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New Advances in the Diagnosis of Canine Visceral Leishmaniasis

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1. Introduction

Visceral leishmaniasis (VL) is a serious public health problem of great medical and veterinary importance. This disease is endemic in Brazil and in many other countries of Latin America, Asia, Africa and Europe (1). According to recent review (2), approximately 0.2 to 0.4 million cases of VL occur each year and although worldwide distributed, higher prevalence of the disease is concentrated in six countries, including India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil, that undertake for more than 90% of the cases. The clinical importance of VL resides in the severity of the disease that results in death of unrecognized cases and even for individuals with treatment access, death occurs in 10 to 20% of the cases [2-8].

Most of the VL cases are caused by the *Leishmania* species from *Leishmania donovani* complex. Parasites from the *Leishmania* genus are diphasic and are transmitted as promastigote form to vertebrate hosts through the bites of female sand flies [9-12]. The genetic similarities between Old World and New World strains, respectively, of *L. infantum* and *L. infantum* (syn. *chagasi*) from different regions in Latin America, indicate these parasites belong to the same origin [11, 12].

The notion that dogs are the main urban domestic reservoir for this *Leishmania* in certain part of the globe is supported by the facts including: i) cases of canine visceral leishmaniasis (CVL) have been reported in 50 of the 88 countries where VL is endemic [13], ii) canine cases precede the onset of human cases [14], iii) high rates of prevalence in dogs, and iv) frequency of parasites is high in dog skin [15-18].

Control strategies include performing accurate and early diagnosis of CVL to identify infected animals [19, 20]. CVL diagnosis is a difficult task since clinical signs of the disease in dogs can be confused with other diseases [19]. In endemic areas, a large percentage of infected animals are asymptomatic or present low number of discrete signs. The role these animals play in parasite transmission is still largely unknown. Several diagnostic strategies have been implemented based on parasitological, serological or molecular methods in association with clinical and epidemiological parameters [21]. Parasite culturing has been considered as gold standard for disease diagnosis [22, 23]. Although offering a high specificity since allows parasite identification, it offers very low sensitivity, besides it is laborious, time-consuming and largely dependent on the expertise of the observer [24, 25].

Serological tests are the most common diagnostic method employed for CVL diagnosis [3]. Several serological methods have been implemented for diagnosis of CVL, including direct agglutination assay (DAT), enzyme linked immunoassay (ELISA) and indirect immunofluorescent antibody test (IFI) [26]. However, most of these classical serological tests present important limitations for CVL diagnosis, including high consumption of time, and lack of sensitivity and specificity, mainly when animals present low antibody titers. This causes underestimation of disease, reflecting in failures in control measures, as well as the maintenance of infected untreated dogs in endemic areas [27, 28]. New methods based on immunochromatography have been implemented for serodiagnosis of CVL and have shown excellent results [29]. These techniques offer several advantages since they are rapid tests easily performed even in field areas, and more specific since they use recombinant DNA technology that additionally facilitates reproducibility and large-scale production. These advantages result in better identification of infected dogs. However, the efficacy of immunochromatographic techniques for CVL diagnosis needs to be improved [30]. In Brazil, a rapid test based in dual path platform (TR DPP®LVC - Biomanguinhos) had been recently implemented as screening test for CVL. This technique seems to be adequate to disease diagnosis in public health system. However, the TR DPP®LVC has shown an excellent performance identifying 98% of symptomatic dogs, it showed less efficacy for diagnosis of asymptomatic dogs (47%) [31]. Since there is evidence that asymptomatic dogs can participate in natural transmission cycle of VL, new strategies should be implemented in order to improve CVL diagnosis [16, 32-34]. For serological diagnosis one strategy can be the development of rapid tests based on impregnation of multi-antigen that would offer more sensitivity, as well specificity.

Finally, it would be important to include more specific confirmatory tests for control strategies that can be advantageous to diagnose inconclusive cases. There is evidence that molecular diagnosis of *Leishmania* spp. provides high levels of sensitivity and specificity when compared to other diagnostic methods [7, 17]. The use of quantitative methods in molecular level allows not only a more accurate detection but also monitoring tissue parasite load in dogs following anti-leishmanial treatment [35-37].

2. Importance of CVL diagnosis

Since the discovery of canine visceral leishmaniasis (CVL) in Tunisia by Nicolle & Comte (1908), several reports have shown that dog and man share a common etiologic agent. The

notion that dog is the main reservoir of visceral leishmaniasis (VL) in urban centers [38] is supported by several evidences including the high cutaneous parasitism observed in dogs infected by *Leishmania* [15-18], the high rates of prevalence observed among dogs in endemic areas, and the observation that the appearance of canine cases precedes the emergence of human cases [14]. Some studies conducted in endemic regions, where VL occurs in a zoonotic cycle, point to a prevalence of approximately 20% of VL-positive dogs, as described in China [39], Greece [40], and Mexico [41]. In other endemic areas, rates of *Leishmania* infection detected in dogs range between 60 and 80% [4, 42-44]. However, in spite of the high prevalence of *Leishmania* infection, not all dogs exhibit signs of clinical disease and sick dogs may display clinical signs of variable intensity [4, 42, 45]. Differences in clinical manifestations among dogs as the number of clinical signs and the time to onset of the disease may vary depending on the individual immune response of the infected dog. Three forms of progression of infection by *L. infantum* have been described: about 46% of infected dogs acquire the infection and develop the disease immediately, another 44% of dogs develop the disease later and 10% of them never develop CVL [46-48]. Similar to that which occurs in humans, characteristics such as genetic factors, age and nutritional status may influence the progression of VL in dogs [13, 49, 50].

Some studies have shown a correlation between the presence of clinical signs in infected animals and transmissibility of the parasite to the vector and, consequently, a correlation with the occurrence of human cases [16, 32, 51]. In accordance with these studies, Travi et al. (2001) and Verçosa et al. (2008) showed that asymptomatic dogs did not transmit the parasite to the vector [38, 51]. There is not a consensus about this idea, since there is a wide variation in the rates of infectivity (70 to 90%) between asymptomatic and symptomatic dogs. Studies show that, regardless of the clinical presentation, any dog has the ability to transmit *Leishmania* to the vector, even though the symptomatic animals are more likely to disseminate the disease [16, 32, 52, 53], being more capable of transmitting the parasite to the vector and, consequently, to another dog or a human [33, 34, 54, 55]. Based on studies showing that the dog can transmit the disease regardless of its clinical form, in some countries such as Brazil, dog culling is recommended as a control strategy. This control measure is not well accepted, having its effectiveness questioned and demanding studies to increase the diagnostic methods performance. Additionally, identification of new antigens will allow not only improvement of diagnosis, but also differentiation of dogs that transmit, from those that do not transmit the parasite in an endemic area.

