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Research paper

Performance of different serological tests in the diagnosis of natural infection by *Leishmania infantum* in dogs

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ABSTRACT

Visceral leishmaniasis (VL) is a zoonosis caused by the parasite *Leishmania infantum* and the dog is its main reservoir in rural and urban areas. The diagnosis of infection is mainly based on the presence of anti-*Leishmania* IgG antibodies in the serum of infected dogs. In this study, the sensitivity and specificity of qualitative rapid tests (RTs) dual path platform (DPP) Bio-Manguinhos, rapid enzyme-linked immunosorbent assay (ELISA) IDEXX, Kalazar Detect and ALERE, as well as quantitative ELISA Bio-Manguinhos and in-house indirect immunofluorescence assay (IFA) tests were analyzed in sera from infected and uninfected dogs. Serial dilutions of the in-house IFA were compared with RTs and ELISA Bio-Manguinhos. The results showed that none of the tests reached 100% sensitivity and specificity. There was no statistical difference between the analyzed RTs. The most sensitive test was the DPP Bio-Manguinhos (97.9%), while the rapid ELISA IDEXX showed higher specificity (100%). In the treatment setting of infected and/or diseased animals, quantitative tests for monitoring the evolution of antibody titers are required, which indicates the maintenance of in-house IFA in animal handling. Furthermore, we demonstrate that the RTs present higher sensitivity in serum samples with superior antibody titers obtained in the in-house IFA. However, the RTs exhibited false negatives in samples with low titers of antibodies. Among the RTs, only the DPP Bio-Manguinhos presented better performance in this situation. Therefore, the use of RTs for the diagnosis of VL in dogs with low titers of antibodies, such as asymptomatic, should be carefully evaluated.

1. Introduction

Visceral leishmaniasis (VL) is a zoonosis caused by the parasite *Leishmania infantum*, which is transmitted by infected sandflies of the genera *Phlebotomus* or *Lutzomyia* in the European and American continents, respectively (WHO, 2010). Dogs are considered the main reservoirs of the parasite and have greater presence of amastigote parasitic forms in the skin, when compared with man and other animals (Alvar et al., 2004).

The diagnosis of infection is mainly based on the presence of anti-*Leishmania* IgG antibodies in the serum of infected dogs. The option for serological tests is based on the exacerbated humoral response present in canine visceral leishmaniasis (CVL), with high levels of immunoglobulins (Alvar et al., 2004; Gomes et al., 2008; Maia and Campino, 2008). The serological tests routinely used to diagnose CVL in Brazil by veterinarians are the indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and rapid tests (rapid ELISA and immunochromatographic) (RTs), presenting variable sensitivity and specificity according to the used antigens (FIOCRUZ, 2008; Coura-Vital et al., 2014; Peixoto et al., 2015).

The advantages of RTs are related to the rapid processing of the samples and obtaining the results; the possibility of their realization in

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the field (place of the collection); analysis of a large number of animals in a reduced time; application in epidemiological surveys; and the relatively low cost, dispensing the laboratory structure (Grimaldi et al., 2012; Woyames Pinto et al., 2016). Among the RTs stand out the dual platform technology test (DPP® canine leishmaniasis rapid test) (Grimaldi et al., 2012), the rapid ELISA test (SNAP *Leishmania*, IDEXX) (Athanasiou et al., 2014), the Kalazar Detect™ rapid test (InBios International, Inc.) (Lemos et al., 2003; Krawczak et al., 2015), and the ALERE® Leishmaniasis AC test kit (Marcelino and Souza Filho, 2015; Souza Filho et al., 2016). All these tests available on the market show that the effective diagnosis of CVL is extremely important both in the veterinary medical routine and in the leishmaniasis surveillance and control program (Brasil, 2014).

Due to the wide variety of serological tests currently available composed of several antigens, studies that evaluate the sensitivity and specificity of these tests, as well as the best combination among them, are extremely important in the diagnosis of the infection caused by *Leishmania*. Therefore, the objectives of this study were to evaluate the sensitivity and specificity of these tests and to analyze the best combination. We evaluated the rapid ELISA test (SNAP *Leishmania*, IDEXX), DPP® canine leishmaniasis rapid test (Bio-Manguinhos), ELISA Bio-Manguinhos, Kalazar Detect™ (InBios International, Inc.), ALERE® Leishmaniasis AC test kit, and an in-house indirect immunofluorescence assay (IFA) in sera from dogs naturally infected with *L. infantum*.

