis to new locations [2]. Perturbations of the natural vector ecosystem caused by urbanization, deforestation, global warming and natural disasters have a serious impact in disease dissemination. Increased people and animal migration, traveling and military operations contribute to disease spreading. Organ transplantation, lack of *Leishmania* screening in the blood bank or immune suppressive therapies can also contribute to disease perpetuation [2, 3].

This disease can be presented in several ways, the most common ones being: Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL) and Visceral Leishmaniasis (VL), also known as kala-azar. Depending on the specific clinic-pathological form of the disease, symptoms range from cutaneous and mucosal tissue lesions to vital visceral organ damage [4, 5].

1.2. Epidemiology

Out of 200 countries and territories reporting to WHO, 97 are endemic for leishmaniasis (**Figure 1**). This includes 65 countries endemic for both VL and CL, 10 countries endemic only for VL and 22 endemic only for CL (**Figure 1**) [1].

Ninety percent of global VL cases were reported from seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. About 95% of CL cases occur in the Americas, the

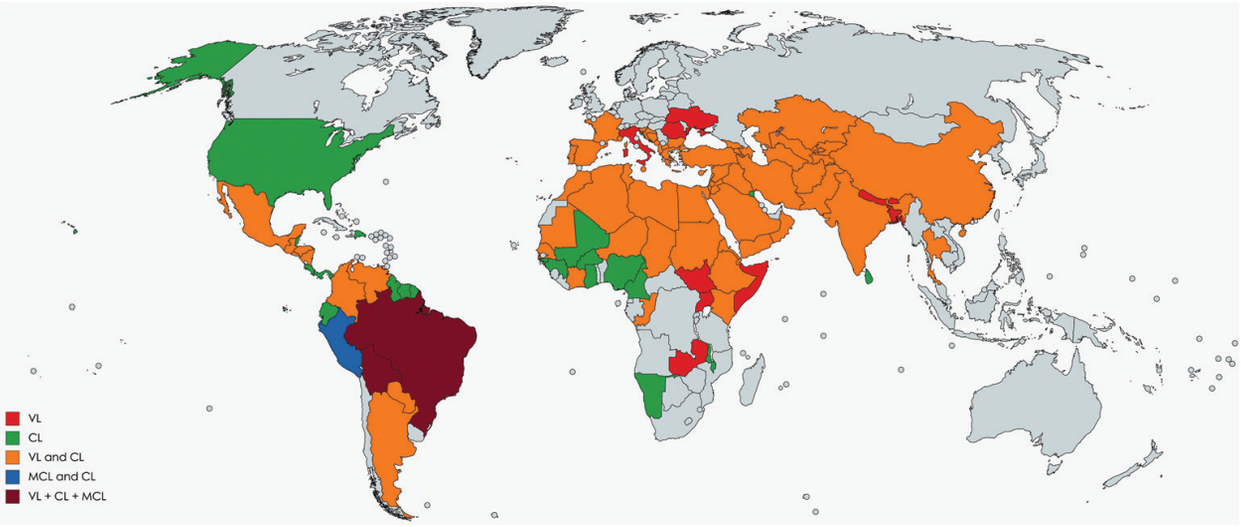

Figure 1. World map: Human leishmaniasis distribution. VL, Visceral Leishmaniasis; CL, Cutaneous Leishmaniasis; MCL, Mucocutaneous Leishmaniasis. Status of endemicity of the different forms of leishmaniasis in 2016, according to the World Health Organization.

Mediterranean basin, Middle East and Central Asia. An estimated 0.6–1 million new cases occur worldwide annually. Over 90% of MCL cases occur in Bolivia, Brazil, Ethiopia and Peru [1].

1.3. Clinical forms of the disease

CL is the most common form of leishmaniasis affecting humans. It is a skin infection that develops as a nodule in the site of inoculation, consequence of macrophage infection in the dermis. This may progress to a dermal granuloma, an ulcer that heals spontaneously or several inflamed ulcers, on the exposed parts of the body, such as the face, arms and legs [1, 5, 6]. These lesions can take months or years to heal and may leave behind atrophic scars with a hyperpigmented halo [2]. The incubation period ranges from 1 week to several months [1].

MCL is less common than CL. Unlike CL, which is confined to small areas of the skin, MCL aggressively spreads to the oral and nasal mucosa and is characterized by progressive ulcers and lesions of the face leading to significant disfiguration. The lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth, throat cavities and surrounding tissues [1]. These progressively destructive ulcers, unlike CL, are not self-healing and can appear months or years after the first episode of CL. The dynamic and evolution of MCL is not completely understood [4, 7]. In endemic areas, it has been noted that in 1–10% of patients, the CL form evolves into MCL 5 years after healing [3].

VL, also known as kala-azar, is the most severe form of leishmaniasis and is fatal if left untreated in over 95% of cases [1, 8]. An incubation period (from 1 month to 2 years) is followed by clinical manifestations such as fever, splenomegaly, hepatomegaly, weight loss, progressive anemia and pancytopenia. These systemic infection symptoms are associated to the dissemination of parasites throughout the blood and reticuloendothelial system and lead to enlarged lymph nodes, spleen and liver [5].

Post-kala-azar Dermal Leishmaniasis (PKDL) is a complication of VL in areas where *Leishmania donovani* (*L. donovani*) is endemic. It is characterized by a hypopigmented macular, maculopapular, and nodular rash in a patient suffering a sequel of VL where the skin is the main focus of the infection. It usually appears 6 months to 1 or more years after apparent cure of the disease but may occur earlier or even simultaneously with visceral leishmaniasis [1, 9]. PKDL is confined to two geographically distinct zones, South Asia (India, Nepal, and Bangladesh) and East Africa (Sudan). In the South Asian variant, polymorphic lesions (macules/patches with papulonodules) are frequent, while the Sudanese variant has papular or nodular lesions. Lesions, especially the papulonodules, are parasite-rich, driving speculation that PKDL plays an important role in anthroponotic transmission of VL [10].

1.4. The vector

Leishmania protozoan parasites are transmitted by the bite of a very small (2–3 mm long) insect vector, known as the phlebotomine sandfly. Solely the female sandfly transmits the parasites, and only 30 from the 500 species of phlebotomine transmit this disease [1]. Female sandflies become infected with *Leishmania* parasites upon a blood meal from an infected person or

animal which they need for egg development. Over a period between 4 and 25 days, the parasites develop in the sandfly. This development period can be extended at low temperatures or shortened at high temperatures [3]. The transmission cycle is completed when the infectious female sandfly feeds and inoculates a host with the parasite [1].

1.5. Life cycle

The parasite life cycle requires the transmission of infectious protozoan between sandflies and a mammalian host. *Leishmania* parasites present two distinct forms: promastigotes and amastigotes. Promastigotes are the flagellated, motile forms of the parasites that develop and morphologically differentiate in the sandfly midgut to an infectious metacyclic form that is transmitted to the mammalian host when the sandfly takes a blood meal. These metacyclic promastigotes are phagocytosed by mono and polymorphonuclear cells and transformed into the ovoid shape with short flagellum known as amastigote. Upon differentiation, they replicate within the host cells. The replication may lead to the rupture of these cells, allowing infection of other phagocytes. The life cycle becomes complete when the sandfly takes another blood meal with amastigotes [11, 12].

Different *Leishmania* species are capable of causing disease and, depending on the species, the mammalian host presents different symptomatology. Although being divided by clinical symptoms, these species can also be divided by their geographical distribution in two main groups: Old World (the Eastern Hemisphere) species and New World species (caused by *Leishmania* species found in Central and South America) [2] (**Table 1**).

Location	Complex	Species	Clinical forms of the disease
Old World	*Leishmania donovani*	*L. donovani*	CL, VL, PKDL, MCL (rare)
		L. infantum	CL, VL (children), PKDL, MCL (rare)
	Leishmania tropica	*L. tropica*	CL, MCL (rare), VL (rare)
		L. major	CL, MCL (rare)
		L. aethiopica	CL, DCL
New World	*Leishmania mexicana*	*L. mexicana*	CL, DCL (rare)
		L. amazonensis	CL, DCL, MCL, VL (rare), PKLD (rare)
		L. venezuelensis	CL, DCL (rare)
	Leishmania (Viannia) braziliensis	*L. braziliensis*	CL, MCL, VL
		L. panamensis	CL, MCL
		L. guyanensis	CL, MCL
		L. peruviana	CL
	Leishmania donovani	*L. infantum*	CL, VL (children), PKDL, MCL (rare)

VL, Visceral Leishmaniasis; CL, Cutaneous Leishmaniasis; MCL, Mucocutaneous Leishmaniasis; PKDL, Post-Kala-azar Dermal Leishmaniasis; DCL, Diffused Cutaneous Leishmaniasis. Adapted from Kevric et al. [2].

Table 1. Geodistribution of the organisms prevalent and disease patterns.

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VL form, the most severe form of leishmaniasis, is caused by *L. donovani* (East Africa and the Indian subcontinent) and *L. infantum* (Europe, North Africa, Latin America). While *L. donovani* infects all age groups, *L. infantum* infects mostly children and immunosuppressed individuals. However, due to increasing prevalence of Human Immunodeficiency Virus (HIV) infection in the Mediterranean basin, HIV-VL coinfection in the adult population is reported frequently [13].

1.6. Transmission pattern

There are two types of disease transmission: zoonotic, where the disease is transmitted from animal to vector and then to human; and anthroponotic, transmitted from human to vector to human. Transmission also varies according to geographical regions, where *L. donovani* is responsible for anthroponotic transmission and *L. infantum* for zoonotic transmission. Traditionally, anthroponotic transmission occurs in the Indian subcontinent and East Africa, and zoonotic transmission is mostly restricted to the Mediterranean basin and South America regions.