3. Visceral leishmaniasis diagnosis in dogs

The diagnosis of VL in the dog must consider the association between clinical, laboratory and epidemiological data. As discussed above, clinical diagnosis is problematic and difficult for veterinarians to perform due to the great variability of clinical signs that *Leishmania*-infected dogs may present, as long as to its similarity with clinical profiles of other diseases. In clinical practice, identification of characteristic manifestations should be confirmed by ascertaining the infection using laboratory techniques [56] that vary in accuracy [57, 58].

There are several laboratorial diagnosis methods for leishmaniasis: i) parasitological methods (detection of the parasite), ii) serological methods (detection of anti-*Leishmania* antibodies), iii) molecular methods (amplification of parasite DNA) and iv) assay of cell-mediated immunity. This last method not being widely applied to routine diagnosis and will not be discussed in the present report. It should be noted that, although there is a wide variety of diagnostic techniques for CVL, none of them offer 100% of sensitivity or specificity [35, 59].

In spite of serological techniques such as enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFAT) being the most widely used methods for the diagnosis of CVL [60] parasitological methods, such as direct examination of slides and isolation from tissue cultures, allow the parasite to be detected and can be used as confirmatory diagnostic methods for CVL [61]. In recent decades, molecular techniques such as polymerase chain reaction (PCR) have been introduced for the diagnosis of CVL, exhibiting high sensitivity and specificity [21]. These techniques detect the genetic material of the parasite, which can be used as confirmatory methods in cases of recently infected or asymptomatic animals, which tend not to be diagnosed serologically, and in most cases, do not show seroconversion, having a low parasite load [4, 60]. In a study conducted in Belo Horizonte-MG, a VL-endemic area in Brazil, among 1,443 dogs evaluated, 15.3% of them were seropositive, while 84.7% showed negative serology. Interestingly, among serologically negative dogs, 24.4% showed up as positive using the molecular diagnostic technique, and most of these (97.6%) would not be diagnosed, since they consist of asymptomatic dogs with negative serology [19].

3.1. Clinical diagnosis

Dogs from endemic areas considered resistant remain clinically normal and asymptomatic without exhibiting clinical signs. There is evidence that the parasites in these animals are effectively eliminated at the infection site [62, 63]. However, in susceptible animals, a large number of parasites are detected in infected tissues. In these animals, the presence of the parasite may occur in multiple organs, accompanied by a granulomatous inflammatory reaction and production of immune-mediated phenomena, probably responsible for the appearance of various types of clinical signs [64].

Initial clinical signs of CVL include: hypertrophy of the lymph nodes, changes in skin appendages such as onychogryphosis, swelling of the footpad, localized alopecia, skin ulcers and nasal and periocular dermatitis. Alopecia and non-pruritic exfoliative dermatitis can spread to other parts of the animal's body. Weight loss may also be present, as well as cachexia, anorexia and conjunctivitis. Internal organs such as spleen, liver, kidney and lymph nodes may also be affected, when kidney injuries are present may lead to the dogs death [13, 65]. Fever, apathy, diarrhea, epistaxis, intestinal bleeding, hepatosplenomegaly, hyperkeratosis, keratoconjunctivitis are also found in affected animals [66-68]. Some clinical signs are more frequent than others; skin lesions are the most frequent manifestations affecting approximately 50 to 90% of symptomatic dogs [4, 67, 69, 70], including non-pruritic exfoliative dermatitis, with or without alopecia, which can be generalized or localized to the muzzle, ears and limbs [67, 71, 72]. Other very common signs are weight loss, observed in 25 to 80% of CVL cases,

including onychogryphosis in 30 to 75%, and ocular abnormalities in 16 to 24% [28]. The most common clinical signs of VL in dogs are depicted in Figure 1.



Figure 1. Major clinical signs associated with CVL. A: alopecia on the muzzle, B: periocular dermatitis with keratoconjunctivitis and hyperkeratosis; C: hyperkeratosis of the nasal mucosa; D: generalized non-pruritic exfoliative dermatitis; E: ulcerated lesion in the ear; F: crust with vascular injury on the tip of the ear; G: lymphadenomegaly of the popliteal lymph node; H: cachexia; I: onychogryphosis. Photos of animals infected by *L. infantum* belong to archives from Laboratory of Pathology and Biointervention (LPBI - CPqGM).

In dogs with CVL, clinical-pathological changes may occur such as intestinal lesions, renal and hepatic abnormalities [73]. The main biochemical laboratory findings from CVL are hyperglobulinemia, mainly due to increased production of antibodies, and hypoalbuminemia, attributed to chronic inflammation, as long as renal and hepatic failure [66]. The result of these changes is a reduction in the albumin/globulin ratio and hyperproteinemia [28]. Additionally, severe CVL is associated with changes in hematological parameters such as severe anemia and leukopenia, associated with lymphopenia, eosinopenia and monocytopenia [66, 74, 75].

Immune-mediated thrombocytopenia also occurs accounting for episodes of bleeding such as epistaxis, hematuria and hemorrhagic diarrhea [76].

Finally, nonspecific signs of illness that are mistaken for other diseases such as babesiosis, ehrlichiosis and canine trypanosomiasis also contribute to make CVL clinical diagnosis imprecise and difficult to perform [13].

3.2. Parasitological diagnosis

The detection by optical microscopy of the parasite by direct observation of stained smears from spleen aspirate, lymph node and bone marrow tissues has high specificity, allowing confirmation of CVL diagnosis [3, 53, 61, 77]. However, the sensitivity of this method is less than 30%, since the direct parasite identification may be limited, especially in mildly and asymptomatic dogs that have low parasitic load, producing false negative results [3, 53, 61, 77].

Another method that can identify the parasite in tissues is the culturing of tissue fragments or aspirates, preferably in a biphasic medium [78], composed by Novy-MacNeal-Nicolle (NNN), or Tobie modified medium or United States Army Medical Research Units (USAMRU) as solid phase medium and, most often, Schneider as liquid phase medium. This parasitological diagnostic method offers high specificity allowing isolation and characterization of parasites, as well as determination of which species and/or variants are circulating in endemic areas [79]. However, the culturing consists of an indirect test, because when the parasites are isolated from various tissues, they are present in amastigote form and during cultivation they transform into the promastigote form. This process may be impaired as a result of parasite death due to a failure of temperature-control during transport of the tissue sample, or contamination during collection or cultivation [13]. Additionally, a culturing is time consuming and may take up to 4 weeks of observation for definitive diagnosis [13, 79]. Furthermore, specific media for promastigote isolation are not easily obtained, being a technique restricted to specialized laboratories [70, 80], in which the outcome also depends on the experience of the observer [24, 25]. Although culturing offers greater sensitivity compared to direct viewing of amastigotes in tissue [35], it still remains at very low levels.

In summary, parasitological techniques have high specificity but low sensitivity, especially for the detection of dogs, recently infected, asymptomatic or those presenting low parasite load. In addition, the need for skilled personnel and the long delays to obtain the results prevent parasitological techniques to be used in epidemiological surveys [4, 23, 61, 81-84].