2. Materials and methods

2.1. Serum samples

A total of 189 dog serum samples were used in the study. These sera were obtained from the serum bank of the Leishmaniasis Study Group of the IRR/FIOCRUZ (Brazil). The samples were collected in research projects approved by the Committee on Ethics in the Use of Animals (CEUA/FIOCRUZ licenses nº P 0119-02 and nº P 44-12-4). Sera were coded and randomized to a blinded study.

Negative sera from dogs without clinical signs of CVL (NC): 47 animals were submitted to a longitudinal study, with five independent collections and intervals of 3 months, in a period of 1 year. All samples were seronegative in the tests: IFA Bio-Manguinhos, ELISA Bio-Manguinhos, DPP® Bio-Manguinhos, Kalazar Detect™ (InBios International, Inc.) and direct agglutination test (DAT-Canis), using *L. infantum* promastigote antigen according to Oliveira et al. (2016). For this study, the samples used were from the third collection of the longitudinal study, so that the dogs were negative in two previous collections and remained negative after two subsequent collections.

Positive dog sera for CVL (PC): 142 animals infected with *L. infantum*. The infection was confirmed by the parasitological method of isolation in NNN/LIT (Novy-MacNeal-Nicolle/Liver infusion tryptose) medium and by characterization of the isolates through *hsp70* PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) (Garcia et al., 2004). To confirm the species causing the infection, a panel of reference strains was also used as a positive control in PCR-RFLP, which included *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), *L. infantum* (MHOM/BR/74/PP75) and *L. guyanensis* (MHOM/BR/75/M4147).

2.2. Diagnostic tests analyzed

We performed the in-house IFA, developed in the Laboratory of Leishmaniasis of the Institute of Biological Sciences of the Federal University of Minas Gerais (ICB/UFMG/Brazil), according to Camargo (1964); besides the tests ELISA Bio-Manguinhos, rapid ELISA IDEXX (SNAP *Leishmania*), Kalazar Detect™ (InBios International, Inc.), ALERE® Leishmaniasis AC test kit and DPP® Bio-Manguinhos. Characteristics of the serological tests performed with each sample are shown in Table 1.

2.3. Reading of diagnostic tests

The in-house IFA was performed by serial dilutions of the sera from 1:40, without final dilution limit until the negativation, being considered a quantitative test. The ELISA Bio-Manguinhos was performed with samples at the recommended dilution by the manufacturer (1:100) and its reading was based on the absorbance reached at that dilution, with reagent or no reagent results.

The RTs were performed according to instructions from each manufacturer and the results obtained were classified by two readers and, in the case of negative and positive disagreement, the result was considered positive. The results of RTs were classified in no reagent (NR) or reagent (RE). These RTs and ELISA Bio-Manguinhos results were compared with the dilutions of the in-house IFA to verify the performance of these tests with serum samples, which showed different antibody titers observed in the in-house IFA.

2.4. Statistical analysis

The McNemar test was used for the comparison of sensitivity and specificity, considering a 95% confidence level and 5% for the probability of type I error and Bonferroni correction for multiple comparisons. Significant differences were considered with $p < 0.05$.

The degree of agreement between the serological tests (in-house IFA, ELISA Bio-Manguinhos and RTs) and the infection status of the serum samples was estimated by kappa index (κ) with 95% confidence interval and classified according to the Fleiss scale: < 0.00, poor; 0.00-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81–1.00, almost perfect agreement (Landis and Koch, 1977).

3. Results

The sensitivity and specificity of the diagnostic tests (in-house IFA, ELISA Bio-Manguinhos, rapid ELISA IDEXX, Kalazar Detect, ALERE and DPP Bio-Manguinhos) were analyzed and the results are presented in Table 2.

Regarding the sensitivity, DPP Bio-Manguinhos was the most sensitive test (97.9%) among those analyzed, presenting a higher probability of identifying seropositive animals, followed by the in-house IFA (95.1%). These tests did not present statistical difference between them in relation to sensitivity. However, both tests were statistically different compared with the rapid ELISA IDEXX, ELISA Bio-Manguinhos, Kalazar Detect and ALERE tests. In turn, the rapid ELISA IDEXX, ELISA Bio-Manguinhos, Kalazar Detect and ALERE tests did not present statistical differences between them (Table 2).