L. donovani is found only in the Old World (except for transmission in patients that have traveled), where it is notoriously associated with VL of the rural poor areas in the northeast of the Indian subcontinent and with VL in East Africa [8, 14]. *L. infantum* is mostly associated to the zoonotic disease with the dog as a natural reservoir.

Domestic animals play a role as new opportunistic hosts, in vector adaptation to urban environments [3]. Animals such as the wild canid, marsupials and rodents have been described as reservoirs of human VL [15]. However, zoonotic VL epidemics have been associated only in areas where Canine Leishmaniasis (CanL) is endemic (**Figure 2**), indicating that dogs are the main reservoir in this type of transmission [16]. Due to the proximity of domestic dogs and human populations, CanL control is more than a veterinary concern, it is also a public health issue due to the zoonotic potential of the disease.

1.7. Canine leishmaniasis

CanL is a major concern for the veterinary community, having high mortality and morbidity rates. Moreover, in endemic areas, domestic dogs are the primary target of infection allowing perpetuation of the life cycle [17].

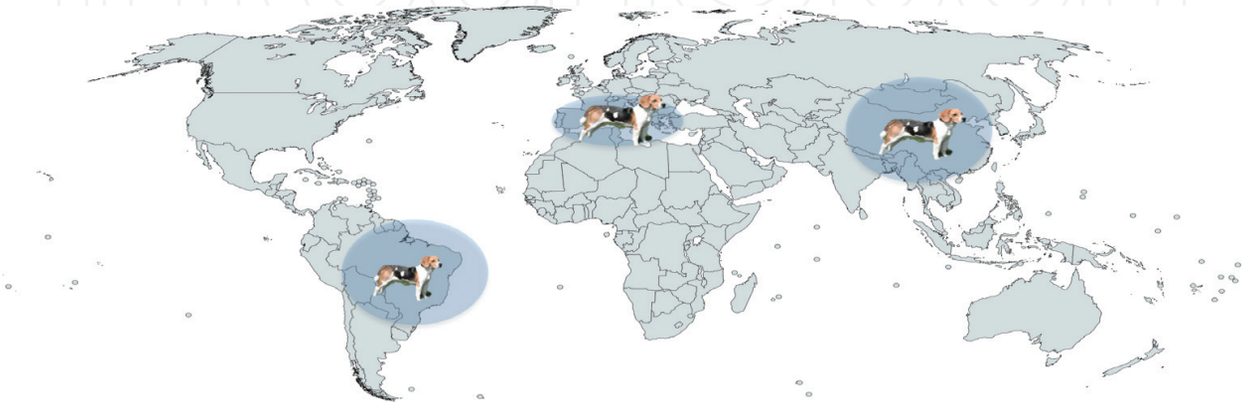

Figure 2. World map: Regions with higher canine leishmaniasis incidence – Brazil, Mediterranean basin and China.

After an incubation period that can range from 3 months to several years, dogs may start presenting clinical signs. However, some dogs remain asymptomatic and never develop any signs [17]. Therefore, dogs can present different forms of the disease: symptomatic, oligosymptomatic and asymptomatic. Dogs that develop few mild symptoms are classified as oligosymptomatic. Even so, it is relevant to mention that it is not consensual that all forms of the disease enable the transmission of the parasite. Infected dogs present non-specific cutaneous alterations such as alopecia, onychogryposis, dermatitis, skin ulceration, anorexia, weight loss and visceral manifestations with splenic, renal and hepatic disorders [18], making an accurate diagnosis impossible.

Given that canine disease usually precedes the appearance of human cases and a clear correlation between canine and human infection rates has been demonstrated, CanL must be considered as a risk for human health [3].

2. Importance of diagnosis

The available therapies for leishmaniasis are far from optimal due to their toxicity, high costs, lack of efficacy, lack of access in certain areas, and emerging drug resistance. Treatment efficacy depends on strains and species and there are currently no effective vaccines available for any form of human leishmaniasis [2].

Some vaccines exist for veterinary use. Being launched in Portugal in 2011, CaniLeish® was the first vaccine for CanL in the European Union. In Brazil, LeishTec® vaccine was also registered but only offers about 40% of protection against infection. LetiFend® has recently been registered in Europe, but there is limited information available [19]. Despite the efforts, *Leishmania* vaccinology still has a lot to improve till an effective and universal vaccine is developed [20].

In the absence of human vaccines and due to the zoonotic character of the disease, accurate detection of infection in humans and dogs is crucial for the control of leishmaniasis [21].

Several specific challenges associated to *Leishmania* infection and leishmaniasis must be overcome. As for any disease, the diagnostic process should be simple, robust, automated, requiring inexpensive reagents and minimal operator intervention without diminishing the fidelity of the results. Notwithstanding, considering that this disease affects mostly poor people in countries with undeveloped and underfinanced health systems, the tests should be cheap and easy to perform in field conditions [22, 23]. Moreover, the detection of asymptomatic infection, often characterized by reduced parasite loads and low specific serology, is essential. In fact, clinical and epidemiological management of *Leishmania* infections can only be fully successful once a diagnostic test with these characteristics is available [24]. The available tools are adequate for detection of disease, (in conjunction with clinical evaluation) but present limitations for diagnosis of infection in asymptomatic patients and dogs. Therefore, the information of real prevalence of infection and overall burden of disease is believed to be underestimated [22].

Coinfection with HIV is common in VL cases and has a disastrous impact since immunocompromised individuals have more severe manifestations and atypical symptoms that complicate treatment [2]. Pregnant women can be considered a risk population, as HIV-infected patients,

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being more susceptible to *Leishmania* relapse and changes in immune response. Immunosuppression by HIV had an enormous impact and highly contributed to the increased number of leishmaniasis cases [3]. Coinfection of *L. donovani* and *L. infantum* with HIV has been identified as a meaningful clinical problem, and presents higher mortality rates. Up to 70% of VL cases in southern Europe are associated with HIV infection. VL is the third most frequent opportunistic infection in many parts of the world, and the coinfection with HIV is now reported in 35 countries [25].

3. Biomarkers to use in diagnosis

Every methodology has a detection limit associated but the specificity of the diagnosis platform is mostly influenced by the antigen used, relying on it to produce high confidence results.

Several antigens were proposed overtime but the most common one is crude soluble antigen (CSA) [13, 26]. Although the sensitivity of CSA in Enzyme-Linked Immunosorbent Assay (ELISA) is high, cross-reactivity with other diseases (trypanosomiasis, toxoplasmosis and tuberculosis) occurs frequently, leading to false positive results [27, 28]. Therefore specificity can be low due to cross-reactivity and is not suitable in detecting seropositivity in asymptomatic dogs [29–31]. This is transversal to other serological techniques like Direct Agglutination Test (DAT) or Indirect Fluorescent Antibody Technique (IFAT) which are based on the recognition of the parasite total antigens. The specificity and sensitivity problems associated to conventional assays (such as IFAT, DAT, and CSA ELISA) may be overcome using recombinant polypeptides containing specific epitopes that are able to induce an immune response in most dogs and humans with VL. Hence, there has been a focus on expressing and purifying *Leishmania* proteins that elicit an immune response in dogs and humans and analyze them and their potential for serological tests [32–34].

The evolution of molecular technology (immunoproteomics) and sequencing of the *Leishmania* genome in 2005 have been powerful tools for the discovery of recombinant *Leishmania* proteins to use in the serodiagnosis of human and canine leishmaniasis [35, 36]. Variability in the humoral response concerning different parasite antigens observed in infection suggests that a combination of recombinant proteins can improve the diagnosis efficiency [37]. In fact, each antigen carries often both specific immune-dominant epitopes and other regions that are not important for serological recognition [38]. Therefore, an ideal test would contain a combination of relevant epitopes in a single recombinant antigen, more specific than crude antigen preparation and more sensitive than single epitope-based ELISA [39]. In fact, chimeric multi-epitope proteins are part of the future strategy to look for novel high specific antigens [40]. Several recombinant proteins with high efficacy have reasonable results for the diagnosis of human and canine VL, but development of suitable antigens for diagnosis is still necessary [34].

The most common used antigens belong to different protein families, such as the kinesin-related proteins, heat shock proteins, acidic ribosomal proteins, nuclear proteins, enzymes and other antigens which are associated with parasite function [41].

3.1. Kinesin-related proteins

Kinesins are a family of motor proteins in eukaryotic cells, and constitute part of the microtubule cytoskeleton in *Leishmania* parasites, important in its growth and differentiation [42–44].

Several *Leishmania* antigens have been characterized, as the recombinant K39 antigen (rK39), a 39-aminoacid-repetitive B-cell epitope of the kinesin-related protein of *L. infantum*. The rK39 ELISA has shown to be capable of detecting human and canine VL.

The K9 and K26 are two other hydrophilic antigens of *L. infantum*, and their recombinant antigens (rK9 and rK26) have been used to diagnose VL. The rK28 is a recombinant fusion protein with tandem repeats of K39 kinesin regions and K26, and has been used to detect high levels of antibody responses in infected patients, using ELISA [13, 37].

The rK39, an antigen used for VL diagnosis, is highly conserved among VL species [15]. Several studies carried out in VL endemic areas have demonstrated that the rK39 ELISA is a sensitive and a specific method for the serodiagnosis of human VL [34, 45–47]. An important aspect of the anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potential for use in predicting response to therapy [48]. The rk39 has also been tested for ELISA and Flow Cytometry (FC) assays showing high sensitivity and specificity in detecting clinical forms of CanL [49]. rK39 is further addressed in Section 5—Successful diagnosis approaches—from laboratory to field conditions.