3.3. Serological diagnosis

Serological tests are based on the presence of specific humoral immune responses against the pathogen or purified fraction or recombinant proteins of the pathogen. These tests allow detection of immunoglobulin (IgG) levels, thus becoming an essential tool for the diagnosis of CVL. These methods are simple to carry out and therefore they are frequently used to determine the prevalence of leishmaniasis in epidemiological studies [85].

A wide variety of serological methods are available for CVL diagnosis, presenting variations in sensitivity and specificity. The performance of these diagnostic techniques varies depending on the type of antigen used and the detection of anti-*Leishmania* antibody system.

The most commonly employed serological tests for the diagnosis of CVL, including ELISA, indirect immunofluorescence test (IFAT), and direct agglutination test (DAT), uses parasite or crude extract of *Leishmania*, as the antigen source. More recently, serological methods based on ELISA or rapid tests have been developed using a purified fraction of the parasite or a specific purified recombinant protein, such as rK39 or a chimeric protein as rK28 (rK9 + rK26 + rK39).

Despite the practicality and simplicity of serological tests, they do not have 100% sensitivity because some dogs, especially those that are resistant or in the early stages of the disease, have negative results. Thus, the results of such tests should be evaluated carefully, always associating test results with epidemiological history, clinical state of the animal, and the result of a more specific diagnostic test [86]. In addition, since titers of anti-*Leishmania* antibodies remain detectable for long periods, serological tests are not a good alternative for assessing healing or monitoring dogs after treatment [71].

- IFAT

IFAT is a test in which anti-immunoglobulin antibodies labeled with fluorochromes react with parasites immobilized in a slide. IFAT is a laborious technique that presents difficulties for both standardization and interpretation of the results. Therefore, detection of antigen-antibody reaction by fluorescence microscopy depends on the observer experience, compromising reproducibility of this test in different laboratories. Thus, it is not considered a simple and practical technique for evaluating a large number of canine sera [57]. In spite of these limitations, it is still being used as a diagnostic method for mass screening of infected dogs [87]. This method varies in its performance, with sensitivity ranging from 68 to 100% and specificity of 60 to 90% [5, 88-90].

In a study evaluating IFAT for the diagnosis of CVL, the efficacy of the test was evaluated using 254 sera from infected and uninfected dogs and sera from animals with other parasitic diseases. The authors observed low sensitivity (72%) and specificity (52%), as well as cross-reactions when sera from dogs infected with other pathologies, such as *Trypanosoma cruzi*, *Leishmania braziliensis*, and *Ehrlichia canis* were tested. In this study, the reproducibility of the results among different laboratories yielded a moderate rate of 0.74 [5]. A similar result was obtained by Lira et al. (2006) that using IFAT, found a low sensitivity and specificity of 68% and 87.5%, respectively [88]. By contrast, it was shown by Alves and colleagues that the IFI-CVL kit, IFAT produced by Biomanguinhos (Brazil), provides high sensitivity (100%), detecting all dogs with CVL, although presenting a low specificity of 70%, levels similar to those observed in studies described above. This low level of specificity was due to false positive results from sera of dogs infected with *Trypanosoma caninum* (48.7%) and healthy dogs (10.3%) [90]. Thus, the Brazilian Ministry of Health withdrew the IFI-CVL kit from the CVL control program, due to data in literature that support IFAT has both low sensitivity, which led to

maintenance of false-negative dogs in the endemic areas, and low specificity, which promoted the culling of false-positive dogs [65, 91, 92].

- DAT

The direct agglutination test (DAT) is an alternative method for the diagnosis of VL, first described in 1975 and adapted for the diagnosis of human and canine infection in the late 1980s [93, 94]. DAT is a method that uses whole stained promastigotes as antigen, either in suspension or freeze-dried [35]. The advantage of this test lies in its low cost when compared with other tests [93]. However, this test is not desirable for screening large numbers of samples, since it is a laborious procedure, due to the production process for crude *Leishmania* antigen, as well as the need to perform serial dilutions of serum [95, 96]. Regarding the accuracy of DAT, there is some controversy in the literature, the sensitivity appears relatively variable between 88 and 93% and specificity of 70 to 100% [5, 59, 96, 97]. It is likely that these variations are related to how the cutoff is defined, since cutoffs using smaller dilutions reduce sensitivity but raise the specificity, while those using higher dilutions increase sensitivity and reduce specificity [98].

Changes to the DAT protocol have been proposed by Gómez-Ochoa *et al.* (2003) in order to decrease the cost of the procedure and the preparation time of the antigen, while maintaining the same sensitivity and specificity of conventional DAT. One of the proposed changes for DAT protocol is the fast agglutination-screening test (FAST), which combines a higher concentration of parasites with a smaller test volume. FAST requires a single serum dilution and the results can be read in 3 hours [95, 96]. However, in spite of the sensitivity of this technique being greater than that of conventional DAT, which varies from 93 to 98%, the specificity values were similar to those from the conventional DAT, between 78-89% [95, 96].

- ELISA

For various reasons, ELISA tests based on whole parasites or crude lysate of parasite antigens for the diagnosis of CVL do not provide satisfactory results, as follows: i) it is a laborious technique, which leads to a delay in the delivery of results and, consequently, the implementation of treatment or the removal of infected dogs from endemic areas [68, 99]; ii) leads to the appearance of cross-reactions with sera from individuals infected with other *Leishmania* species or even with a variety of other pathogens such as *Toxoplasma gondii*, *Ehrlichia canis* [23, 24, 29, 100-103], and parasites of the Trypanosomatidae family such as *Trypanosoma cruzi* [5, 29] or *Trypanosoma caninum* [90] iii) there are reports of low reproducibility between batches of ELISA based on whole parasites or crude antigens, since different isolates of *Leishmania* sp. were used and depending on the culturing batch, distinct antigenic compositions can be detected [104].

A study using 234 domesticated dogs in an endemic area for CVL assessed the efficacy of ELISA, IFAT and DAT for the diagnosis of CVL. In this study, dogs were also parasitologically evaluated for identification of *Leishmania* amastigotes in both skin samples and bone marrow aspirates. The sensitivity of IFAT was 72% and ELISA was 95%. When the tests were evaluated

against sera from dogs infected with other pathogens, *T. cruzi*, *E. canis* and *L. braziliensis*, the specificity of these tests shown to be very low 52% for IFAT and 64% for ELISA [5].

Using sera from dogs with CVL, a comparison of an ELISA test using crude soluble antigen of *L. infantum* (SLA) and another ELISA test based on ribosomal protein of *L. infantum* (LRP) was made. The sensitivity of the two tests was similar when evaluated against sera from symptomatic animals (100%), but the ELISA based on LRP showed also higher sensitivity, reaching 100% in the detection of asymptomatic dogs, when compared with ELISA using SLA (19%). Additionally, the LRP-based ELISA showed high specificity (98%), with no cross-reactivity with sera from dogs with other diseases [102].

