Review Article

The Immunochromatography Use in Canine Visceral Leishmaniasis in Brazil: A "Quick Solution" of a Complex Diagnostic? Rapid Test in Dogs with Leishmaniasis

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Abstract

Diagnosis of Canine Leishmaniasis is complex, once there is no test able to detect infection period / disease. Serological methods can have different results when performed by different methods and misinterpretations may result in hasty diagnosis. Rapid tests can be considered attractive, from a clinical point of view, because of its easy carrying and reading these results. However, the use of rapid testing alone for the diagnosis of canine leishmaniasis in Brazil should be considered dangerous, especially in asymptomatic dogs. In addition, knowledge of the technique and the different commercial assays available (which differ in the composition) must be considered essential for better performance and interpretation of results. The objective of this study is to show the main characteristics of rapid tests for diagnosis of canine visceral leishmaniasis, used in Brazil, and demonstrate the effectiveness of these in previous studies associated with other methods of serological diagnosis and evaluating the sensitivity and specificity of these tests.

INTRODUCTION

The diagnosis of Canine Visceral Leishmaniasis (CVL) is challenging and complex for clinical veterinaries. In fact, there not exist tests capable of detecting the period of infection / disease. Serological methods have different results when performed in epidemiological surveys by public institutions and those held in private laboratories [1]. These differences are associated with different techniques and protocols used by different laboratories. The diagnosis of CVL becomes even more complex when based on clinical manifestations of dogs living in endemic areas and the asymptomatic dogs become more difficult to detect the infection than symptomatic ones [2].

Many infected dogs may never exhibit clinical signs, thus making it difficult to detect CVL early [3,4]. Thus, asymptomatic dogs infected with *Leishmania Infantum* can develop progressed disease along with high antibody titers with suppressed cellular immune response or can exhibit protective immunity keeping a seronegative state remaining parasite positive. In these cases, the use of individual tests and interpretations should be avoided [5]. In symptomatic dogs predominates humoral immune response with production of high titers of antibodies and high parasite loads. In these cases the serological tests can confirm disease when used quantitative tests [2,6,7]. False-negative results, false positives and misinterpretation can lead to hasty conduct leading dogs to death or unnecessary and incomplete treatments. This indicates that the sensitivity of a serological test is likely to chance during the clinical course of the infection, depending on both host immune system and the virulence of the parasite.

The performance of serological tests is dependent on several factors such as: quality kits and preservation of samples; equipment and technical quality and interpretation and observation results [8-10].

An innovative colloidal gold-based Immunochromatography assay was recently developed by Chembio Diagnostic Systems, Inc. (Medford, NY, USA) and it is now manufactured in Brazil (Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil) as a Dual-Path Plataform (DPP®) CVL rapid test, to detect antibodies against the rK26/rK39 fusion protein. The DPP® test become the diagnostic method for screening in Brazil, followed by ELISA as a serological confirmatory test (BRASIL 2011).

A recent study has demonstrated high sensitivity using DPP® CVL in symptomatic dogs, but they still found low sensitivity in asymptomatic dogs [11].

DPP®CVL belongs to others diagnostic methods that detect anti *L. Infantum* antibodies, named as Rapid Tests (RT). As advantages the RT have speed in their realization and achievement of results, can be realized in a number of dogs for epidemiological surveys and low cost for taking laboratory structure [11]. However, the RT should be used with caution especially in negative results [2]. The main objective of this review is elucidate characteristics of rapid tests for diagnoses of canine visceral leishmaniasis, used in Brazil, and demonstrates the effectiveness of these in previous studies associated with other methods of serological diagnosis, evaluating the sensitivity and specificity of these tests and its application in diagnosis of CVL.