VL patients also have a strong antibody response to K26, which can complement rK39 in a better and more accurately diagnosis of human VL [50]. Specific and independent antibody reactivity to each of these antigens (rK9, rK26 and rK39) have been studied and tested in serodiagnosis of canine VL [31, 51].

Another antigen that has been successfully used in the diagnosis of human VL is rK28. This recombinant fusion antigen was used for ELISA and has shown sensitivity and specificity values similar or higher than those obtained for rK39. Therefore, this antigen has been proposed as a new choice, especially regarding regions where rK39 has shown low levels of sensitivity [52]. In Sudan and in India [53], human serological surveys using rK28 antigen have shown good performance with high sensitivity and specificity [54]. ELISA with rK28 antigen was 100% sensitive and specific in serological diagnosis of CanL [54]. The sensitivity of rK28 was slightly higher than rK39, although no significant differences in the detection of positive dogs were observed, indicating that both antigens may be useful for diagnosing CanL, as previously observed in VL in human [54, 55].

KE16 is another kinesin gene derived from the C-terminus of the kinesin protein from an isolate of *L. donovani* [56] that has been successfully cloned and expressed. It is used in the rKE16 dipstick test, a one-step rapid immunochromatographic test. Results showed that this antigen can be used as a highly specific and sensitive tool for VL diagnosis, although, in dogs, the average of positive cases among asymptomatic dogs was low [34, 56, 57].

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3.2. Heat shock proteins

Heat Shock Proteins (HSPs) are a family of proteins that are produced by cells in response to stressful conditions. They are highly conserved molecules that play important roles in protein folding, assembly of protein complexes, and translocation of proteins across cellular compartments.

The *L. donovani* HSP70 was identified after screening of a cDNA library with serum from a patient with VL [58]. It is found in prokaryotic and eukaryotic cells and is a highly conserved protein. HSP70s from other *Leishmania* species (*L. infantum* and *L. braziliensis* [59]) have also been characterized.

rHSP70 had high sensitivity and specificity values, being superior to CSA in the diagnosis of CL [60]. The antigenicity of heat shock proteins in general and of HSP70 specifically is not unexpected, since antibodies against the parasite recombinant HSPs have been found in sera of patients with different parasitic diseases [61, 62]. Other authors have described rHSP70 as a preeminent antigen present in *Leishmania* [63]. Specific antibodies against *L. braziliensis* rHSP70 or rHSP83 were found in 95% of sera from ML or CL patients and a high recognition of rHSP70 by sera from the same patients has been detailed [59].

L. infantum-rHSP83 is a possible antigen to be used in the serodiagnosis of leishmaniasis, due to its high specificity and sensitivity and insignificant cross-reactivity with other infectious diseases [48]. Members of the HSP83/90 family have also been described as immunodominant antigens during infections caused by *L. donovani*, *L. braziliensis* and *L. infantum* [64].

Another gene, *LmSti1*, encoding a stress-inducible protein STI1 [65] was strongly recognized in sera from human patients with CL, VL and post-kala azar CL [48].

3.3. Ribosomal proteins

Ribosomal proteins have been described to induce antibodies in animal and humans infected with the parasite, making them a favorable candidate to assess its diagnosis potential.

Studies have described some *Leishmania* ribosomal proteins function as immunoregulatory molecules [41, 59]. As a matter of fact, the eukaryotic ribosome is composed of four RNA molecules and more than 70 ribosomal proteins [66]. Furthermore, the acidic ribosomal proteins (P-proteins) have been detailed as prominent antigens during *Leishmania* infections [41, 67].

The large subunit of ribosomes contains various copies of a protein called P protein. P1, P2 and P0 form a complex notorious for having an essential role in protein synthesis. Three *L. infantum* antigens (LiP2a, LiP2b and LiP0), homologous to acidic ribosomal proteins, were identified after immunoscreening of a cDNA expression library with CanL serum [68]. They are also recognized by sera from patients with either VL or MCL [41, 69].

The ribosomal proteins LiP2a, LIP2b, LiP0/LcP0 and LeIF, all conserved antigens of *Leishmania*, have been shown to be recognized by the immune system of the host [70].

The *L. infantum* acidic ribosomal proteins, LiP2a and LiP2b, have disease-specific antigenic determinants identified by more than 80% of canine VL [70]. Engineered LiP2a and LiP2b recombinant proteins are shown to be useful as tools to discriminate between VL and Chagas disease [71]. *L. infantum* P0 ribosomal protein is also recognized by a high percentage of the sera from dogs with VL [70].

The acidic ribosomal proteins (P1, P2 and P0) are recognized by antibodies frequently found in sera from systemic lupus erythematosus (SLE) patients. These autoantibodies recognize an amino acid sequence located at the C-terminal ends of the three P proteins [72]. Furthermore, patients with chronic Chagas heart disease produce a strong humoral response against the C-terminal of *Trypanosoma cruzi* ribosomal P proteins. *Leishmania* ribosomal P2a and P2b C-terminal end of the proteins is also well conserved and these proteins are recognized by sera from both SLE and Chagas disease patients [69]. The anti-P antibodies produced during the *Leishmania* infection do not recognize the conserved C-terminal domain of the P proteins. Therefore, to avoid cross-reactivity, engineered versions of the recombinant proteins LiP2a and LiP2b, without the C-terminal ends, have been found to be useful for the diagnosis of MCL and VL [69].

3.4. Peroxiredoxins

Tryparedoxins belong to a particular class of oxidoreductases related to thioredoxins and found in trypanosomatids. They bear oxidoreductase activity toward disulfide bridges and are crucial to the parasite, as they are expressed in all stages of development [73]. These proteins are believed to be involved in *Leishmania* detoxification of peroxides.

LiTXN1 protein is present in the cytosol and upregulated in the infectious forms of the parasite, indicating that it plays an important role during infection. LiTXN1 preferentially reduces the cytosolic *L. infantum* peroxiredoxins, LicTXNPx1 and LicTXNPx2 [73].

LicTXNPx antigen is highly immunogenic during both human and canine infections [74]. High antibody titers are found during the *Leishmania* infection and these decrease after its resolution [75].

Anti-LicTXNPx antibodies are present in both symptomatic and asymptomatic experimental canine infections, making this antigen a good candidate marker and a prognostic indicator for monitoring the response to CanL treatment [75]. An ELISA with both LicTXNPx and rK39 antigens (LAM-ELISA) was performed to improve specificity and sensitivity of this methodology and presented promising results. This test associated with DAT may be a valuable tool for screening CanL [22, 30].

3.5. Cysteine proteinases

This family of proteins is associated to disease progression. Activity of cysteine proteases can be found in parasite surface or inside the macrophage endoplasmatic reticulum. Domains of cysteine proteinases (CP), type I (CPB) and type II (CPA), were used to diagnose active and

recovered cases of VL in both humans and dogs. *Leishmania* cysteine proteinases had already been used as vaccines targets and in chemotherapy [76].

A recombinant cysteine proteinase from *L. chagasi*, rLdccys1, has shown to be a good biomarker for the different stages of both human and CanL. ELISA assays showed high sensitivity and specificity. Moreover, the fact that it is possible to detect clinical and subclinical forms of the disease in canines indicates that this biomarker is important in the control of CanL in endemic areas [77, 78].

3.6. Nuclear proteins

Histones are structural proteins involved in the organization and function of DNA within the eukaryotic nucleus. There are four main classes of histones: H2A, H2B, H3 and H4 which form the nucleosomal core unit of chromatin. These proteins are among the most conserved proteins in eukaryotic organisms, maintaining sequence and function in trypanosomatids. Histones are prominent antigens found in animals during *Leishmania* infection that trigger a specific immune response and antibody production [39]. Also, sera from children infected with VL specifically recognize the *L. infantum* H2A and H2B histones, with high specificity and sensitivity in ELISA assays.

3.7. A2 proteins

A2 proteins of *L. donovani* are only present in the amastigote stage, and help visceralization of parasites in the mammalian host. They are overexpressed in the amastigote stage in *L. donovani* as well as *L. mexicana* species complex, including *L. amazonensis*, but not in *L. tropica* or *L. braziliensis* species complexes. Anti-A2 antibodies were detected in human and dog sera suffering from active VL [79, 80]. Also, it was found that rA2 is more sensitive when compared to the rK39 and rK26 antigens, for serological detection of asymptomatic infection in dogs [34].

3.8. *Leishmania* homolog of receptors for activated C kinase

Leishmania homolog of receptors for activated C Kinase (LACK) was identified after a search for parasite antigens recognized by a protective Th1 clone derived from the spleen of BALB/c mice that had been vaccinated with a soluble extract of *L. major* promastigotes. It has been proposed that the LACK protein contains an immunodominant epitope that acts as a target of early immune responses [41, 81].

3.9. rKLO8

Sudan has the highest number of reported cases in East Africa [82], in particular in eastern and central regions. Field tests based on rK39 commonly have a high reliability in various countries [83, 84]; however, the low sensitivity in Sudan limits its use in this region. A novel *L. donovani*-derived recombinant immunodominant protein (rKLO8) was tested for detection of VL in Sudan.