Thus, the search for tests with higher sensitivity and specificity for dogs with a variety of conditions became necessary for control of CVL, which would lead to a reduction of errors in actions taken for treatment or control. In countries that adopt culling of seropositive dogs as a control measure, low sensitivity of diagnostic tests can lead to the maintenance of dogs that transmit disease and lack of specificity can result in unnecessary culling of healthy dogs. The identification of new proteins of *Leishmania sp.* in order to compose diagnostic tests for CVL can improve both sensitivity and specificity of diagnostic techniques and allow infected dogs to be differentiated from the uninfected ones [5, 29, 87, 105, 106].

Another way to overcome the obstacles of ELISA based on whole parasites or crude parasite antigen was the development of ELISA tests based on parasite fractions such as that using the parasite surface molecule, fucose-mannose ligand antigen (FML). The FML-based ELISA showed a high sensitivity, which was similar in detecting either oligosymptomatic (90%) or symptomatic (90%) dogs. Regarding specificity, ELISA using crude parasite antigen for the diagnosis of oligosymptomatic dogs was superior, achieving 100% in comparison to FML-based ELISA that was 93.3%. However, for symptomatic dogs the specificity of the FML-based ELISA showed similar results of 96.7% compared to that obtained by ELISA based on crude parasite antigen (93.3%) [101].

Other ELISA assays based on recombinant antigens such as rA2 from *L. donovani*, rK26 or rK39 from *L. infantum* have been developed. When compared to an ELISA test based on crude antigen, taking IFAT as the gold standard, and tested against sera from symptomatic dogs, sensitivities was higher for ELISA based on rK26 or rK39 from *L. infantum*, respectively, of 94% and 100% in comparison to 88% for ELISA based on crude antigen and 70% for ELISA based on rA2 from *L. donovani*. However, ELISA based on rA2 showed the highest specificity value of 96% in comparison to those from ELISA based on crude parasite antigen or parasite recombinant antigens rK26, rK39, that showed values of, respectively, 87%, 90%, and 85% [29]. Although good enough for diagnosis of symptomatic dogs, the use of ELISA tests based on recombinant antigens for the diagnosis of asymptomatic dogs seems to be disadvantageous. ELISA based on rA2 gave the lowest sensitivity of 30%, followed by rK26 and rK39 that sensitivity yielded greater, but still stayed low with value of 66% for both tests, in comparison to the sensitivity of 88% for ELISA based on crude parasite antigen. By contrast, another in a multicenter study conducted in 5 regions of Italy using IFAT as gold standard, ELISA based on the recombinant antigen rK39 gave higher levels of sensitivity and specificity of 97.1% and

98.8%, respectively [107]. It is possible that the differences found in relation to the performance of the ELISA containing these proteins may be related to the study design, characterization of serum samples and the experimental protocols of the ELISA assays.

Interestingly, the association of the recombinant proteins enhanced test performance both for detection of symptomatic and asymptomatic infected dogs. Indeed, using IFAT as the gold standard, ELISA based on the mix of rK9, rK26 and rK39 from *L. donovani* showed sensitivity of 95-100% and specificity of 100% against a panel of serum from parasitologically positive dogs, using parasitologically and serologically negative dogs as controls [108]. Furthermore, ELISA containing a chimeric protein rK28, containing reactive epitopes of the three recombinant proteins described above (rK9, rK26 and rK29), shown to present high levels of sensitivity and specificity of 99% and 96%, respectively when tested against a panel of serum from dogs parasitologically positive or serologically positive using IFA, and sera from parasitologically negative dogs as controls [87]. In this study, the authors didn't evaluate rK28-based ELISA against sera from asymptomatic dogs.

The combination of these findings reinforces the notion that the use of multiple antigens in diagnostic tests enhances test performance and the need to search for new antigens that may compose a diagnostic test able to better diagnose asymptomatic dogs.

New recombinant proteins are being evaluated. Faria *et al.* (2011) performed predictions on B-cell epitopes, identifying 360 peptides that were synthesized onto nitrocellulose membranes [105]. The 10 most reactive were evaluated in an ELISA platform. The sensitivity and specificity of the ELISA based on these peptides varied from 70.96% to 88.7% and 55% to 95.0%, respectively, a better result than that obtained with EIE-CVL kit, which gave a sensitivity of 13.8% and specificity of 100%.

Another study evaluated the performance of the ELISA based on another recombinant antigens of *L. infantum*, rLci1A and rLci2B, against a panel of canine sera (n = 256). ELISA based on rLci1A gave sensitivity of 96% and specificity of 92% for rLci1A and sensitivity of 100% and specificity of 95% for rLci2B. The recombinant antigens showed no cross-reactivity with sera from dogs infected with *Trypanosoma caninum*, *Babesia canis* and *Ehrlichia canis*. Cross-reaction against sera from dogs infected with *Leishmania braziliensis* was observed for rLci1A-based ELISA (11.7%) and for rLci2B-based ELISA (2.9%) [109].

In summary, most studies using ELISA suggest that in comparison to tests based on crude antigen, those based on recombinant antigens improves accuracy, increasing sensitivity and specificity for the diagnosis of symptomatic dogs. Although improved, test accuracy is still low for the detection of asymptomatic animals.

- Rapid tests

Recently, rapid immunodiagnostic tests have begun to be employed as routine laboratory tests for detection of diseases such as leishmaniasis. The recombinant antigens of the parasite are impregnated onto nitrocellulose membranes and serum samples are applied in the rapid test platform. Antigens impregnated in nitrocellulose membranes are recognized by specific immunoglobulin present in the serum of infected individuals. This reaction is revealed by

the interaction of protein A coupled to colloidal gold particles, with the Fc portion of the immunoglobulins associated with the recombinant antigens. The use of immunochromatographic assays as diagnostic methods has the main advantages of being rapid, completed in around 15 minutes, easy to carry out and can dispense with the need for equipment to read the results [110]. Furthermore, these tests are easily stored, and test supplies and samples do not need to be maintained at low temperatures and can it even be performed at the place of collection. These tests are already widely used to detect HIV [111] and H1N1 [112] infection. For the diagnosis of CVL and human VL, among the tested and commercially available recombinant proteins, the most widely used for composing immunochromatographic tests is the recombinant protein rK39. This protein contains repetitive sequences of 39 amino acids from a protein related to kinesin of kinetoplast from *L. infantum*, and has been widely used in ELISA platform as described above [30, 31, 91, 96, 97, 110, 113-119]. The efficacy of rK39-based rapid lateral flow test was compared with the IFAT against sera of parasitologically positive dogs from Italy. Both tests offered similar sensitivities of 97% for the lateral flow test in the dipstick format and 99% for the DAT, as well as the maximum specificity of 100% in both tests [120]. Similar results were obtained in other study which detected sensitivity of 97% and specificity of 100% using the rK39 dipstick for diagnosis of CVL, indicating that the rK39 dipstick is promising for both large-scale screening in endemic areas and for veterinary clinical practice [121]. However, other studies also conducted in endemic areas for CVL showed that the dipstick based on the recombinant protein rK39 offered inferior performance to that of the studies described above, with sensitivity values of 61-75% and specificity of 72-84.9% [122, 123]. These differences in performance between these studies using dipsticks tests based on rK39 may be related to the use of sera from dogs with different conditions and therefore with varying concentrations of specific immunoglobulins [29-31, 118, 122]. Effectively, another study employing the rapid test based on the recombinant protein rK39 dipstick offered a sensitivity of 96.7% for sera from symptomatic dogs, compared to a sensitivity of 52.9% when evaluating sera from asymptomatic dogs [119]. Similarly, another study found that using sera from asymptomatic dogs that the sensitivity of the dipstick based on rK39 was 75% compared to sensitivities of 88% and 84% for sera from oligosymptomatic and polysymptomatic dogs, respectively [118]. In the same study, an ELISA based on crude parasite antigens yielded a sensitivity of 94% tested using sera from asymptomatic and oligosymptomatic and 95% using sera of polysymptomatic dogs.