Immunochromatography

Serological diagnosis techniques have emerged from the 1960s decade, considered for screening, easily handling and a technique able to provide qualitative results under a presence of antibodies or antigens from a serum-analyzed sample. Thus, it has been currently used for diagnosing infectious diseases in both human and veterinarian medicine. This technique consists of a nitrocellulose membrane matrix embedded with antigens or antibodies as dot shapes or lines enclosed in delimitated matrix regions. For staining revelation of these antigens, insoluble gold or colloidal silver over the membrane is employed. Testing samples may be whole blood, plasma, other body fluids and secretions (mucous) whereas it goes through the membrane reaching the impregnation antigen or antibody regions. This process occurs as spontaneous antigen-antibody interactions [12-16] (Figure1).

Some models of Rapid Tests used in Brazil in the diagnosis of Canine Visceral Leishmaniasis

Dual-path platform (dpp®) Biomanguinhos: Fiocruz (Bio-Manguinhos Unit, Rio de Janeiro, Brazil) produces in Brazil one RT using the Dual-Path Platform technology consisting of a plastic cassette containing two nitrocellulose strips connected a 'T' shape. The test requires about 5μl of sample (serum, plasma or whole fresh blood) from the animals. In the sample deposition platform (sample well) is added to the sample along with two drops of buffer (included in kit). The diluted sample then migrates to the second strip having a test line containing the recombinant proteins impregnated in the nitrocellulose strip (test line) and a control line containing anti-dog IgG antibodies. Subsequently, with the addition of four drops of the buffer solution, the combined release of dry particulate colloidal gold, coupled to Protein A/G, which facilitates their migration along the second strip into the test area. These gold particles then react with the antibodies bound to the antigens that are present in the test line, creating an immunological complex for viewing. In the absence of specific antibodies, the test line is not visible in the window [11] (Figure 2).

Snap® Leishmania Idexx: The SNAP® *Leishmania* (Iddex Laboratories Inc., USA) is a RT-based enzyme immunoassay for in vitro detection of antibodies to *L. donovani* and *L. infantum* in whole blood, serum or plasma. The SNAP® *Leishmania* is used as RT because it is based on ELISA technique, but it differs from Immunochromatography tests. For this method we must use a pipette supplied by the manufacturer transferring two drops of sample (whole blood, serum or plasma collected from animals) for the sample bottle. In a vertical position, in the sample bottle containing the collected sample, we have to add six drops of conjugate antibody solution homogenizing it three to five times.

Then, with the device (test plate) placed on a flat surface, in each well, is added the flask contents, which it flows through "the result window" until the Activation Circle in 30-60 seconds. After, you must firmly press the activator until it is flush with the device body. After six minutes the result is read [17].

kalazar detect ™ Inbios: The Kalazar Detect™ (INBIOS International, Seattle, WA, USA) is an Immunochromatography assay qualitative antibody test against *L. Infantum* rK39 antigen. This is a test on a single strand of lateral flow nitrocellulose detecting anti-*Leishmania* antibodies. For their realization must remove the tape without touching the test area by adding 20μl of whole blood or serum sample canine below the test strip of the arrow. Then one should place the tape in a test tube, add 40 to 60μl of buffer solution equivalent to three drops waiting 10 to 15 minutes for reading. We should not interpret the results after 15 minutes of running, because they may generate misleading results [18].

Alere™ leishmaniasis ac test kit: The Alere Leishmaniasis Ac Test Kit (Alere, São Paulo, Brazil) is an Immunochromatography test using the rK9 antigens, and rK39 rK26 based on a nitrocellulose strip. Serum samples, plasma or whole blood obtained from animals may be used. To perform the test we should add 10μl of whole blood, serum or plasma using a capillary pipette into the device well marked with the letter "S", provided by the manufacturer. Then, we add two drops of this buffer in the kit waiting the result in 20 minutes. It is not recommend interpreting the results after 30 minutes, because it may generate misleading results. According to the manufacturer the sensitivity is 97.2% and the specificity is 99.8%.

Sensitivity and Specificity of Immunochromatography in the diagnosis of CVL in Brazil: There is a wide explanation for the variable sensitivity and specificity values for RT. For example: symptomatic and/or asymptomatic dogs carry on different antibody titers. Otherwise, different researchers use different antigens and/or distinct protein concentrations, besides studies have showed a high specificity of the recombinant proteins rk39 and rk26 than others in the literature [18-21].