The results presented with rKLO8 show increased reactivity with patient sera as compared to rK39 ELISA [85]. The rKLO8 ELISA is more sensitive than the DAT and rK39 strip test, and VL patients from Sudan were tested and have decreased immune responses to rK39, confirming the low sensitivity of rK39 strip test in Sudan. Malaria is common in VL endemic regions of Africa and Asia [86] and is known to be a major cause of cross reactivity to rK39 [84]. Sera of malaria patients were tested and did not give a signal in the rKLO8 ELISA. In conclusion, rKLO8 is a novel recombinant protein of *L. donovani* with increased reactivity to VL sera from Sudan and a valuable candidate to be used in diagnosis in this area [84].

3.10. Secreted proteins

Traditionally the intrinsic intracellular and surface proteins of the parasites were targeted as primary source of antigens. Recently, secreted proteins are considered an untapped source of possible antigens and are being exploited using combinations of bioinformatic and immunoproteomic approaches [87, 88] .

3.11. Lipids

Lipid levels are known to vary in acute and chronic infections. In these infections, there is typically a decrease of total cholesterol levels and an increase in the concentration of triglyceride-rich lipoproteins; mainly very low-density lipoproteins. Moreover, apolipoprotein A1, apolipoprotein B and low-density lipoprotein cholesterol levels decrease. In leishmaniasis, this lipid concentration difference may have a prognostic and diagnostic role, as lipids play an important role in the innate and adaptive immune response. Although these have potential as clinical markers, several factors have to be taken into account when interpreting lipids values, such as: genetic and environmental factors, malnutrition, reduced food intake during acute infection, and acute kidney injury and/or acute liver failure (many times associated with this neglected disease), all which influence lipids parameters. More efforts have to be put in the study of these molecules as disease markers [89].

4. Diagnosis approaches

Due to its wide range of manifestations, leishmaniasis diagnosis can be difficult [90]. Clinical manifestations can also be confused with other illnesses, often common in *Leishmania* endemic areas, such as malaria, toxoplasmosis and tuberculosis [91]. Age, medical history and host immune system response are crucial parameters for diagnosis.

Diagnosis of diseased patients and animals can be confirmed with conventional laboratorial techniques, such as visualization of the parasite in tissues by microscopic examination of a stained specimen, or *in vitro* culture of the parasite from biopsies or aspirates from lesions, lymph nodes, spleen and bone marrow. Other diagnosis methods include molecular detection of parasitic DNA in tissue samples and serological tests that detect anti-*Leishmania* antibodies [6]. Overall, the available techniques can detect active disease. Still, leishmaniasis has intrinsic

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particularities that require not only the detection of symptomatic (diseased) conditions but also asymptomatic (infected, not diseased). This control is important, especially in the context of CanL, as the asymptomatic animals act as reservoirs, and as mentioned above, increase the risk for the human variant of the disease. An already complex scenario rendered more complicated by the need to distinguish treated, exposed and vaccinated. Therefore, leishmaniasis is a very particular disease and one of the many examples where ongoing research for a new and affordable diagnosis approaches is necessary.

4.1. Parasitological diagnosis

The gold standard for diagnosis is the microscopic observation of parasites in tissue samples. Parasite rich localizations like bone marrow, skin lesions, liver, lymph nodes and spleen are preferred. Identification of amastigotes by direct examination of aspirates is also possible but must be done by experts since the results are often dependent on the observer [6]. It can also originate false negative results due to the low number of parasites in some samples, particularly in asymptomatic cases. Most often, diagnostic is obtained by observation of the *Leishmania* amastigote forms in stained microscopic preparations with Giemsa [8]. The best results are obtained with parasite rich regions like spleen aspirates. With bone marrow aspirates, the sensitivity decreases considerably and lymph nodes aspirates have the worst sensitivity ranges [4]. This method requires trained personnel and involves invasive sampling, a risky procedure that can lead to fatal hemorrhage, which can only be performed in a place with access to appropriate medical facilities. Lymph node and bone marrow aspirates are safer; however, material obtained is less concentrated and therefore less sensitive, elevating the risk of false negatives [92]. All these methods include invasive sampling, are time-consuming and impracticable to be performed on a large scale [3].

The culture of infected tissues is another classical diagnostic test, although the major problem with this technique is that different species of *Leishmania* can have different growth requirements and contaminations are recurrent. The culture is performed through the inoculation of the triturated tissue in adequate media. Both techniques (microscopic exam and culture) have an overall sensitivity of around 85% [3]. The best results are obtained with spleen aspirates (93–98% of sensitivity). When it comes to bone marrow aspirates, the sensitivity comes down to 60 to 85% and worse results are obtained with lymph nodes aspirates (sensitivity ranges between 52 to 58%) [4]. Despite being more sensitive than microscopic examination, it is time consuming and expensive, therefore rarely used for clinical diagnosis [93].

4.1.1. Xenodiagnosis

A positive serological test result is not enough to prove that an infected dog is capable of transmitting the pathogen to the vector. Lack of sensitivity contributes to the lack of diagnostic control efficiency. The only technique that evaluates if an infected mammalian host can transmit the parasite by natural means to the vector is xenodiagnosis [94–96]. Sandflies are placed inside specific containers and placed in contact with dogs for a certain period of time for a blood meal to occur. After the feeding, the sandflies are separated and the feeding rate is calculated. The presence of parasites in the sandfly can be evaluated by direct observation of the promastigote

forms by optical microscopy or Polymerase Chain Reaction (PCR). This test can be highly specific and sensitive, depending on parasite load and procedure implementation [96].

4.1.2. Montenegro skin test

The Montenegro Skin Test evaluates the late cellular hypersensitivity response. A solution with promastigotes is injected intradermally, and a positive result consists in the appearance of a hardened papule, equal or greater than 5 mm after 48 hours of injection. The test is low cost and highly sensitive (can reach over 90%). A positive result is possible within 3 months after infection, and relates to the disease evolution. Specificity is low (around 75%) due to the overall large number of false positive results in cases of unapparent infection and cross-reactivity with some pathologies, as well as technical problems [97].

4.2. Molecular methodologies

PCR technology has become an indispensable tool for the diagnosis of many parasitic diseases, including leishmaniasis. It suffers similar limitations to the parasitological tests, where the success is limited by the quality of the sample (parasite rich specific locations), as these techniques are based on the amplification of specific parasitic DNA sequences. Although several DNA targets exist, like rRNA or ITS-1 gene, the most common DNA target is the *Leishmania* kinetoplast (kDNA) present in the minicircles of the parasite [98]. The diagnostic sensitivity of these approaches is above 95% [99, 100] . In asymptomatic dogs the reported efficacy is lower, less than 70% [100]. *Leishmania* DNA has been found to be present in the canine oral mucosa, and can possibly be used for diagnostic. Oral swabs have shown positive results in molecular diagnosis of infected dogs. Although, in samples from asymptomatic dogs, the diagnosis test showed lack of sensitivity. Oral swabs combined with conjunctival ones can be used for detection of *L. infantum* in asymptomatic dogs. The big advantage of this test is that the sample collection is non-invasive, and combining this two swab methods, can significantly contribute for detecting different stages of the infection [101]. The sensitivity of a PCR assay depends on three factors: the physicochemical conditions of the reaction, the concentration of the DNA target and the selected PCR primers [38, 102, 103]. Amplification-based methods include the conventional PCR and qPCR (quantitative polymerase chain reaction) [104]. Real-time PCR (qPCR) is an innovative approach of target DNA quantification, faster than conventional PCR [102].

Although these techniques have a higher sensitivity for asymptomatic cases and early-stage infections when compared to serological methods, they imply high costs due to sophisticated equipment, reagents and specialized personnel [103, 105, 106].

4.3. Serological diagnosis

Serological tests are based on the screening of antigen or antibodies. Antigen detection should be more specific for diagnosis that antibody-based immunodiagnostic tests. Thus, antigen levels are expected to correlate with parasite burden, being useful when antibody prediction is deficient. This approach should avoid cross-reactivity and distinguish active from past

infections [8]. However, this technique is still unreliable (lack of specificity and variable sensitivity). Efforts are being made to improve this tool, as it stands as a promising approach.

Currently, most clinical and surveillance laboratories use serological techniques to detect pathogen-specific antibodies, since direct methods are often either invasive, potentially fatal or expensive. *Leishmania* infection is characterized by the presence of a significant humoral response, leading to the production of antibodies against *Leishmania* species. Serological methods to detect these anti-*Leishmania* antibodies are useful as alternative diagnosis tests for both human and canine *Leishmania* infections [107]. The presence of anti-*Leishmania* antigens/antibodies in both asymptomatic and symptomatic infected dogs has allowed the development of agglutination tests, immunofluorescent serologic tests (such as Western blotting), immunochromatographic tests, and enzyme-linked immunosorbent assays (ELISAs). The sensitivity depends on the methodology but the specificity will depend on the antigen used. Serological tests are the current tests of choice to diagnose CanL. Although, these tests often lack sensitivity and specificity due to dog low specific antibody titers in the early stages of infection. Significant cross-reactivity has also been reported [108]. Additionally, after a successful treatment, antibody levels take a while to decrease. This can mask a relapse, making it impossible to diagnose it. The introduction of commercial vaccines against CanL raised concerns since it can lead to antibody production and might prevent accurate results in serological tests for the diagnosis. This may not allow differentiating between Infected and Vaccinated Animals (DIVA) [19]. Most commercially available vaccines against CanL are not DIVA. This difficults the serological identification of animals that become sick or infected in the future [19].

4.3.1. Direct agglutination test

Originally created as an alternative to the risky procedure of splenic aspirates, the Direct Agglutination Test (DAT) was the first antibody detection test used in VL diagnosis and has been used for more than 25 years. DAT is a simple semi-quantitative diagnostic tool, with a high sensitivity (91–100%), specificity (72–100%), accuracy, reliability and inexpensiveness [4, 109].