The rapid ELISA IDEXX showed higher specificity (100%), but it was not compared with the other tests through the McNemar test, since it did not present false positives. However, comparing the 95% confidence intervals (95% CI), the specificity of the rapid ELISA IDEXX showed a statistically significant difference compared with the in-house IFA, but not with the other tests. The other tests had no statistical difference between them and neither with the in-house IFA test (Table 2).

Similarity levels observed among the test results revealed that there was a high similarity between the rapid ELISA IDEXX and ALERE tests and less similarity between the DPP Bio-Manguinhos and rapid ELISA IDEXX, and DPP Bio-Manguinhos and ALERE (Table 2).

When evaluating the agreement of the test results with the infection status of each sample, we observed that DPP Bio-Manguinhos was the test with the highest kappa concordance index (0.92), while ALERE was the test that obtained the lowest index (0.57) (Table 2). In addition, DPP Bio-Manguinhos was the only test with almost perfect agreement classification.

We also observed that DPP Bio-Manguinhos recognized the highest number of true positive, presenting only 3 false negatives, and reached 96.8% accuracy. The second most accurate test was the in-house IFA

Table 1
Characteristics of the tests used in the present study according to information from the manufacturer's laboratories.

Characteristics	In-house IFA	ELISA Bio-Manguinhos	DPP Bio-Manguinhos	Rapid ELISA IDEXX	Kalazar Detect	ALERE
Material for examination	Serum	Serum	Serum, plasma, blood	Serum, plasma, blood	Serum, blood	Serum, plasma, blood
Antigen	*L. infantum*	*Leishmania* sp.	rk28	*L. infantum*	rk39	rk28
Type of test	Immuno fluorescence	ELISA	Immunochro-matography	Rapid ELISA	Immunochro-matography	Immunochro-matography
Identified Ig	IgG	IgG	IgG	IgG	IgG	IgG
Sensitivity (%)	ND	95.54	92.9-100.0	96.3	> 90	97.2
Specificity (%)	ND	91.76	87.5-91.7	99.2	> 90	99.8
Execution time	Variable	ELISA reader	10-25 minutes	6 minutes	10 minutes	20 minutes

ND: not determined; IgG: immunoglobulin G.
ELISA: enzyme-linked immunosorbent assay.

(91.0%), followed by the ELISA Bio-Manguinhos (87.0%). The other tests had lower accuracy: 83.6% for Kalazar Detect, 82.5% for rapid ELISA IDEXX, and 79.6% for ALERE test.

To verify the performance of the RTs and ELISA Bio-Manguinhos in relation to the concentration of antibodies present in the canine sera analyzed, all 189 samples were classified according to the results of the quantitative in-house IFA test (Table 3). The DPP Bio-Manguinhos was the test that presented better performance in the lower dilutions of the in-house IFA (up to 1:160), in which the antibody titers are lower. The other tests presented greater number of recognition failures in these in-house IFA dilutions.

Antibody titers higher than 1:160 in the in-house IFA showed greater agreement with the results of RTs. In 111/189 (58,7%) samples that showed dilutions of 1:320 to 1:10,240 in the in-house IFA, all were from PC group and we observed that the DPP Bio-Manguinhos obtained complete concordance with the in-house IFA results (Table 3). The remaining tests maintained false negative results until high dilutions of in-house IFA, although in a small number. The ELISA Bio-Manguinhos revealed false negative results until 1:5,120 dilution by in-house IFA. In the other RTs, false negative reactions were observed until the dilution 1:1,280. In the dilutions 1:2,560 and 1:5,120 no false negative reactions happened in anyone and only the ALERE test presented one false negative reaction in dilution 1:10,240 (Table 3).

We verified that none of the serological tests was able to identify all positive samples. Although the 1:40 to 1:160 dilutions of in-house IFA are capable of identifying all positive animals, they showed false-positive reactions (Table 3). False-positive reactions were observed in the in-house IFA up to the dilution of 1:160 and in the RTs up to the 1:80 titers, except for the rapid ELISA IDEXX.

In this scenario, the in-house IFA and DPP Bio-Manguinhos tests were more sensitive in recognizing seropositive animals, although with false positive samples. In-house IFA had the highest number of false positive samples, presenting the lowest specificity (78.7%) among the evaluated tests (Table 2). In contrast to in-house IFA, rapid ELISA IDEXX, Kalazar Detect, ALERE and ELISA Bio-Manguinhos presented higher specificity than sensitivity (Table 2), and a high percentage of false negatives at the lower dilutions of the in-house IFA (Table 3).