As known RT has been currently employed for human and canine infections disease diagnoses [22]. In a RT sensitivity study in Rio de Janeiro, Brazil, authors have found a significant difference in the evaluation in symptomatic than asymptomatic dogs. In this study, all canine serum samples had positive parasitological results (reference standard). In the group of symptomatic dogs, the sensitivity was 98%, whereas in asymptomatic group, the sensitivity did not exceed 50%. The TR specificity was 96% [11]. Considered the use of rK39 and rK26 protein as possible markers for severe disease, but they concluded that the TR method should not be carried out alone in the field as serological survey. However, other studies [18] revealed that RT containing only rK39 can achieve 83% of sensitivity, whereas the ELISA technique show 95% of sensitivity using the same type of antigen. Thus, authors suggested the use of these tests in combination (RT + ELISA) for epidemiological surveys.

In a longitudinal and meta-analysis study of CVL diagnoses revealed RT sensitivity for distinct experimental group. They found 46% of sensitivity in asymptomatic dogs, 77% in symptomatic dogs and 78% in animals able to transmit the parasite (xenodiagnoses), respectively. Moreover, for asymptomatic or low infectivity dogs, the author concludes
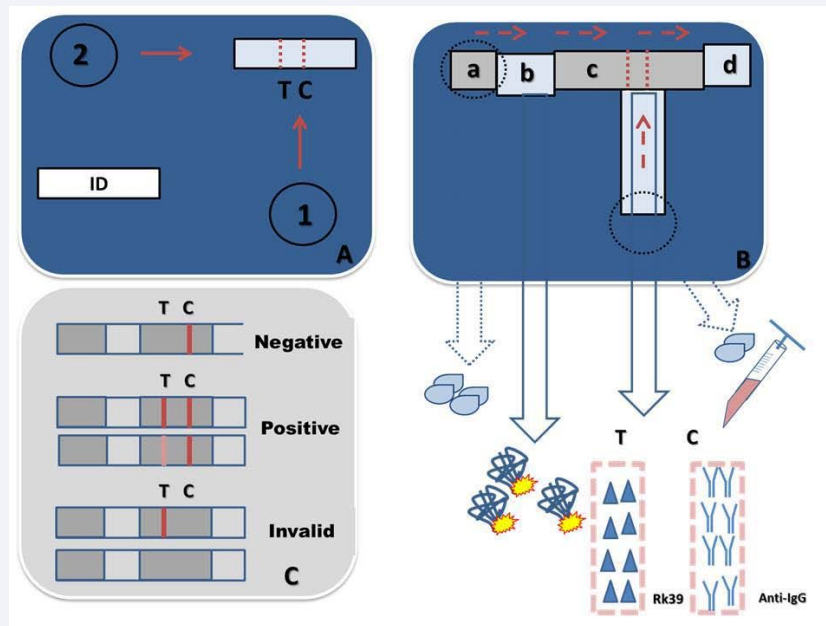
Figure 1 Schematic drawing of an Immunochromatography test with two strips of nitrocellulose. Schematic drawing of an immunochromatography test with two strips of nitrocellulose (AB) and the interpretation of results (C). Note in (A) the plastic tape template containing the sample wells + buffer (1) and buffer (2) the sample identification area / animal (ID) and the reading window displaying the test lines (T) and control (C). In (B) watching the tape of the internal structure containing two strips of nitrocellulose. After adding the sample in the sample well + buffer that runs through the first nitrocellulose strip. In the membrane "a" are added four drops of the buffer solution to traverse lateral flow membrane "b" where there are colloidal gold particles associated protein A/G. The membrane "c" is defined by the presence of anti-dog IgG antibodies that will make the test and control lines consequently. In "d" the residual absorption membrane. In (C) observe the possible interpretations of the results of reading in the test window.
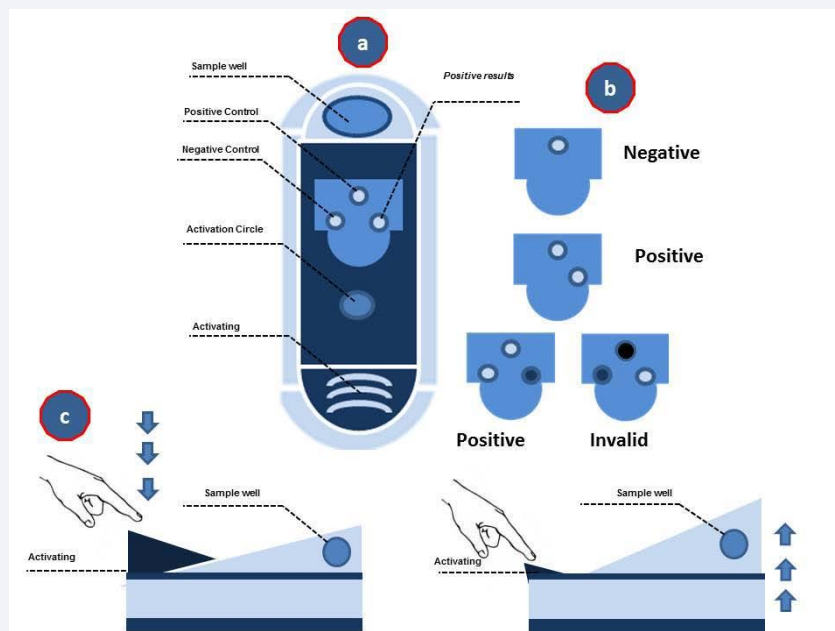
Figure 2 Schematic drawing of a SNAP® *LEISHMANIA IDEXX.* Schematic diagram of the test platform SNAP® Leishmania (AC) and the interpretation of results (C). Observe in (AC) plastic cassette model containing the sample wells and just below the test window with the points in the scatter Activation circle where the sample or blue color is displayed. The activator is where to press the platform on a flat surface. (B) The visualization of positive or negative is a representation of a reactant or non-reactant test result. However, the point of view called Leishmania Sample Node serves as a protection against false positives and helps indicate that the test was run properly. If the staining of the Leishmania sample spot is darker than the negative control spot, the result is positive for this node. If the staining in the negative control spot is equal to or darker than sample spot Leishmania, the test is invalid for this sample spot.