DAT detects parasite antibodies in infected blood or serum through direct agglutination. It is a semi-quantitative method based on visual agglutinations obtained by the increased dilution of blood or serum mixed with stained, killed parasites in V-shaped wells [110]. If the result is negative (absence of anti-*Leishmania* antibodies) DAT antigen accumulates at the bottom of the plate. If there are anti-*Leishmania* antibodies present, the antigens form a film over the well (positive result) [6, 111]. DAT has some disadvantages; it requires moderate technical expertise (the interpretation of the results depend on the person analyzing the results, creating inter-observer discrepancy) [112], serial dilutions must be done (requiring a considerable volume of antigen) and has a relatively long incubation time [112]. DAT results remain positive long after the patient is cured (anti-*Leishmania* antibodies can persist for years as a result of a VL infection), making this test inappropriate for detecting relapses [8].

4.3.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is an important serodiagnosis technique used in almost all infectious diseases, including VL. ELISA assays are based on the detection of

antibodies present in blood or serum. An antigen/recombinant protein is used to coat the plate and samples are added and incubated. A secondary antibody conjugated with an enzyme recognizes and binds to the primary antibody. After adding a substrate, product susceptible to colorimetric determination is developed and the results can be measured by optical density techniques.

This tool is frequently used to detect anti-*Leishmania* antibodies due to its high sensitive and specificity as well as good reproducibility and high throughput screening of large number of samples, at an affordable expense. However, the specificity of this technique depends on the antigen used. ELISA's major disadvantage is its inadaptability in poor-resource field conditions and requiring specialized operators.

4.3.3. Indirect fluorescent antibody technique

Indirect Fluorescent Antibody Technique (IFAT) is an extremely valuable tool that allows assessment of anti-*Leishmania* antibody titers produced by infected individuals. It is a quite useful method in epidemiological studies and in clinical practice [113]. Promastigote or amastigote forms have been used as a source for antigens used by IFAT [25]. IFAT is based on the use of fluorophores to detect antibodies that recognize the parasites. It involves the use of a primary antibody that binds to the antigen and allows the formation of an antigen-antibody complex. Posteriorly, the binding of a fluorophore-conjugated secondary antibody results in an amplified signal that can be examined by fluorescence microscopy. IFAT is the serological gold standard for diagnosis of CanL in most countries. This analytical methodology differs from ELISA as it resorts to the whole body parasite as antigen [113]. Although having a high specificity and sensitivity (100% and 90%, respectively), IFAT sensitivity is lower for asymptomatic infections when compared to ELISA [29, 114].

4.3.4. Immunochromatographic tests

Immunochromatographic (IC) tests are recurrently used in large-scale screenings. The results obtained from IC tests consider the epidemiological context and do not require antibody titration [115]. IC test is based on chromatographic and relies on capillary action. An antigen-antibody complex is formed and entrapped on the membrane. The sample migrates across this membrane, the antibodies present in the blood recognize the antigen and the labeled antibody present in the membrane resulting in the formation of a colored product. IC tests are inexpensive, practical, rapid and suitable for field use. However, these tests frequently lead to false positives with patients in endemic regions that have already been infected in the past. Resultantly, this technique does not work in the diagnosis of relapsed patients [115]. Much like ELISA, the specificity and sensitivity of IC test is dependent on the antigen used.

4.3.5. Flow cytometry

Flow Cytometry (FC) is an emerging technique for the diagnostic of several infectious diseases [116]. It has a high throughput capacity, possibility of quantification, high reproducibility and sensitivity and potential for multiplexing [49]. This technique analyses and sorts cells or particle suspensions in a controlled fluid stream, through the measurement of fluorescence and scatter induced illumination, allowing the acquisition of structural and functional data.

The flow cytometry technique can quantify the antibodies against *Leishmania* surface antigens, restraining potential cross-reactivity against more conserved intracellular structures [117] and showing potential to monitor post chemotherapy VL, in order to evaluate the success of treatment [118].

As described before, every diagnosis technique has its downsides. Parasite demonstration in tissue smears and culture provide definitive diagnosis of VL, but generally have a lower sensitivity than serologic methods with a high risk associated. Microscopy techniques lacks sensitivity, whereas culture requires a longer time to obtain a result and is vulnerable to contamination [119].

Molecular diagnostic tools like PCR and real-time PCR are quite sensitive and specific although difficult to perform in field conditions and costly [8]. Its use remains largely restricted to some hospitals and research centers [90].

Serological tests are often not sensitive enough to detect asymptomatic individuals, having to be combined with classical methods of diagnosis to confirm. ELISA, IFAT, DAT and rK39 immunochromatographic strip test (ICT) are highly sensitive and specific when analyzing active VL in immunocompetent individuals. This does not happen when titers decline and parasite charge is lower. Due to this, false negative results frequently happen in immunocompromised patients and asymptomatic *Leishmania* infections [120].

In that way, the big challenge in the field is to isolate, recombine, produce and identify new proteins capable of detecting asymptomatic leishmaniasis cases and early stages of infection in VL endemic regions, using safe techniques that are easy, fast, sensitive and with a fair cost.

The importance of serological methods has been rising in vector-borne diseases, due to the low sensitivity associated to the microscopic methods [52]. Molecular biology developments over the years have led to advances in techniques that aided in VL control and had significant progress in surveillance and diagnosis of this disease [52].

The main problem with leishmaniasis diagnosis is the identification of markers that can detect the presence of low titres of antibodies. Thus, new markers are needed to contribute for a more accurate diagnosis capable of detecting asymptomatic cases and early stages of disease in large screening studies [38, 121].

5. Successful diagnosis approaches-from laboratory to field conditions

Currently, the scientific community is investing in developing VL tests based on antigen detection, such as rapid diagnostic tests.

Rapid diagnostic tests (RDTs) are equipment-free diagnostic devices that are adequate for field conditions. The results of this test can be read easily and in a short amount of time. Most RDTs work by capturing either an antigen or an antibody on a solid surface and then attaching molecules that allow detection by the naked eye.

5.1. Immunochromatographic Tests

These Immunochromatographic Tests (ICTs) usually based on immunochromatography with a dipstick, are used for VL diagnosis using protein from *Leishmania* as the antigen [110]. The RDTs for VL, above all but not exclusively the rK39-based ICTs, seem to be the present solution for field diagnosis in field settings due to their ease of use, convenience and cost, making them potentially favorable to increase patients' access to VL diagnosis and treatment [110]. There is a limited number of commercially available RDTs for VL [110] (**Table 2**).

The scientific technological advances in recombinant antigens as reagents for the serological diagnosis of VL have resulted in high sensitivity and specificity of the serological tests. Several recombinant proteins have been shown to be useful for the diagnosis of *Leishmania* infection both in humans and dogs [34]. Recombinant antigens such as the above mentioned rK9, rK16, rK26, rK28 and rk39 have been evaluated for its potential use in rapid diagnostic tests in field conditions.

Of all commercial RDTs, the rK39 is the most widely used. The rK39 rapid immune-chromatographic dipstick test was developed to meet the need for diagnosis in field conditions. Among the recombinant antigens, this one showed promising diagnosis and has been extensively tested in the last 5 years with IC tests in several leishmaniasis endemic areas [60, 122], being associated with several commercial applications (**Table 2**). It is an easy and qualitative test able to detect anti-*Leishmania* circulating antibodies, not requiring scientifically trained personnel. The test procedure involves adding the patient's blood or serum with diluent buffer on the strip [110]. These rK39 ICTs give an immediate result (typically between 10 and 20 minutes) and give a binary reading (positive or negative) [110]. This test has been approved by the Food and Drug Administration and tested by the World Health Organization in many endemic countries. The rapid rK39 immunochromatographic dipstick test is both

Application	Product name	Bound antigen	Manufacturer
Human	DiaMed-IT LEISH	rK39	Bio-Rad Laboratories
	OnSite *Leishmania* Ab Rapid Test	rK39	CTK Biotech, Inc
	Crystal KA (Kala azar)	rKE16	Span Diagnostics, Lda
	Kalazar Detect™	rK39	InBios International Inc
Dog	Canine Visceral Leishmaniasis Dipstick*	rK39	InBios International Inc
	Anigen Rapid *Leishmania* Ab Test Kit	Na	Vtrade
	Anigen Rapid *Leishmania* Ab Test Kit	Na	BIONOTE
	Speed Leish K	Kinesin Capture Complex	Virbac Animal Health
	DPP® Canine Leishmaniasis	rK39	Bio-Manguinhos/Fiocruz
	ImmunoRun	Na	Biogal, Galed Labs.
	Canine *Leishmania* Antibody Test	HRPO Conjugate	Quicking Biotech
	Leishmania Ab	na	EcoDiagnóstica

Data representative of available information in 2016.

Table 2. Immunochromatographic tests available for *Leishmania* diagnosis.

sensitive (67–100%) and specific (70–100%) [34]. In the Indian subcontinent (India and Nepal), the test proved to have a high sensitivity and specificity but in East Africa (Sudan, Kenya and Ethiopia) the results were not so remarkable [34, 123]. These results in East Africa are not entirely understood; however, lower antibody levels for rK39 in this region might be the reason for these decreased values of sensitivity and specificity [31]. Several studies have been carried out using the dipstick form of rK39 in dogs. The results revealed an overall good sensitivity in symptomatic dogs, although the number of studies for a proper analysis is too small for a detailed comparison [34, 124]. This recombinant antigen can also be used in a latex agglutination test, where latex is used for antigen absorption. The attachment of molecules to latex particles can be accomplished through either physical adsorption or covalent coupling. However, the sensitivity of this latex test is relatively low [34]. In comparison to DAT and ELISA, dipstick rK39 is a better choice for field conditions, sufficiently sensitive and highly specific method for the diagnosis of active VL in humans as well as in dogs [34].