Recently, a meta-analysis was performed in order to broadly assess the performance of rapid tests using rK39 as the antigen in the diagnosis of CVL. The combined analysis of 16 studies using rapid tests based on rK39 offered a sensitivity of 86.7% (95% CI: 76.9–92.8%) for the detection of clinical disease and 59.3% (95% CI: 37.9–77.6%) for identification of *Leishmania* infection with a specificity of 98.7% (95% CI: 89.5–99.9%). In summary, this study supports the idea that the rapid test based on rK39 is useful to confirm the disease in dogs with clinical suspicion. However, given its low sensitivity for the diagnosis of asymptomatic dogs, its use is not recommended for large-scale epidemiological studies or VL control programs [30].

First author and year of publication	Serology		Sample			Results	
	Technique	Antigen	(n) Infected dogs confirmed by other techniques	(n) Non-infected dogs	(n) Dogs infected with others pathologies	Sensitivity (%)	Specificity (%)
Harith <i>et al.</i> , 1989	DAT	<i>L. infantum</i> promastigotes	44	6	176	98.9	100
Barbosa-de-Deus <i>et al.</i> , 2002	ELISA	LMS	188	1582	55	98.0	95.0
Scalone <i>et al.</i> , 2002	ELISA	K39	209	81	62	97.1	98.8
Schallig <i>et al.</i> , 2002	DAT	<i>L. donovani</i> promastigotes	79	67	24	88.6	96.7
	FAST	<i>L. donovani</i> promastigotes	79	67	24	93.6	89.0
Rosati <i>et al.</i> , 2003	ELISA	K26	202	20	0	100	100
	ELISA	K9	202	20	0	95	95
	ELISA	K39	202	20	0	95	95
Mohebbali, <i>et al.</i> , 2004	Dipstick	rK39	268*	0	0	70.9	84.9
Boarino <i>et al.</i> , 2005	ELISA	K9-K39-K26 chimera	232	362	0	95.8	99.1
Mettler <i>et al.</i> , 2005	Rapid test	rK39	47	50	26	A: 52.9 S: 96.7	94
	IFAT	<i>L. infantum</i> promastigotes	47	50	26	A: 29.4 S: 90.0	100
Lira <i>et al.</i> , 2006	EIE* - LVC	<i>L. major</i> lysate	25	16	11	72.0	87.5
	IFI* - LVC	<i>L. major</i> like promastigotes	25	16	11	68.0	87.5
Ferreira <i>et al.</i> , 2007	EIE* - LVC	<i>L. major</i> lysate	234*	20	20	96.0	100
	IFI* - LVC	<i>L. major</i> like promastigotes	234*	20	20	72.0	100
	DAT	<i>L. donovani</i> promastigotes	234*	20	20	93.0	100
Ferroglio <i>et al.</i> , 2007	SNAP* CLATK CTA		59	124	0	91.1	99.0
Porrozzi <i>et al.</i> , 2007	ELISA	rK26	100	25	14	A: 66.0 S: 94.0	90.0

First author and year of publication	Serology		Sample			Results	
	Technique	Antigen	(n) Infected dogs confirmed by other techniques	(n) Non-infected dogs	(n) Dogs infected with others pathologies	Sensitivity (%)	Specificity (%)
	ELISA	rK39	100	25	14	A: 66.0 S: 100	85.0
	ELISA	rA2	100	25	14	A: 88.0 S: 70.0	96.0
	ELISA	CTA	100	25	14	A: 30.0 S: 88.0	87.0
Cândido <i>et al.</i> , 2008	ELISA	CTA	60	30	0	O: 86.7 P: 90.0	O: 100 P: 93.3
	ELISA	FML	60	30	0	O: 90.0 P: 86.7	O: 93.3 P: 96.7
Lemos <i>et al.</i> , 2008	RDTs	rK39	76	33	0	83	100
	ELISA	<i>L. chagasi</i> lysate	76	33	0	95	100
Babakhan <i>et al.</i> , 2009	FAST	<i>L. infantum</i> promastigotes	73	74	0	98.6	78.7
Coelho <i>et al.</i> , 2009	ELISA	LRP	111	47	14	100	98.2
	ELISA	CTA	111	47	14	96.0	100
Troncarelli <i>et al.</i> , 2009	IFAT	<i>L. major</i> like promastigotes	51	0	0	83.0	92.5
Figueiredo <i>et al.</i> , 2010	EIE° - LVC	<i>L. major</i> lysate	305*	0	0	100	96.6
	IFI° - LVC	<i>L. major</i> like promastigotes	305*	0	0	22.2	97.0
de Lima <i>et al.</i> , 2010	ELISA	CTA	52	52	0	91.5	94.7
	RDTs	rK39	52	52	0	100	91.2
Marcondes <i>et al.</i> , 2011	SNAP° CLATK	CTA	283	86	31	94.7	90.6
Alves <i>et al.</i> , 2012	EIE° - LVC	<i>L. major</i> lysate	39	39	39	100	68.0
	ELISA	<i>L. chagasi</i> lysate	39	39	39	100	93.6
	IFI° - LVC	<i>L. major</i> like promastigotes	39	39	39	100	70.5
	IFAT	<i>L. chagasi</i> lysate	39	39	39	100	61.5

First author and year of publication	Serology		Sample			Results	
	Technique	Antigen	(n) Infected dogs confirmed by other techniques	(n) Non-infected dogs	(n) Dogs infected with others pathologies	Sensitivity (%)	Specificity (%)
Grimaldi <i>et al.</i> , 2012	DPP* - LVC	rK28	39	39	39	100	97.5
	DPP* - LVC	rK28	120	59	11	A: 47.0 S: 98.0	96.0
Souza <i>et al.</i> , 2012	ELISA	rLci1A	138	119	86	96.0	92.0
	ELISA	rLci2B	138	119	86	100	95.0
Barral-Veloso <i>et al.</i> , 2013	ELISA	<i>L. infantum</i> promastigotes fixed with β -mercaptoethanol	31	37	45	93.5	97.6
	ELISA	<i>L. infantum</i> promastigotes fixed with trypsin	31	37	45	87.1	100
Quinnell <i>et al.</i> , 2013	RDTs	rK39	322	59	0	46.0	98.7

* Dogs from an endemic area for CVL without confirmed diagnosis.