that the sensitivity of RT is not effective for control and/or epidemiological study programs [20]. Dogs which highlight that potentially suspect should be questioned on results that tested negative and a combination of serological and parasitological tests for CVL. Recent research from Brazil, the sensitivity and specificity of RT are described in Table (1).

Comparison of serological methods used for the diagnosis associated with rapid test

ELISA, IFAT and Immunochromatography are the main serological tests currently used from public and private institutions in Brazil. The last literature decade, IFAT has been considered the reference standard test [23-26]. This is a quantitative method, which results expressed as the dilution of antibodies in serum samples of dogs with CVL. Dilutions readings start at 1:40 (cut-off) to 1:10240 or more. The titles found can aid in the diagnostic conclusion given that dogs can vary significantly in antibody titers at different times. Brazilian researchers have pointed out that high titers of antibodies are confirmatory for the diagnosis of infection/disease [6,27,28] emphasize that in Brazil, results that have titers of antibodies equal or greater than 1:160 are confirmatory of infection/disease, independent of animal clinical status. However, results with low antibody titers (1:40 to 1:80) should be considered suspect (Figure 3).

Authors working with sera from dogs of Marajó Island (Pará, Brazil) using ELISA with crude antigen (ELISA$_{sla}$) found that 85% of infected dogs were positive while only 45% were positive for

Table 1: Sensitivity and specificity of different Rapid Tests (RT) for diagnosis of Canine Visceral Leishmaniasis in Brazil.