The diagnosis of CanL remains a problem due to lack of sensitivity or specificity in the current diagnostic tests. Nevertheless, rapid tests like the immunochromatographic-dipstick test using rK39 and rK26 proteins of *L. infantum* [32] as antigens seem to be most appropriate for diagnosis of symptomatic cases of CanL but lack sensitivity for asymptomatic dogs. Research should continue in order to develop a more sensitive and specific recombinant assay able to detect asymptomatic cases [31].

5.2. Latex agglutination kit

Latex Agglutination Kit (KATex) is a simple, noninvasive, rapid, reliable and easily executable extensively used in diagnosis of *L. donovani* in endemic areas as Sudan, India and Nepal. It is not commonly used in *L. infantum* endemic areas [125]. Latex beads coated with anti-*Leishmania* antibodies detect the presence of a low molecular weight, heat-stable glycoconjugate antigen in urine [126]. This antigen is present in both promastigote and amastigote forms of VL patients with active infection. It has a high specificity (from 82 to 100%) and a sensitivity that varies from 47 to 95%. Although, for immunocompromised patients, the sensitivity and specificity have been reported at 85–100% and at 96–100%, respectively. The antigen may be detectable from one to 6 months after treatment. The method is appropriate for the diagnosis of primary VL, for monitoring the efficacy of treatment and for the detection of sub-clinical infection. KAtex test was also tested with oral fluids, which demonstrated the usefulness of oral-fluid collection in the detection of both *Leishmania* antibodies and DNA [125]. Sensitivity was higher in saliva that in urine, but specificity was lower. The immunoassay probably detects the same antigen present in urine [125].

6. Conclusion and remarks

Currently, several biomarkers for diagnosis are available in research laboratories, with expensive and sophisticated equipment requiring trained operators, precluding their direct application for field use.

Due to the specific characteristics associated to serological methodologies, they are accepted as the ideal diagnosis approach for both human and animal use, enabling epidemiological studies. Still, a significant limitation of serological tests is their capacity to detect asymptomatic cases. Further challenges associated to the advent of vaccination still need to be addressed.

Ultimately, the goal is to find biomarkers that detect different clinical forms of the disease that can be used universally in a rapid diagnosis kit.

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References

[1] World Health Organization. Leishmaniasis. Available from: http://www.who.int/leishmaniasis/en/ [Accessed: 2017-11-30]

[2] Kevric I, Cappel MA, Keeling JH. New world and old world Leishmania infections: A practical review. Dermatologic Clinics. 2015;**33**:579-593

[3] Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, et al. Leishmaniasis: A review. F1000Research. 2017;**6**:750

[4] Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: What are the needs for diagnosis, treatment and control? Nature Reviews. Microbiology. 2007;**5**:873-882

[5] Roberts MTM. Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. British Medical Bulletin. 2005;**75–76**:115-130

[6] WHO. Technical Report of the TDR Disease Reference Group on Chagas Disease HT and L. Research Priorities for Chagas Disease, Human African Trypanosomiasis and Leishmaniasis; 2012. p. 100

[7] Strazzulla A, Cocuzza S, Pinzone MR, et al. Mucosal leishmaniasis: An underestimated presentation of a neglected disease. BioMed Research International. 2013. Epub ahead of print 2013. DOI: 10.1155/2013/805108

[8] Daniell H. NIH Public Access. 2012;**76**:211-220

[9] Murray HW, Berman JD, Davies CR, et al. Advances in leishmaniasis. [Review] [199 refs]. Lancet 9496;**366**:1561-1577

[10] Mukhopadhyay D, Dalton JE, Chatterjee M. Post kala-azar dermal leishmaniasis : An unresolved mystery PKDL: Current scenario a role for antimonial drugs. Trends in Parasitology. 2015;**2015**:11-14

[11] Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. International Journal for Parasitology. 2007;**37**:1097-1106

[12] Kaye P, Scott P. Leishmaniasis: Complexity at the host–pathogen interface. Nature Reviews. Microbiology. 2011;**9**:604-615

[13] Gontijo CMF, Maria MN. Laboratory diagnosis of visceral leishmaniasis laboratory diagnosis of visceral leishmaniasis. Clinical and Diagnostic Laboratory Immunology. 2002;**9**:951-958

[14] Report WH. The Wo World rld Health Report 2004 Changing History

[15] Schallig HDFH, da Silva ES, van der Meide WF, et al. Didelphis marsupialis (common opossum): A potential reservoir host for zoonotic leishmaniasis in the metropolitan region of Belo Horizonte (Minas Gerais, Brazil). Vector Borne and Zoonotic Diseases 2007; 7: 387–393.

[16] Coelho EAF, Ramírez L, Costa MAF, et al. Specific serodiagnosis of canine visceral leishmaniasis using Leishmania species ribosomal protein extracts. Clinical and Vaccine Immunology. 2009;**16**:1774-1780

[17] Gomes YM, Paiva Cavalcanti M, Lira RA, et al. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. Veterinary Journal. 2008;**175**:45-52

[18] Solcà MDS, Bastos LA, Guedes CES, et al. Evaluating the accuracy of molecular diagnostic testing for canine visceral leishmaniasis using latent class analysis. PLoS One;**9**. Epub ahead of print 2014. DOI: 10.1371/journal.pone.0103635

[19] Solano-Gallego L, Cardoso L, Pennisi MG, et al. Diagnostic challenges in the era of canine *Leishmania infantum* vaccines. Trends in Parasitology. 2017;**33**:706-717

[20] Gradoni L. Canine Leishmania vaccines: Still a long way to go. Veterinary Parasitology. 2015;**208**:94-100

[21] Pejcic B, Marco RD, Parkinson G. The role of biosensors in the detection of emerging infectious diseases. Analyst. 2006;**131**:1079

[22] Santarém N, Silvestre R, Cardoso L, et al. Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. Journal of Clinical Microbiology. 2010;**48**:1866-1874

[23] Soper SA, Brown K, Ellington A, et al. Point-of-care biosensor systems for cancer diagnostics/prognostics. Biosensors & Bioelectronics. 2006;**21**:1932-1942

[24] Jani IV, Janossy G, Brown DWG, et al. Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor settings. The Lancet Infectious Diseases. 2002;**2**:243-250

[25] Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of visceral leishmaniasis. Journal of Vector Borne Diseases. 2016;**53**:8-16

[26] Desjeux P. Leishmaniasis: Current situation and new perspectives. Comparative Immunology, Microbiology and Infectious Diseases. 2004;**27**:305-318

[27] Porrozzi R, Santos MV, Teva A, et al. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant Leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clinical and vaccine immunology. . Epub ahead of print 2007. DOI: 10.1128/CVI.00420-06

[28] Kumar R, Pai K, Pathak K, et al. Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. Clinical and Diagnostic Laboratory Immunology. 2001;**8**:1220-1224

[29] Mettler M, Grimm F, Capelli G, et al. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests and gel tests for serological diagnosis of symptomatic and asymptomatic Leishmania infections in dogs evaluation of enzyme-linked immunosorbent A. Journal of Clinical Microbiology. 2005;**43**:5515-5519

[30] Sousa S, Lopes AP, Cardoso L, et al. Seroepidemiological survey of *Leishmania infantum* infection in dogs from northeastern Portugal. Acta Tropica. 2011;**120**:82-87

[31] Pattabhi S, Whittle J, Mohamath R, et al. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. PLoS Neglected Tropical Diseases;**4**. Epub ahead of print 2010. DOI: 10.1371/journal.pntd.0000822

[32] Burns JM, Shreffler WG, Benson DR, et al. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proceedings of the National Academy of Sciences. 1993;**90**:775-779

[33] Carvalho FAA, Charest H, Tavares CAP, et al. Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant Leishmania donovani A2 antigen. Diagnostic Microbiology and Infectious Disease. 2002;**43**:289-295

[34] Farahmand M, Nahrevanian H. Application of recombinant proteins for serodiagnosis of visceral leishmaniasis in humans and dogs. Iranian Biomedical Journal. 2016;**20**:128-134

[35] Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. Veterinary Parasitology. 2008;**158**:274-287

[36] Ivens AC. The genome of the Kinetoplastid parasite, Leishmania major. Science (80-). 2005;**309**:436-442

[37] Boarino A, Scalone A, Gradoni L, et al. Development of recombinant chimeric antigen expressing immunodominant B epitopes of *Leishmania infantum* for serodiagnosis of visceral leishmaniasis. Clinical and Vaccine Immunology. 2005;**12**:647-653

[38] Porrozzi R, Santos Da Costa MV, Teva A, et al. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clinical and Vaccine Immunology. 2007;**14**:544-548

[39] Soto M, Requena JM, Quijada L, et al. Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis. Journal of Clinical Microbiology. 1998;**36**:58-63

[40] Faria AR, de Castro Veloso L, Coura-Vital W, et al. Novel recombinant multiepitope proteins for the diagnosis of asymptomatic *Leishmania infantum*-infected dogs. PLoS Neglected Tropical Diseases. 2015;**9**:13-16

[41] Requena JM, Alonso C, Soto M. Evolutionarily conserved proteins as prominent immunogens during Leishmania infections. Parasitology Today. 2000;**16**:246-250

[42] Albers K, Fuchs E, Biol JC, et al. Frontiers in cell biology: Articles kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science (80-). 2005;**279**: 519-526