4. Discussion

In this study, the results were analyzed according to the degree of agreement, using as gold standard, persistently negative samples in serological tests obtained in a previous longitudinal study to calculate the specificity, and positive samples in parasitological tests for the calculation of sensitivity, similar to that developed by Marcondes et al. (2011) and Souza Filho et al. (2016). The decrease in the level of significance imposed by multiple comparisons of RTs may have caused difficulty in detecting the differences between RTs. A tendency to present different results can be observed by comparing confidence intervals.

In this study, the DPP Bio-Manguinhos presented a higher sensitivity (97.9%) than demonstrated by Grimaldi et al. (2012), in animals without signs of disease (47%). However, these authors found sensitivity of 98%, when they analyzed animals with clinical signs of CVL. In our study, samples from dogs with and without clinical signs were used together to evaluate test performance. Laurenti et al. (2014) reported a sensitivity of 90.6% of the DPP Bio-Manguinhos test in animals with or without signs of disease. Similar results were also observed by Mendonça et al. (2017). Schubach et al. (2014) obtained the sensitivity of 87.5% and 88% in the visual reading of whole blood and serum, respectively, and of 88% in the electronic reading, using the DPP Bio-Manguinhos test. In the present study, the reading was visual and had higher sensitivity than those found by these authors.

For indirect immunofluorescence assays, Laurenti et al. (2014), using IFA Bio-Manguinhos and in-house IFA, reported sensitivity of 96.4% and 89.4%, respectively, which were results close to those obtained in the present study. We also observed superior results to those reported by Assis et al. (2010) and Peixoto et al. (2015), which found

Table 2
: Diagnostic performance of the different serological tests used in the study.

	Sensitivity			Specificity			Kappa[#]		
Test	n	Point value (%)	95% CI	n	Point value (%)	95% CI	κ	95% CI	Agreement
In-house IFA	142	95.1[B]	[89.7 to 97.8]	47	78.7[A]	[63.9 to 88.8]	0.75	[0.64 to 0.86]	Substantial
ELISA Bio-Manguinhos	139	84.2[A]	[76.8 to 89.6]	45	95.6[A]	[83.6 to 99.2]	0.69	[0.58 to 0.80]	Substantial
DPP Bio-Manguinhos	142	97.9[B]	[93.5 to 99.5]	47	93.6[A]	[81.4 to 98.3]	0.92	[0.85 to 0.98]	Almost perfect
Rapid ELISA IDEXX	142	76.8[A]	[68.8 to 83.3]	47	100[*]	[93.6 to 100.0]	0.62	[0.51 to 0.73]	Substantial
Kalazar Detect	142	79.6[A]	[71.8 to 85.7]	47	95.7[A]	[84.3 to 99.3]	0.63	[0.52 to 0.74]	Substantial
ALERE	140	73.6[A]	[65.3 to 80.5]	46	97.8[A]	[87.0 to 99.9]	0.57	[0.45 to 0.68]	Moderate

[*] As the specificity was 100%, the McNemar test was not performed. There is statistical difference between rapid ELISA IDEXX and in-house IFA, comparing their confidence intervals (CI). [A,B] Values with different superscripts within the column are significantly different ($p < 0.05$) and with same superscripts within the column have no significant difference. Serum samples with indeterminate result in the ELISA Bio-Manguinhos were disregarded in the calculations of sensitivity and specificity of the test. [#]The kappa index of each test was calculated in relation to the infection status of each sample. CI: confidence interval.

Table 3
Descriptive analysis of the relationship between serial dilutions of in-house indirect immunofluorescence assay (IFA), with the infection status, the results of the serological tests ELISA Bio-Manguinhos and the rapid tests DPP Bio-Manguinhos, rapid ELISA IDEXX, Kalazar Detect, and ALERE.