A: asymptomatic dogs; S: dogs with clinical signs related to CVL; O: oligosymptomatic dogs (presenting one to three clinical signs of CVL); P: polysymptomatic dogs (presenting more than three clinical signs of CVL).

RDTs = Kalazar Detect - Canine Rapid Test

LMS = *L. major* like promastigotes lysate

CTA = Crude Total Antigen - antigenic fractions of *L. infantum* promastigotes form

FML = Fucose-Mannose Ligand antigen - glycoprotein complex isolated from the surface of *L. donovani*

LRP = *Leishmania* species ribosomal proteins

Table 1. Sensitivity and specificity of some serological techniques by type of antigen, and evaluated population

Efforts have been made to improve the efficacy of rapid tests by developing more sensitive and specific method that could be used in mass screening for the diagnosis of CVL. An alternative proposal is to use a mixture of recombinant proteins or chimeric proteins. The protein rK28 chimeric for the relevant epitopes of three antigens, rK9, rK26 and rK39 [87, 108] that showed promising efficient results in an ELISA based test [124], was recently used to compose a new rapid test in DPP format. This format consists of a double track platform that offers greater sensitivity and specificity [125]. In addition, this rapid test has advantages over previously used serological methods due to greater precision, simplified interpretation of the

data, minimal use of sample volumes, and compatibility with different types of body fluids such as blood, serum, saliva, plasma and urine. In contrast to these advantages, recently Grimaldi et al (2012) showed that rK28-based DPP despite its high sensitivity (98%) and specificity (96%) towards sera from symptomatic dogs, showed low sensitivity of only 47% towards sera from dogs with no signs [31]. With regard to sera from dogs with other diseases, the observed specificity was 96%, with false-positive reactions mainly for some sera of dogs infected with *L. braziliensis*. Thus, *L. infantum* may not be detected in serological investigations of asymptomatic infected dogs, leading for perpetuation of the parasite cycle and, consequently, hampering the efficacy of the disease control measures. This limitation is reason for concern because several studies indicate that asymptomatic dogs are involved in transmission of infection to the vector, although this occurs less frequently than with symptomatic dogs [16, 32-34]. Therefore, it is necessary to implement more effective serodiagnostic tests so that there is broader detection of animals infected with *L. infantum* by public health services, contributing to more efficient control of CVL.

3.4. PCR

In recent decades, due to advances in molecular biology techniques and reduced implementation costs, the polymerase chain reaction (PCR) began to be used in VL diagnosis [23, 126]. Its use has demonstrated superior results to those obtained by ELISA, IFA and culture in detecting animals infected with *Leishmania* sp., exhibiting high sensitivity and specificity [23, 126].

PCR is a technique based on the principle of complementary bases pairing of the DNA molecule, allowing amplification and detection of a particular region of the target genome using a pair of specific oligonucleotide primers. The reaction can produce tens of billions of DNA fragments from a single molecule, and has high sensitivity small quantities of samples to be used. This type of PCR, hereafter referred as "conventional PCR" (cPCR) needs electrophoresis in agarose or polyacrylamide gels along with dyes such as ethidium bromide, SYBR Green or silver nitrate to view the amplified product. This approach is usually qualitative, with analysis of the presence or absence of bands, or semi-quantitative, when densitometry of bands is used in comparison with known standards. Since it uses qualitative or semi-quantitative analysis, it is imprecise and generates false negatives with some frequency.

A variant of cPCR called "quantitative real-time PCR" (qPCR) became popular in the 2000s. It uses a quantitative approach that allows real-time monitoring of the amplification of the target PCR fragment using fluorophores that bind to double stranded DNA or linked to probes. The most commonly used method is SYBR Green: fluorophore binds to double stranded DNA molecules produced during amplification of the target fragment, leading to the emission of fluorescence during the PCR. This method has the disadvantage of not being able to directly discriminate the amplification of nonspecific DNA fragments, which is usually solved by analyzing the dissociation curve. In contrast, the TaqMan method uses a probe containing between 13 and 30 nucleotides, specifically for the target sequence and combined with a fluorophore and a fluorescence inhibitor. During polymerization of the target fragment, DNA

polymerase degrades the probe and fluorescence is emitted. The use of this technique enables an increase in the specificity of this method.

Various PCR-based protocols have been developed for the detection of parasite's DNA and CVL diagnosis. However, the methods used may vary with respect to several parameters, such as fluorophores, probes, target regions and tissue used for detection of target DNA (Table 2), making it difficult to do a comparative analysis between the different protocols. It is known that the sensitivity and specificity of PCR for detection of *Leishmania* sp. depends on many factors such as the physico-chemical conditions of the reaction, the concentration and nature of the sample DNA, the probes, and oligonucleotide primers selected for the target region [44, 127, 128]. The protocols standardization based on changes in previous parameters is the key step to increased sensitivity, specificity and reproducibility of the tests.

The PCR protocol sensitivity is also affected by the type of tissue used in the detection of *Leishmania* sp. DNA. A wide variety of clinical samples can be used such as blood, lymph node, bone marrow, conjunctiva, skin and spleen. The sensitivity tends to vary, since the parasitic load is not equally distributed in all tissues [4, 7, 129-133]. However, studies have shown that PCR can detect the presence of *Leishmania* DNA in a similar way, even in different tissues as demonstrated by Manna et al. (2004) in a study using 56 dogs naturally infected with *L. infantum*, which evaluated samples from lymph nodes, skin and blood by cPCR obtaining positivity values of 99%, 95% and 94% respectively [133]. Similarly, Ferreira et al. (2013), using qPCR, obtained positivity values on the order of 90% for blood samples, 97% for skin biopsy, 98% for conjunctival swab samples and 100% for bone marrow samples [134]. Other studies showed better results for sensitivity to certain tissue, such as Maya et al. (2009) that evaluated dogs with different clinical profiles and demonstrated that the use of cPCR for parasite DNA detection on lymph node aspirate would be ideal for the early CVL diagnosis in symptomatic animals [132], a finding later corroborated by Lombardo et al. (2012) and Belinchon-Lorenzo (2013) [135, 136]. However, in the absence of lymphadenopathy, other studies showed that bone marrow aspirates offered better sensitivity, since it has a higher parasite load in relation to lymph nodes [132, 137]. Studies indicate bone marrow as the tissue in which PCR has greater sensitivity; as reported by Andrade et al. (2002), where bone marrow aspirate gave a sensitivity of 100% [138]. However Ferreira et al (2008) obtained positivity values of only 50% for bone marrow aspirates from asymptomatic and 77% from symptomatic dogs using cPCR followed by hybridization, using cloned kDNA-probes from mini circles of *L. infantum* [139].