Rapid Test	Sample/Dogs	Other Tests Used.	Sensitivity TR		Specificity TR	References
	(n)		(%)		(%)	
			Symptomatic	Assymptomatic		
		· Bone Marrow aspirate (cytology) $_{(S, A)}$				
	60$_{(S)}$	· Histopathology (Necropsy)$_{(S, A)}$				
	60$_{(A)}$	· ELISA rK39, rK26, A2$_{(C)}$	98	47	96	
DPP®	59$_{(C)}$	· Electrophoreses and ELISA$_{(RC)}$				Grimaldi et al., 2012
	11$_{(RC^1)}$					
		· ELISA *major-like*				
DPP®		· ELISA *L.chagasi*				
	144*	· IFAT major-like	93*		92	Silva et al., 2013
		· IFAT *L.chagasi*				
		· Cultural				
	148	· ELISA$_{(SLA)}$				
RAPYDTEST®		· PCR	72 – 77* **		61 – 75* **	Reithinger et al., 2002
Kalazar Detect™	99$_{(S)}$		77$_{(S)}$			
	39$_{(PO)}$	· ELISA$_{(SLA)}$	89$_{(PO)}$	32.6	100	Quinnell et al., 2013
	60$_{(OLI)}$		69$_{(OLI)}$			
	178$_{(A)}$					
TR	52$_{(S)}$	· ELISA$_{(SLA)}$	92	-	95	
	52$_{(C)}$	· PCR				Lima et al., 2010
	47$_{(S)}$	· ELISA				
DPP®	38$_{(A)}$	· IFAT	89.4	92.1	95.1	Laurenti et al. 2014
	18$_{(C)}$					
	283*	· IFAT		94.7	90.6	Marcondes et al. 2011
SNAP®	86$_{(C)}$	· ELISA				

The related works are studies evaluated serum samples (frozen) or experimental groups.

$_{(S)}$ – symptomatic $_{(SLA)}$ – Soluble *Leishmania* antigen $_{(RC^1)}$ – cross reactivity: *L. braziliensis*ou*Leptospirainterrogans*

$_{(A)}$ – asymptomatic   * – there is no specification on clinical manifestation of dogs (symptomatic and asymptomatic) $_{(RC^2)}$ – cross reactivity: *T. cruzi, T. gondi. N. caninum, B. canis*e *E. canis*

$_{(C)}$ – control   **– Sensitivity based on comparative data to ELISA and PCR. $_{(PO)}$ –polisymptomatic

$_{(Infec.)}$ - infected $_{(OLI)}$ – oligosymptomatic

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rapid test using sample bone marrow PCR as reference standard test. However, authors report that the ELISA test performed with 52 serum samples symptomatic dogs had 100% sensitivity compared to 91% of TR [20]. However, researches working with comparison between TR, ELISA and IFAT observed sensitivity of 90% in serum resident dogs from endemic areas and a specificity of 95% for TR used (DPP®). A similar result was seen in ELISA (Bio-Manguinhos®) that observed 90% sensitivity of the samples. However the same specificity ELISA was 70%. As for the IFAT (Bio-Manguinhos®) the sensitivity of dog serum samples was 96.5%. However, the specificity of it was 69.1%. Taken together these results authors concluded that the use of DPP® as serologic test was able to detect both asymptomatic and symptomatic dogs and TRs should be used instead of the IFAT, even if its sensitivity is not completely satisfactory. Also, the use of TRs associated with ELISA (massive research survey method used in Brazil) increases the accuracy and sensitivity compared to the truly positive dogs, especially in endemic area [29].

In general, ELISA and IFAT, quantitative serological tests, provide easily CVL diagnoses when associated to high titers of serum antibodies mainly in patients with clinical signs of the disease. However, the performance and accuracy of these serological methods are dependent on each laboratory, as well as standardization of the technique, especially when the ELISA and IFAT is used for diagnosis in patients suspected of CVL (Maia and Campino 2008)[26]. Another important fact is that in cases of patients with low titers of antibodies. ELISA cannot be trusted alone requiring additional tests for conclusive diagnosis [2].