In-house IFA dilution	Infection status	ELISA Bio-Manguinhos	DPP Bio-Manguinhos	Rapid ELISA IDEXX	Kalazar Detect	ALERE
44 (NR)	37 NC	41 (NR)	39 (NR)	43 (NR)	42 (NR)	43 (NR)
	7 PC	3 (RE)*	5 (RE)**	1 (RE)	2 (RE)***	(43 exams)
15 (1:40)	6 NC	11 (NR)	6 (NR)	14 (NR)	14 (NR)	14 (NR)
	9 PC	2 (RE)	9 (RE)	1 (RE)	1 (RE)	(14 exams)
		2 (IN)				
8 (1:80)	3 NC	6 (NR)	1 (NR)	8 (NR)	7 (NR)	6 (NR)
	5 PC	2 (IN)	7 (RE)****		1 (RE)*****	2 (RE)******
11 (1:160)	1 NC	4 (NR)	1 (NR)	7 (NR)	7 (NR)	9 (NR)
	10 PC	7 (RE)	10 (RE)	4 (RE)	4 (RE)	2 (RE)
11 (1:320)	11 PC	11 (RE)	11 (RE)	3 (NR)	3 (NR)	4 (NR)
				8 (RE)	8 (RE)	7 (RE)
27 (1:640)	27 PC	1 (NR)	27 (RE)	3 (NR)	27 (RE)	4 (NR)
		26 (RE)		24 (RE)		22 (RE)
						(26 exams)
41 (1:1,280)	41 PC	1 (IN)	41 (RE)	2 (NR)	1 (NR)	1 (NR)
		40 (RE)		39 (RE)	40 (RE)	40 (RE)
10 (1:2,560)	10 PC	1 (NR)	10 (RE)	10 (RE)	10 (RE)	10 (RE)
		9 (RE)				
10 (1:5,120)	10 PC	1 (NR)	10 (RE)	10 (RE)	10 (RE)	10 (RE)
		9 (RE)				
12 (1:10,240)	12 PC	12 (RE)	12 (RE)	12 (RE)	12 (RE)	1 (NR)
						11 (RE)

NC: negative control; PC: positive control; NR: no reagent; RE: reagent; IN: indeterminate.
*2 False positives / **1 False positive / ***1 False positive / ****2 False positives / *****1 False positive / ******1 False positive.

56% and 88% sensitivity in IFA, respectively. Mendonça et al. (2017) observed 96% sensitivity in the IFA Bio-Manguinhos kit, at 1:40 dilution, close to that obtained in this study. However, Lira et al. (2006), using the same kit, verified a sensitivity of 68%, significantly lower than that we observed in the present study. The IFA Bio-Manguinhos kit is no longer commercialized and the IFA method used in the present study and by Assis et al. (2010) were developed in-house.

In the present study, the clinical classification of the animals of the positive group was not performed. Quinnell et al. (2013) discriminated animals with and without signs of disease and concluded that the Kalazar Detect test showed higher sensitivity (86.7%) when carried out on sick animals. Thus, the lower sensitivity (79.6%) observed in our study with this test may be related to the presence of dogs without clinical signs in the positive group.

The ELISA Bio-Manguinhos test showed a lower sensitivity than that found by Figueiredo et al. (2010) and Laurenti et al. (2014). However, our result of ELISA Bio-Manguinhos was higher than the 72% observed by Lira et al. (2006). According to the laboratory manufacturer, Bio-Manguinhos, the sensitivity from serum samples is higher than the results that we found. However, for the sensitivity calculations, the laboratory manufacturer used the IFA as gold standard, different from the reference used in the present study, which was the parasitological test.

The sensitivity of the rapid ELISA IDEXX test (76.8%) was lower than those obtained by Ferroglio et al. (2007) (91.1%) and Athanasiou et al. (2014) (89.23%). This difference may be associated to the fact that these studies had the IFA as the gold standard and not the parasitological results. However, the results also differ from the study by Marcondes et al. (2011) that used parasitological tests to include positive serological animals. A question to be raised in this study is the possibility that the animals analyzed had elevated antibody titers, which increases the sensitivity of the test, as verified by Athanasiou et al. (2014) and also in the present study.

The sensitivity obtained in the ALERE test (73.6%) was lower than the 85% found by Souza Filho et al. (2016) that used parasitological tests as gold standard. In this study, we observed a high similarity between the rapid ELISA IDEXX and ALERE tests. These results differ from those found by Dantas Torres et al. (2018), when sensitivity levels of the ALERE test were significantly lower than those of the rapid ELISA IDEXX.

The sensitivities found in the RTs differ from the results recorded in the product labels. Only the DPP Bio-Manguinhos presented a sensitivity (97.9%) within the range described by the manufacturer (92.9–100%). For the other tests, the sensitivities reported by manufacturers of the rapid ELISA IDEXX (96.3%), Kalazar Detect (> 90%) and ALERE (97.2%) were higher than those obtained in the present study.