[43] Lawrence CJ, Dawe RK, Christie KR, et al. A standardized kinesin nomenclature. The Journal of Cell Biology. 2004;**167**:19-22

[44] Gerald NJ, Coppens I, Dwyer DM. Molecular dissection and expression of the LdK39 kinesin in the human pathogen, Leishmania donovani. Molecular Microbiology. 2007;**63**: 962-979

[45] Zijlstra EE, Daifalla NS, Kager PA, et al. rK39 enzyme-linked immunosorbent assay for diagnosis of Leishmania donovani infection. Clinical and Diagnostic Laboratory Immunology. 1998;**5**:717-720

[46] Ozensoy S, Ozbel Y, Turgay N, et al. Serodiagnosis and epidemiology of visceral leishmaniasis in Turkey. The American Journal of Tropical Medicine and Hygiene. 1998;**59**: 363-369

[47] Zerpa O, Ulrich M, Benitez M, et al. Epidemiological and immunological aspects of human visceral leishmaniasis on Margarita Island, Venezuela. Memórias do Instituto Oswaldo Cruz. 2002;**97**:1079-1083

[48] Celeste BJ, Arroyo Sanchez MC, Ramos-Sanchez EM, et al. Recombinant *Leishmania infantum* heat shock protein 83 for the serodiagnosis of cutaneous mucosal, visceral leishmaniases. The American Journal of Tropical Medicine and Hygiene. 2014;**90**:860-865

[49] Sousa S, Cardoso L, Reed SG, et al. Development of a fluorescent based immunosensor for the serodiagnosis of canine leishmaniasis combining immunomagnetic separation and flow Cytometry. PLoS Neglected Tropical Diseases. 2013;**7**:1-7

[50] Bhatia A, Daifalla NS, Jen S, et al. Cloning, characterization and serological evaluation of K9 and K26: Two related hydrophilic antigens of *Leishmania chagasi*. Molecular and Biochemical Parasitology. 1999;**102**:249-261

[51] Rosati S, Ortoffi M, Profiti M, et al. Prokaryotic expression and antigenic characterization of three recombinant Leishmania antigens for serological diagnosis of canine leishmaniasis. Clinical and Diagnostic Laboratory Immunology. 2003;**10**(6):1153

[52] Negri V, Sinagra AJ, Romagosa CM, et al. An rK28-based immunoenzymatic assay for the diagnosis of canine visceral leishmaniasis in Latin America. The American Journal of Tropical Medicine and Hygiene. 2016;**95**:92-98

[53] Reithinger R, Quinnell RJ, Alexander B, et al. Rapid detection of *Leishmania infantum* infection in dogs: Comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. Journal of Clinical Microbiology. 2002; **40**:2352-2356

[54] Venturin GL, Bragato JP, Silva KLO, et al. Recombinant K28 antigen in ELISA in the diagnosis of canine visceral leishmaniosis. Parasite Immunology. 2015;**37**:670-673

[55] Manuscript A, Blood W, Count C. NIH Public Access 2009;49:1841-1850

[56] Sivakumar R, Sharma P, Chang K-P, et al. Cloning, expression, and purification of a novel recombinant antigen from Leishmania donovani. Protein Expression and Purification. 2006;**46**:156-165

[57] Sivakumar R, Dey A, Sharma P, et al. Expression and characterization of a recombinant kinesin antigen from an old Indian strain (DD8) of Leishmania donovani and comparing it with a commercially available antigen from a newly isolated (KE16) strain of *L. donovani*. Infection, Genetics and Evolution. 2008;**8**:313-322

[58] MacFarlane J, Blaxter ML, Bishop RP, et al. Identification and characterisation of a Leishmania donovani antigen belonging to the 70-kDa heat-shock protein family. European Journal of Biochemistry. 1990;**190**:377-384

[59] Skeiky YAW, Benson DR, Guderian JA, et al. Immune responses of leishmaniasis patients to heat shock proteins of Leishmania species and humans. Infection and Immunity. 1995;**63**:4105-4114

[60] Mohammadiha A, Haghighi A, Mohebali M, et al. Canine visceral leishmaniasis: A comparative study of real-time PCR, conventional PCR, and direct agglutination on sera for the detection of *Leishmania infantum* infection. Veterinary Parasitology. 2013;**192**:83-90

[61] Hedstrom R, Culpepper J, Harrison RA, et al. A major immunogen in Schistosoma mansoni infections is homologous to the heat-shock protein Hsp70. The Journal of Experimental Medicine. 1987;**165**:1430-1435

[62] Zügeli U, Kaufmann SHE. Immune response against heat shock proteins in infectious diseases. Immunobiology. 1999;**201**:22-35

[63] De Andrade CR, Kirchhoff LV, Donelson JE, et al. Recombinant Leishmania Hsp90 and Hsp70 are recognized by sera from visceral leishmaniasis patients but not Chagas' disease patients. Journal of Clinical Microbiology. 1992;**30**:330-335

[64] Justicia J, Rosales A, Bañuel E, et al. To print this article, please use the print button in the bottom toolbar of the web reader. Journal of Clinical Nursing. 2004;**19**:3274-3290

[65] Romero GAS, Boelaert M. Control of visceral leishmaniasis in latin America - a systematic review. PLoS Neglected Tropical Diseases;**4**. Epub ahead of print 2010. DOI: 10.1371/journal.pntd.0000584

[66] Wilson DN, Cate JHD. The structure and function of the eukaryotic. Cold Spring Harbor perspectives in biology. 2012;**4**:1-18

[67] Cordeiro-da-silva A, Borges MC, Guilvard E, et al. Dual role of the Leishmania major ribosomal protein S3a homologue in regulation of T- and B-cell activation. Infection and Immunity. 2001;**69**:6588-6596

[68] Soto M, Requena JM, Garcia M, et al. Genomic organization and expression of two independent gene arrays coding for two antigenic acidic ribosomal proteins of Leishmania. The Journal of Biological Chemistry. 1993;**268**:21835-21843

[69] Soto M, Requena JM, Quijada L, et al. Specific serodiagnosis of human leishmaniasis with recombinant Leishmania P2 acidic ribosomal proteins. Clinical and Diagnostic Laboratory Immunology. 1996;**3**:387-391

[70] Wallace GR, Ball AE, MacFarlane J, et al. Mapping of a visceral leishmaniasis-specific immunodominant B-cell epitope of Leishmania donovani Hsp70. Infection and Immunity. 1992;**60**:2688-2693

[71] Quijada L, Requena JM, Soto M, et al. Mapping of the linear antigenic determinants of the *Leishmania infantum* Hsp70 recognized by leishmaniasis sera. Immunology Letters. 1996;**52**:73-79

[72] Elkon K, Bonfa E, Llovet R, et al. Properties of the ribosomal P2 protein autoantigen are similar to those of foreign protein antigens. Proceedings of the National Academy of Sciences of the United States of America. 1988;**85**:5186-5189

[73] Castro H, Sousa C, Novais M, et al. Two linked genes of *Leishmania infantum* encode tryparedoxins localised to cytosol and mitochondrion. Molecular and Biochemical Parasitology. 2004;**136**:137-147

[74] Santarém N, Silvestre R, Tavares J, et al. Immune response regulation by Leishmania secreted and nonsecreted antigens. Journal of Biomedicine & Biotechnology. 2007. Epub ahead of print 2007. DOI: 10.1155/2007/85154

[75] Santarém N, Tomás A, Ouaissi A, et al. Antibodies against a *Leishmania infantum* peroxiredoxin as a possible marker for diagnosis of visceral leishmaniasis and for monitoring the efficacy of treatment. Immunology Letters. 2005;**101**:18-23

[76] Rafati S, Nakhaee A, Taheri T, et al. Expression of cysteine proteinase type I and II of *Leishmania infantum* and their recognition by sera during canine and human visceral leishmaniasis. Experimental Parasitology. 2003;**103**:143-151

[77] De Souza Dias S, Da Costa Pinheiro PH, Katz S, et al. A recombinant cysteine proteinase from Leishmania (Leishmania) chagasi suitable for serodiagnosis of American visceral leishmaniasis. The American Journal of Tropical Medicine and Hygiene. 2005;**72**:126-132

[78] Henrique P, Nunes A, Hayde J, et al. A recombinant cysteine proteinase from Leishmania (Leishmania) chagasi as an antigen for delayed-type hypersensitivity assays and serodiagnosis of canine visceral leishmaniasis. Veterinary Parasitology. 2009;**162**:32-39

[79] Ghedin E, Zhang WW, Charest H, et al. Antibody response against a Leishmania donovani amastigote-stage-specific protein in patients with visceral leishmaniasis. Clinical and Diagnostic Laboratory Immunology. 1997;**4**:530-535

[80] Zhang WW, Charest H, Ghedin E, et al. Identification and overexpression of the A2 amastigote-specific protein in Leishmania donovani. Molecular and Biochemical Parasitology. 1996;**78**:79-90

[81] Mougneau E, Altare F, Wakil a E, et al. Expression cloning of a protective Leishmania antigen. Science. 1995;**268**:563-566

[82] Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One;**7**. Epub ahead of print 2012. DOI: 10.1371/journal.pone.0035671

[83] Maalej IA, Chenik M, Louzir H, et al. Comparative evaluation of ELISAs based on ten recombinant or purified Leishmania antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. The American Journal of Tropical Medicine and Hygiene. 2003;**68**: 312-320

[84] Abass E, Bollig N, Reinhard K, et al. rKLO8, a novel Leishmania donovani - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. PLoS Neglected Tropical Diseases;**7**. Epub ahead of print 2013. DOI: 10.1371/journal.pntd.0002322