As mentioned at all, Brazilian laboratories have used ELISA method as a routine for diagnosing CVL. However, the results are presented to veterinarian clinicians qualitative without antibodies title levels. In conclusion, authors declare the importance of clinicians ask the laboratories the quantitative serologic data (antibody levels). In fact in Europe and in Brazil, report that the antibody titers through quantitative tests are essential in clinical practice for diagnosis of dogs with suspect

cases or progression of CVL [6]. On the other hand, some works suggests the use of ELISA associated with DPP® as serological tests for epidemiological inquiry, dismissing the IFAT. However, it seems worrying for routine veterinary clinicians because the use of ELISA as a qualitative test (as is done in the routine of the main CVL diagnostic laboratories in Brazil) associated with RT, the serological diagnosis of CVL becomes exclusively qualitative, disregarding titles and antibody levels of each individual [28].

The use of rapid test in the diagnosis of CVL in Brazil

The Immunochromatography in Brazil should be used with caution, especially in cases of asymptomatic dogs [20,6]. However, some authors discussed the use of TR and DPP® consequently, in association with ELISA for epidemiological surveys because of the sensitivity and specificity satisfactory [19,28]. Currently, the Immunochromatography is indicated as a screening test that will indicate whether or not suspicious cases for further tests such as serological and parasitological tests in seeking confirmation of CVL, as directed by the official program. However, it is necessary the use of serological tests complementary to RT as the ELISA and IFAT [1].

Under the individual clinical perspective of the patient, we cannot forget that the diagnoses of CVL go through a triad of serological, parasitological and molecular exams. Even though serological positive exams it is necessary other techniques for systemic evaluation of the disease as: (1) clinical aspiration assays of bone marrow and/or lymph nodes, (2) Immunohistochemistry of skin ear and nose biopsies, (3) PCR for bone marrow aspirate samples [2,29,6]. Moreover, the RT, and the parasitological test may show greater sensitivities in patients compared to asymptomatic dogs. Considered in asymptomatic dogs, the use of RT should be questioned even in negative in view of the possible low antibody titers (Figure 4).

The rt as a pre-vaccination serologic testing

In Brazil, vaccination trials of dogs living in endemic areas are indicated as an important tool in controlling the CVL [30].
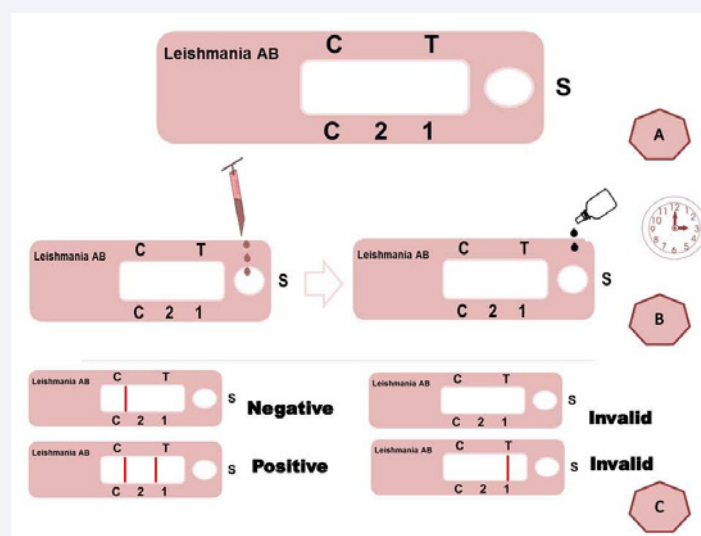
Figure 3 Schematic drawing of a *Kalazar DETECT™ INBIOS.* Schematic drawing of Kalazar Detect™ Rapid Test (AB) and the interpretation of results (C). In (A) to observe the tape nitrocellulose test model. In (B) observe the performance of steps: add whole blood sample or dog serum in the sample area of the strip; subsequently placing the tape in a test tube so that the tape is facing downward; After add three to four drops (40-60μl) buffer and waiting for between 5 and 10 minutes for reading. In (C) observe the possible outcomes and their interpretation.