Considering the specificity, the results indicate that the rapid ELISA IDEXX test as the best confirmatory test of the infection, because it did not record false positive results. In this regard, the rapid ELISA IDEXX test had superior performance than the one described by the manufacturer (99.2%). The lower specificity was observed in the in-house IFA, since this test had the highest occurrence of false positives, which may result in inadequate treatment or unnecessary euthanasia when used alone (Alves and Bevilacqua, 2004; Lira et al., 2006; Ribeiro et al., 2009; Mendonça et al., 2017). However, this test can be used to monitor the antibody titers from dogs under treatment that assists in the staging of CVL.

The ELISA Bio-Manguinhos presented better specificity (95.6%) than predicted by the manufacturer (91.76%). To establish its specificity, the manufacturer used the IFA test as gold standard, produced by the laboratory itself. Results of lower specificity were also observed by others authors (Lira et al., 2006; Laurenti et al., 2014). Interestingly, Mendonça et al. (2017) observed that the specificity of this test varied with the prevalence of canine infection in the evaluated areas. The results of Figueiredo et al. (2008) (96.6%) and Lemos et al. (2008) (100%), using an in-house test, were higher than those verified in this study.

In the present study, DPP Bio-Manguinhos demonstrated 93.6% specificity, which is a lower result than that found in the study developed by Grimaldi et al. (2012) (96%). In a group of dogs with negative parasitological tests, Mendonça et al. (2017) observed specificity of 60% in an area with high canine infection index and 98% in an area of low endemicity. Specificity values lower than those observed in this study were obtained by Schubah et al. (2014), in the visual reading of whole blood (73.3%) and serum samples (69.2%) and using the electronic reading (68.2%). In our study, DPP Bio-Manguinhos presented better specificity than the range predicted by the manufacturer (87.5–91.7%).

For the Kalazar Detect test, the specificity value (95.7%) was within the range predicted by the manufacturer (> 90%) and was lower than that obtained in the study developed by Lemos et al. (2008) (100%). Mendonça et al. (2017) observed 79% specificity in an area of high infection level and 98% in an area of low prevalence. Comparing the results of this study with those obtained by Quinnell et al. (2013), the specificity in infected group varied from 89.5% to 99.9%, which are values that encompass the result of this study. Krawczak et al. (2015) detected 98.7% specificity, a superior index to that found in this study, and also verified that there were no cross-reactions with *Ehrlichia canis* and *Babesia canis*. This aspect was not addressed in our study.

For the ALERE test, we observed 97.8% specificity, close to the values indicated by the manufacturer (99.8%) and observed in the study developed by Souza Filho et al. (2016).

Antibody titers higher than 1:160 in the in-house IFA showed greater agreement with the results of RTs. Athanasiou et al. (2014), using the rapid ELISA IDEXX, observed that their sensitivity increased in higher IFA titers, reaching 100% for titers equal or greater than 1:200. Despite this, the present study also showed that ELISA Bio-Manguinhos, rapid ELISA IDEXX, Kalazar Detect and ALERE tests presented false negative reactions up to higher dilutions. This demonstrates the importance that such tests should not be used alone as a dog screening test (verification of negative results) and vaccination practice, since these tests presented low sensitivity, despite having high specificity.

Results of this study corroborate previous findings that consider mistaken the dog euthanasia based on the criterion of positivity by IFA at the cut-off point 1:40 (Alves and Bevilacqua, 2004; Ribeiro et al., 2009; Mendonça et al., 2017). According to the data of this study, the in-house IFA, up to the 1:160 dilution, can still present false positive samples, which was also observed by Ferroglio et al. (2007). In this context, animals with a dilution of in-house IFA up to 1:160 should be investigated before confirming the infection.