[85] Abass E, Kang C, Martinkovic F, et al. Heterogeneity of Leishmania donovani parasites complicates diagnosis of visceral leishmaniasis: Comparison of different serological tests in three endemic regions. PLoS One. 2015;**10**:1-13

[86] van den Bogaart E, Berkhout MMZ, Adams ER, et al. Prevalence, features and risk factors for malaria co-infections amongst visceral leishmaniasis patients from Amudat hospital, Uganda. PLoS Neglected Tropical Diseases;**6**. Epub ahead of print 2012. DOI: 10.1371/journal.pntd.0001617

[87] Pinedo-Cancino V, Laurenti MD, Kesper N, et al. Evaluation of Leishmania (Leishmania) infantum excreted-secreted antigens for detection of canine leishmaniasis. Acta Tropica. 2016;**161**:41-43

[88] Lima BSS, Fialho LC, Pires SF, et al. Immunoproteomic and bioinformatic approaches to identify secreted *Leishmania amazonensis*, *L. braziliensis*, and *L. infantum* proteins with specific reactivity using canine serum. Veterinary Parasitology. 2016;**223**:115-119

[89] Filippas-Ntekouan S, Liberopoulos E, Elisaf M. Lipid testing in infectious diseases: Possible role in diagnosis and prognosis. Infection. 2017;**45**:575-588

[90] Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: Current status and future applications. Journal of Clinical Microbiology. 2007;**45**:21-25

[91] Silvestre R, Santarém N, Teixeira L, et al. Evaluation of Leishmania species reactivity in human serologic diagnosis of leishmaniasis. The American Journal of Tropical Medicine and Hygiene. 2009;**81**:202-208

[92] Srividya G, Kulshrestha A, Singh R, et al. Diagnosis of visceral leishmaniasis: Developments over the last decade. Parasitology Research. 2012;**110**:1065-1078

[93] Andrade RA, Reis AB, Gontijo CMF, et al. Clinical value of anti-Leishmania (Leishmania) chagasi IgG titers detected by flow cytometry to distinguish infected from vaccinated dogs. Veterinary Immunology and Immunopathology. 2007;**116**:85-97

[94] Courtenay O, Carson C, Calvo-Bado L, et al. Heterogeneities in *Leishmania infantum* infection: Using skin parasite burdens to identify highly infectious dogs. PLoS Neglected Tropical Diseases. 2014;**8**:26

[95] Courtenay O, Quinnell RJ, Garcez LM, et al. Infectiousness in a cohort of Brazilian dogs: Why culling fails to control visceral leishmaniasis in areas of high transmission. The Journal of Infectious Diseases. 2002;**186**:1314-1320

[96] Magalhães-Junior JT, Mota TF, Porfirio-Passos G, et al. Xenodiagnosis on dogs with visceral leishmaniasis: Canine and sand fly aspects related to the parasite transmission. Veterinary Parasitology. 2016;**223**:120-126

[97] Gomes CM, de Morais OO, Roselino AM, et al. Complementary exams in the diagnosis of American tegumentary leishmaniasis. Anais Brasileiros de Dermatologia. 2014;**89**:701-709

[98] Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of Leishmania. Experimental Parasitology. 1990;**71**(3):267-275

[99] Moreira MAB, Luvizotto MCR, Garcia JF, et al. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. Veterinary Parasitology. 2007;**145**:245-252

[100] Mohammadiha A, Mohebali M, Haghighi A, et al. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania (Leishmania) infantum* infection in human and dog blood samples. Experimental Parasitology. 2013;**133**:89-94

[101] Aschar M, de Oliveira ETB, Laurenti MD, et al. Value of the oral swab for the molecular diagnosis of dogs in different stages of infection with *Leishmania infantum*. Veterinary Parasitology. 2016;**225**:108-113

[102] Carson C, Quinnell RJ, Holden J, et al. Comparison of Leishmania OligoC-TesT PCR with conventional and real-time PCR for diagnosis of canine Leishmania infection. Journal of Clinical Microbiology. 2010;**48**:3325-3330

[103] Bastien P, Procop GW, Reischl U. Quantitative real-time PCR is not more sensitive than ' conventional' PCR. Journal of Clinical Microbiology. 2008;**46**:1897-1900

[104] Sudarshan M, Weirather JL, Wilson ME, et al. Study of parasite kinetics with antileishmanial drugs using real-time quantitative PCR in Indian visceral leishmaniasis. The Journal of Antimicrobial Chemotherapy. 2011;**66**:1751-1755

[105] Espy MJ, Uhl JR, Sloan LM, et al. Real-time PCR in clinical microbiology : Applications for routine laboratory testing. Clinical Microbiology Reviews. 2006;**19**:165-256

[106] Espinosa D, Boggild AK, Deborggraeve S, et al. Leishmania OligoC-TesT as a simple, rapid, and standardized tool for molecular diagnosis of cutaneous leishmaniasis in Peru. Journal of Clinical Microbiology. 2009;**47**:2560-2563

[107] Palatnik-De-Sousa CB, Dos Santos WR, França-Silva JC, et al. Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. The American Journal of Tropical Medicine and Hygiene. 2001;**65**:510-517

[108] Coura-Vital W, Marques MJ, Veloso VM, et al. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. PLoS Neglected Tropical Diseases;**5**. Epub ahead of print 2011. DOI: 10.1371/journal.pntd.0001291

[109] Islam MZ, Itoh M, Mirza R, et al. Direct agglutination test with urine samples for the diagnosis of visceral leishmaniasis. The American Journal of Tropical Medicine and Hygiene. 2004;**70**:78-82

[110] Boelaert M, Verdonck K, Menten J, et al. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. Cochrane Database of Systematic Reviews. 2014;**6**:CD009135

[111] Schallig HDFH, Canto-Cavalheiro M, Da Silva ES. Evaluation of the direct agglutination test and the rK39 dipstick test for the sero-diagnosis of visceral leishmaniasis. Memórias do Instituto Oswaldo Cruz. 2002;**97**:1015-1018

[112] Adams ER, Jacquet D, Schoone G, et al. Leishmaniasis direct agglutination test: Using pictorials as training materials to reduce inter-reader variability and improve accuracy. PLoS Neglected Tropical Diseases. 2012;**6**:1-6

[113] Alvar J, Cañavate C, Molina R, et al. Canine leishmaniasis. Advances in Parasitology. 2004;**57**:1-88

[114] Otranto D, Paradies P, Sasanelli M, et al. Rapid immunochromatographic test for serodiagnosis of canine leishmaniasis. Journal of Clinical Microbiology. 2004;**42**:2769-2771

[115] Otranto D, Paradies P, De Caprariis D, et al. Toward diagnosing *Leishmania infantum* infection in asymptomatic dogs in an area where leishmaniasis is endemic. Clinical and Vaccine Immunology. 2009;**16**:337-343

[116] Nolan JP, Mandy F. Multiplexed and microparticle-based analyses? Quantitative tools for the large-scale analysis of biological systems. Cytometry Part A. 2006;**69**:318-325

[117] Silvestre R, Santarém N, Cunha J, et al. Serological evaluation of experimentally infected dogs by LicTXNPx-ELISA and amastigote-flow cytometry. Veterinary Parasitology. 2008;**158**:23-30

[118] Lemos EM, Gomes IT, Carvalho SFG, et al. Detection of anti-Leishmania (Leishmania) chagasi immunoglobulin G by flow cytometry for cure assessment following chemotherapeutic treatment of american visceral leishmaniasis. Clinical and Vaccine Immunology. 2007;**14**:569-576

[119] Cañavate C, Herrero M, Nieto J, et al. Evaluation of two rK39 dipstick tests, direct agglutination test, and indirect fluorescent antibody test for diagnosis of visceral leishmaniasis in a new epidemic site in highland Ethiopia. The American Journal of Tropical Medicine and Hygiene. 2011;**84**:102-106

[120] Biglino A, Bolla C, Concialdi E, et al. Asymptomatic *Leishmania infantum* infection in an area of Northwestern Italy (piedmont region) where such infections are traditionally nonendemic. Journal of Clinical Microbiology. 2010;**48**:131-136

[121] Goto Y, Coler RN, Guderian J, et al. Cloning, characterization, and serodiagnostic evaluation of *Leishmania infantum* tandem repeat proteins. Infection and Immunity. 2006;**74**:3939-3945

[122] Mirsamadi N, Naeini KM, Mamishi S, et al. Application of direct agglutination test (DAT) for the diagnosis and Seroepidemiological studies of visceral leishmaniasis in Iran. Iranian Journal of Parasitology. 2006;**1**:15-25

[123] Miró G, Cardoso L, Pennisi MG, et al. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: Part two. Trends in Parasitology. 2008;**24**:371-377

[124] Quinnell RJ, Carson C, Reithinger R, et al. Evaluation of rK39 rapid diagnostic tests for canine visceral leishmaniasis: Longitudinal study and meta-analysis. PLoS Neglected Tropical Diseases;**7**. Epub ahead of print 2013. DOI: 10.1371/journal.pntd.0001992

[125] Ben-Abid M, Galaï Y, Habboul Z, et al. Diagnosis of Mediterranean visceral leishmaniasis by detection of Leishmania-related antigen in urine and oral fluid samples. Acta Tropica. 2017;**167**:71-72

[126] Sarkari B, Chance M, Hommel M. Antigenuria in visceral leishmaniasis: Detection and partial characterisation of a carbohydrate antigen. Acta Tropica. 2002;**82**:339-348

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