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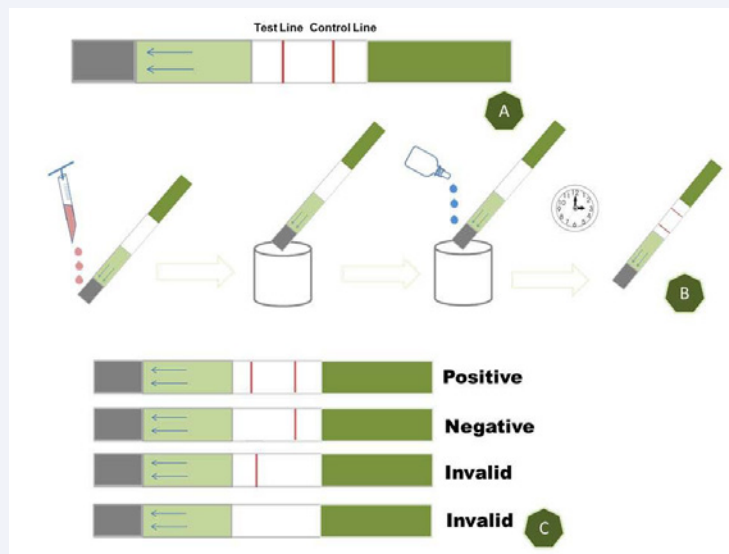
Figure 4 Schematic drawing of Alere ™ Leishmaniasis Ac Test Kit. Schematic drawing of the Alere™ Leishmaniasis Ab Test Kit (AB) and the interpretation of results (C). In (A) observe the cassette test model. In (B) observe the performance of steps: add whole blood sample or dog serum in the sample area of the strip (S); subsequently adding two drops (40µl) buffer and interpret results in 20 minutes. In (C) observe the possible outcomes and their interpretation.

Vaccination is recommended for dogs living in endemic areas over four months, asymptomatic and with negative serological results for CVL [31]. Thus, RT could be used as pre-vaccine trial in dogs living in endemic areas. However, several studies suggest that the use of RT should be used as a screening and in combination with other serological tests for the diagnosis of CVL (such as the IFAT and ELISA) because of the importance of quantitative evaluation, particularly in asymptomatic dogs.

CONCLUSIONS

The diagnosis of CVL is considered complex under a lack of testing with higher sensitive and specific. Moreover, it has been claimed tests with low cost, commercially available and, of course, no laborious. Under this reality, RT are currently considered screening tests that allow the veterinarian to exclude healthy dogs in epidemiological surveys. However, the use of RT for asymptomatic dogs in endemic areas diagnoses should be cautious, because of its lower sensitivity. Thus, the quantitative serological tests should be chosen to assess the true serological profile of dogs with positive RT.

The Immunochromatography method in the diagnosis of CVL is practical and quick alternative, in diagnostic screening in suspected or resident dogs in endemic areas. However, more studies are necessary regarding the sensitivity and specificity of these tests, together with other serological tests as well as in-depth studies on the use of recombinant proteins, RT use in dogs of various clinical manifestations.

Studies aimed at evaluating the sensitivity and specificity of RT are important, linking and comparing the serological tests used in routine as well as the evaluation of these in groups of dogs with other infections and residents of non-endemic areas to establish findings about false positives and false-negative, respectively. For best interpretation of veterinarians in Brazil it is necessary that laboratories join RT, ELISA and/or IFAT in a single scan profile and that there is standardization between laboratories. Laboratories which employ RT associated with

ELISA in routine testing (profile) should offer clinical results of quantitative ELISA so there is standardization in the use of RT as screening and serological examination in sequence to RT provide more comprehensive results to serological diagnosis of CVL.

COMPETING INTERESTS

The authors declare that they have no conflict of interest related to this research. We have no commercial interest or competition with other research.

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