The selection of target region in the parasite genome is important because the variation in the number of copies, depending on the region, influences the sensitivity for detecting the parasite's DNA and for quantification of parasite load. The highly conserved and repetitive regions are the most commonly employed, such as the gene for subunit ribosomal RNA (rRNA) or minicircle kinetoplast DNA (kDNA) [21, 23, 127, 140, 141], that has 40-200 copies per cell, while the kDNA minicircles have about 10,000 copies distributed among 10 different classes of sequences. Using this as a target region confers high sensitivity to PCR [142]. For quantification of the parasitic load is recommended to normalize the amount of parasite gene amplification in relation to a constitutive gene derived from the host genome in order to correct distortions caused by errors in the DNA used in the PCR reaction [127].

First author and year of publication	qPCR technique		qPCR internal control			Sample		Results	
	Method and target	Detection limit	Inhib. ^a	Norm. ^b	Control gene employed	(n)	Tissue evaluated	Study design*	Sensitivity**
Ferreira et al. 2012	Syber α pol DNA	NI	Yes	Yes	β - canine actin	(80) Infected dogs	Conjunctival swab, blood, bone marrow and skin	Comparative ^{1,2}	Skin > Bone marrow > Conjunctival swab > Blood
Solcà et al. 2014*	TaqMan kDNA	0.01 parasites/ reaction	Yes	Yes	18S eukaryotic rRNA	(51) Dogs	Bone marrow, conjunctival swab, lymph node, skin and spleen	Comparative ^{1,2}	Spleen > Blood > Lymph node > Skin > Bone marrow > Conjunctival swab
Belinchón-Lorenzo et al. 2013	TaqMan kDNA	0.0079 parasites/ reaction	Yes	Yes	18S eukaryotic rRNA	(28) Dogs	Blood, hair and lymph node	Comparative ²	Lymph node > Hair = Blood
Ferreira et al. 2013	Syber α pol DNA	NI	Yes	Yes	β - canine actin	(62) CVL positive dogs	Conjunctival, nasal and ear swab, blood, Bone marrow and skin	Comparative ^{1,2}	Skin = Nasal swab and bone marrow > Conjunctival swab > Oral swab > Ear swab
Geisweid et al. 2013	Syber kDNA	NI	Yes	No	Canine NCX1	(74) CVL suspected dogs	Conjunctival swab, blood, bone marrow and lymph node	Comparative ²	Bone marrow > Conjunctival Swab
Reis et al. 2013	Syber α pol DNA	NI	Yes	No	G3PDH	(60) Seropositive dogs	Skin and spleen	Comparative ^{1,2}	Spleen > Skin
Pennisi et al. 2005	NI kDNA	NI	No	No	---	(6) Treated dogs	Blood, lymph node and skin	Not comparative	---
Francino et al. 2006	TaqMan kDNA	0.001 parasites/ reaction	Yes	No	18S eukaryotic rRNA	(15) Dogs with clinical signs suggestive of CVL	Blood and bone marrow	Comparative ^{1,2}	Bone marrow > Blood
Rodriguez-Cortez et al. 2007	TaqMan kDNA	0.001 parasites/ reaction	Yes	Yes	18S eukaryotic rRNA	(6) Experimentally infected dogs	Blood, bone marrow, liver, lymph node, skin and spleen	Not comparative	---
Solano-Gallego et al. 2007	Syber kDNA	7 parasites/ml	Yes	No	Canine GAPDH	(10) Symptomatic	Blood, bone marrow and urine	Comparative ²	Bone marrow > Blood

First author and year of publication	qPCR technique		qPCR internal control			Sample		Results	
	Method and target	Detection limit	Inhib. ^a	Norm. ^b	Control gene employed	(n)	Tissue evaluated	Study design [*]	Sensitivity ^{**}
						dogs naturally infected			> Urine
Manna <i>et al.</i> 2008	TaqMan kDNA	0.001 parasites/ml	Yes	Yes	β - actin	(18) Naturally infected treated dogs	Blood, lymph node and skin	Comparative ²	Lymph node > Skin > Blood
Manna <i>et al.</i> 2009	TaqMan kDNA	NI	Yes	Yes	β - actin	(56) Dogs	Blood and lymph node	Not comparative	---
Quaresma <i>et al.</i> 2009	Syber kDNA	0.1pg DNA/ml	Yes	Yes	β -canine globin	(35) Dogs	Blood and bone marrow	Comparative ²	Blood = Bone marrow
Maia <i>et al.</i> 2010	TaqMan kDNA	1 parasite / reaction	Yes	No	β - canine actin	(12) Experimentally infected dogs	Blood, bone marrow, buffy coat, liver, lymph node, skin and spleen	Comparative ^{1, 2}	Spleen / Buffy coat / Liver / Lymph node / Bone marrow / Skin > Blood
Galletti <i>et al.</i> 2011	TaqMan kDNA	0.03 parasite/ reaction	No	No	---	(88) Dogs	Conjunctival swab, Lymph node, bone marrow and blood	Comparative ¹	---
Lombardo <i>et al.</i> 2011	TaqMan kDNA	NI	No	No	---	(138) Dogs	Blood, conjunctival and oral swabs and lymph node	Comparative ¹	---
Naranjo <i>et al.</i> 2011	TaqMan kDNA	NI	Yes	No	18S eukaryotic rRNA	(22) Sick dogs	Main lacrimal gland, tarsal gland and nictitating membrane gland	Comparative ¹	---
* Study design: Comparative ¹ : aim to compare different diagnostic techniques results; Comparative ² : aim to compare different canine tissue sensitivity; ** Sensitivity: evaluation of <i>Leishmania</i> sp. detection in the different canine tissues; NI: Not informed; # submitted; ^a : qPCR inhibition control; ^b : qPCR normalization control.									

Table 2. List of papers using the qPCR technique for *Leishmania* sp. detection in different canine tissues, comparing the different methodologies and the use of internal control of the reaction

In a cytological study, Reis *et al.* (2006) showed that the spleen is a major organ where parasite density is high throughout the course of CVL in both symptomatic and asymptomatic dogs [74]. Saldarriaga *et al.* (2006) demonstrated in a study using dogs submitted to intradermal experimental infection with promastigotes of *Leishmania* sp. ninety-six hours after inoculation,

parasites were found in the lymph nodes and spleen of infected animals [148]. Another study revealed varying degrees of splenomegaly in most dogs infected with *Leishmania* sp. [132]. Reis et al. (2013) found 100% of positivity in spleen samples of 37 infected animals [149]. These findings make the spleen the best choice for the CVL diagnosis employing molecular techniques [150]. Nevertheless, obtaining spleen samples, even if tolerated by the animal, can incur a risk of hemorrhage and internal lesions [150]. However, in a study performed by Barrouin-Melo et al. (2006) in which 257 splenic punctures were performed, only two animals had the intestinal loop aspirated and one animal experienced bleeding at the puncture site [151]. These risks can be minimized by organ visualization using ultrasound devices, which allow a guided and safe collection of tissue sample [152]. In a recent study performed by the present authors, 1,200 dogs were punctured with the aid of ultrasound machine without any incident (unpublished data).