Based on our results, to find all true positive animals, the best combination of two tests is the use of the DPP Bio-Manguinhos, which has the highest sensitivity (97.9%), as a screening test, followed by the use of the rapid ELISA IDEXX, which has the highest specificity (100%), as confirmatory test. In addition, positive animals in the DPP Bio-Manguinhos and negatives in the rapid ELISA IDEXX should be monitored, as well as new tests should be performed, such as molecular and parasitological tests and staging of the disease. In order to follow the treatment of the dogs, IFA is the only quantitative method that allows the monitoring of the antibody curve presented by the animal throughout the treatment, being this one of the criteria for the infection staging (Solano-Gallego et al., 2011). Considering also that DPP Bio-Manguinhos is a method not available to the private market, IFA becomes important for the clinical veterinarian, since its sensitivity is high and did not have statistical difference when compared with the DPP Bio-Manguinhos. However, the specificity of in-house IFA was low when compared with all other tests, although it showed a statistical difference only from the rapid ELISA IDEXX. Currently, Brazilian Ministry of Health, according to technical note 01/2011, suggests for the diagnosis of CVL the use of two tests: DPP Bio-Manguinhos, which has high sensitivity, as a screening test; and ELISA Bio-Manguinhos, as a confirmatory test. However, ELISA Bio-Manguinhos demonstrated inferior specificity, which can generate false positive reactions and thus lead to the erroneous death of healthy animals.

In this scenario, the serological diagnosis of CVL needs to be evaluated by means tests that demonstrate a lower number of false positives and negatives, beside reliable confirmatory parasitological or molecular tests. Tests that are capable of identifying the etiological agent of the disease are important, since other species of *Leishmania*, such as *L. braziliensis* and *L. amazonensis*, have been confirmed in enzootic environments of *L. infantum*, and can generate cross reactions as described by Madeira et al. (2006); Marcondes et al. (2011); Grimaldi et al. (2012) and Paz et al. (2018).

5. Conclusions

Based on the evidence obtained in this study, we can conclude that none of the tests reached 100% sensitivity and specificity, which indicates the need for an association of tests with the best combination of sensitivity and specificity. In this context, this study demonstrated that the tests with higher sensitivity levels were DPP Bio-Manguinhos and in-house IFA, with no statistical difference between them, and the rapid ELISA IDEXX was the only test that presented difference in relation in-house IFA in specificity, but not in relation the ALERE, Kalazar Detect, ELISA Bio-Manguinhos and DPP Bio-Manguinhos. However, for the treatment of infected and/or diseased animals, quantitative tests accompanying the evolution of antibody titers are required, which indicates the maintenance of IFA in animal handling. We also demonstrate that all RTs, except the DPP Bio-Manguinhos, present greater specificity than sensitivity, and should not be used alone as the only screening test of dogs with suspected CVL.

Declaration of Competing Interest

The authors declare no conflicts of interest related to the study.

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References

Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Adv. Parasitol. 57, 1–88.

Alves, W.A., Bevilacqua, P.D., 2004. Reflexões sobre a qualidade do diagnóstico da leishmaniose visceral canina em inquéritos epidemiológicos: o caso da epidemia de Belo Horizonte, Minas Gerais, Brasil, 1993-1997. Cad. Saúde Pública. 20, 259–265.

Assis, J., Queiroz, N.M.G.P., Silveira, R.C.V., Nunes, C.M., Oliveira, T.M.F.S., Noronha Junior, A.C.F., Neves, M.F., Machado, R.Z., Buzzetti, W.A.S., 2010. Estudo comparativo dos métodos diagnósticos para leishmaniose visceral em cães oriundos de Ilha Solteira. SP. Rev. Bras. Parasitol. Vet. 19, 17–25.

Athanasiou, L.V., Petanides, T.A., Chatzis, M.K., Kasabalis, D., Apostolidis, K.N., Saridomichelakis, M.N., 2014. Comparison of two commercial rapid in-clinic serological tests for detection of antibodies against *Leishmania* spp. in dogs. J. Vet. Diagn. Invest. 26, 286–290.

Brasil, 2014. Manual de vigilância e controle da leishmaniose visceral: normas e manuais técnicos. Ministério da Saúde, Brasília, pp. 120.

Camargo, M.E., 1964. Improved technique of indirect immunofluorescence for serological diagnosis of toxoplasmosis. Rev. Inst. Med. Trop. São Paulo. 6, 117–118.

Coura-Vital, W., Ker, H.G., Roatt, B.M., Aguiar-Soares, R.D.O., Leal, G.G.A., Moreira, N.D., Oliveira, L.A.M., de Menezes Machado, E.M., Morais, M.H.F., Corrêa-Oliveira, R., Carneiro, M., Reis, A.B., 2014. Evaluation of change in canine diagnosis protocol adopted by the Visceral Leishmaniasis Control Program in Brazil and a new proposal for diagnosis. PLoS One 9, e91009.