Splenic collection, bone marrow and lymph node aspirates are considered invasive procedures [153] in addition to having an elevated cost compared to blood collection. Thus, it can be recommended to use samples obtained less invasively, such as blood and conjunctival swabs [136, 154, 155]. These samples are quick and easy to obtain, and it is low-cost compared to more invasive procedures, in addition to their higher acceptance by animal owners [132, 154, 155].

Some studies have shown that detection of parasites in the peripheral blood is less sensitive compared to other tissue samples such as spleen, bone marrow, lymph nodes and skin and tends to have variable parasitic load in accordance with the stage of infection [129, 141, 156]. However, depending on the technique and the target, blood can be used for detection of *Leishmania* sp. infection even when there is a low parasitic load [142, 157]. Maia et al. (2009) showed that the use of peripheral blood samples, extracted from soaked filter paper, from 29 infected dogs showed 93.1% positivity in cPCR of kDNA, suggesting that this tissue can be used as a complement for serological diagnosis [132]. However, despite blood has the advantage of being less invasive than spleen, bone marrow and lymph node punctures, this tissue contains hemoglobin that may acts as inhibitor to the PCR reaction, which are usually neutralized by the addition of albumin, and also present a high variability in parasite detection, due to the fluctuation of parasitemia according to the stage of infection.

According to Solano-Gallego et al. (2001) in comparison to other tissues, skin has the greatest sensitivity [4]. In a study involving 80 naturally infected dogs, Ferreira et al. (2012) showed that skin is the tissue with the higher parasitic load, showing no significant difference between symptomatic and asymptomatic animals [139]. Reis et al (2013) used skin samples from 37 animals and by means of qPCR obtained a sensitivity of 97.3% in identifying infected dogs. Nevertheless, other noninvasive samples are being tested for molecular diagnosis of CVL, such as conjunctival swabs [149]. Using this type of sample with cPCR, Ayali-Strauss et al (2004) were able to detect 92% of the symptomatic animals evaluated in the study, using spleen or lymph node aspirates, they detected 86% whereas using peripheral blood or white blood cells they detected, respectively, only 17% and 57% of the infected dogs [158]. Leite et al (2010) also succeeded in detecting the parasite DNA from conjunctival swabs of asymptomatic animals by the technique of cPCR followed by kDNA-probe hybridization [159]. Lombardo (2012) using qPCR technique with conjunctival swabs obtained similar results to those obtained with

more invasive samples such as lymph node aspirates [136]. Ferreira *et al.* (2012) used cPCR followed by kDNA-probe hybridization of blood samples, skin, marrow and conjunctival swabs. The use of swabs gave the best results for detection of infection in both symptomatic and asymptomatic dogs, showing to be a suitable sample for the molecular diagnosis of CVL. Furthermore, Di Muccio *et al* (2012) argue that the use of nested PCR from conjunctival swabs shown to be the least invasive procedure with the best performance for the diagnosis of CVL in asymptomatic animals [156].

Among other less invasive sample types investigated, Solano-Gallego *et al* (2007) evaluated urine samples with qPCR technique, but the results described showed positivity only in dogs with severe renal injury [160]. Naranjo *et al.* (2012) identified the presence of *Leishmania* sp. DNA in lacrimal glands of infected dogs using qPCR, with positive correlation between positivity and the presence of ocular lesions [161]. Recently, Belinchon-Lorenzo (2013), using qPCR, demonstrated the presence of *Leishmania* kDNA in the hair and keratinocytes of infected animals. According to the authors, the use of the non-invasive hair sample for the diagnosis of CVL would be advantageous because it is easy to collect, handle, transport, and store [135]. However, further studies should be conducted to determine the sensitivity of this method.

Laboratory – Country	Tests Price			
	Serology	cPCR	qPCR	qPCR and Citology
Elleviti – Torino, Italy	26.80*	---	63.00*	---
Scanelis - Toulouse, France	---	---	60.30*	---
Laboratoire d'Anatomie Pathologique Vétérinaire du Sud-Ouest – Toulouse, France	---	---	---	127.30*
Laboratório Veterinário INNO – Braga, Portugal	20.60*	54.40*	---	---
Instituto Nacional de Investigação Agrária e Veterinária, I.P. – Lisboa, Portugal	28.00*	41.20*	---	---
Centro de Investigación y Análisis Biológicos – Madrid, Spain	13.60*	60.30*	73.70*	---
Texas Veterinary Medical Diagnostic Laboratory – San Antonio TX, USA	19.20	---	---	---
Cornell University - Ithaca NY, USA	22.50	60.00	---	---
Hermes Pardini - Belo Horizonte MG, Brazil	17.20*	60.20*	---	---
Análisis Biológicos– Chapecó SC, Brazil	9.40*	42.15*	72.25*	--
Laborlife - Rio de Janeiro RJ, Brazil	30.10*	77.40*	---	---

*Prices in Euros (€) and Brazilian Real (R\$) converted in US dollars (U\$), quotation of November 14th of 2013. €1 = aprox. U\$ 1.34 - €1 = aprox.R\$ 0.43

Table 3. Cost of the main diagnostic test for CVL in different laboratories consulted in the second semester of 2013 in U\$

Despite the high sensitivity and specificity, the use of molecular methods for the CVL diagnosis presents some limitations to its use in epidemiological surveys: i) it has higher costs than other techniques (Table 3) used in the CVL diagnosis, including reagent and equipment costs; ii) it presents relative complexity in its implementation, requiring personnel with training in the execution of PCR reactions. However, this method has advantages in terms of sensitivity and specificity when compared to other diagnostic techniques, which justify its use in confirming cases screened by serology [24, 132]. Particularly due to the possibility of quantifying target DNA, qPCR may be used to monitor the parasitic load of the animal during the experimental infection, or during and after treatment in countries where it is permitted [35-37, 162]. Compared with cPCR, qPCR enables a reduction in the probability of false positives resulting from amplification artifacts and greater speed in obtaining results, once electrophoresis is no longer performed [163].

4. Conclusion

In summary, detailed clinical evaluation complemented with highly sensitive test allows proper identification of infected dogs in an endemic area. Evidence shows that the use of a rapid serological test associated with a molecular diagnostic test with high specificity, such as qPCR, is required for identification of all infected dogs, both asymptomatic and symptomatic. On the other hand, for sick dogs a correct diagnosis is necessary either to perform dog culling in countries where this measure is used as a control strategy of VL or to define treatment. In this case, a detailed clinical evaluation should be associated with biochemistry and hematological tests to identify signs of renal and hepatic failure, in conjunction with a serological test to confirm animal clinical condition.

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