Dantas Torres, F., Sales, K.G.S., Silva, L.G., Otranto, D., Figueredo, L.A., 2018. Level of agreement between two commercially available rapid serological tests and the official screening test used to detect *Leishmania* seropositive dogs in Brazil. Vet. J. 234, 102–104.

Ferroglio, E., Centaro, E., Mignome, W., Trisciuoglio, A., 2007. Evaluation of an ELISA rapid device for the serological diagnosis of *Leishmania infantum* infection in dog as compared with immunofluorescence assay and western blot. Vet. Parasitol. 144, 162–166.

Figueiredo, F.B., Madeira, M.F., Nascimento, L.D., Abrantes, T.R., Mouta-Confort, E., Passos, S.R.L., Schubach, T.M.P., 2010. Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples. Rev. Inst. Med. Trop. São Paulo. 52, 4.

Fiocruz, 2008. EIE - Leishmaniose Visceral Canina Bio-Manguinhos. Fiocruz. In:. http://www.bio.fiocruz.br/images/stories/pdfs/manuais/leishmaniose/BM_014_08Bk_EIEleish_can.pdf.

Garcia, L., Kindt, A., Bermudez, H., Llanos-Cuentas, A., De Doncker, S., Arevalo, J., Tintaya, K.W.Q., Dujardin, J.C., 2004. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J. Clin. Microbiol. 42, 2294–2297.

Gomes, Y.M., Cavalcanti, M.P., Lira, R.A., Abath, F.G., Alves, L.C., 2008. Diagnosis of canine visceral leishmaniasis: biotechnological advances. Vet. J. 175, 45–52.
Grimaldi, G.J.R., Teva, A., Ferreira, A.L., Dos Santos, C.B., Pinto, I., De-Azevedo, C.T., Falqueto, A., 2012. Evaluation of a novel chromatographic immunoassay based on dual-path platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 106, 54–59.
Krawczak, F.S., Reis, I.A., Silveira, J.A., Avelar, D.M., Marcelino, A.P., Werneck, G.L., Labruna, M.B., Paz, G.F., 2015. *Leishmania*, *Babesia* and *Ehrlichia* in urban pet dogs: co-infection or cross-reaction in serological methods? Rev. Soc. Bras. Med. Trop. 48, 1–5.
Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biom. 33, 159–174.
Laurenti, M.D., Leandro, J.R.S., Tomokane, T.Y., De Lucca, H.R.L., Aschar, M., Souza, C.S.F., Silva, R.M., Marcondes, M., Matta, V.L.R., 2014. Comparative evaluation of the DPP CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. Vet. Parasitol. 205, 444–450.
Lemos, E.M., Carvalho, S.F.G., Corey, R., Dietze, R., 2003. Avaliação do teste rápido utilizando o antígeno recombinante K39 no diagnóstico da leishmaniose visceral no Brasil. Rev. Soc. Bras. Med. Trop. 36 (suplemento II).
Lemos, E.M., Laurenti, M.D., Moreira, M.A.B., Reis, A.B., Ginchetti, R.C., Raychaudhuri, S., Dietze, R., 2008. Canine visceral leishmaniasis: performance of a rapid diagnostic test (Kalazar Detect™) in dogs with and without signs of the disease. Acta Trop. 107, 205–207.
Lira, R.A., Cavalcanti, M.P., Nakazawa, M., Ferreira, A.G.P., Silva, E.D., Abath, F.G.C., Alves, L.C., Souza, W.V., Gomes, Y.M., 2006. Canine visceral leishmaniosis: a comparative analysis of the EIE-leishmaniose visceral canina Bio-Manguinhos and the IFI-leishmaniose visceral canina Bio-Manguinhos kits. Vet. Parasitol. 137, 11–16.
Madeira, M.F., Schubach, A.O., Schubach, T.M., Pereira, A.S., Figueiredo, F.B., Baptista, C., Leal, C.A., Melo, C.X., Confort, E.M., Marzochi, M.C., 2006. *Post mortem* parasitological evaluation of dogs seroreactive for *Leishmania* from Rio de Janeiro. Rev. Bras. Parasitol. Vet. 138, 366–370.
Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. 158, 274–287.
Marcelino, A.P., Souza Filho, J.A., 2015. Instruções para a realização do teste rápido imunocromatográfico ALERE para diagnóstico da leishmaniose visceral canina. IOM FUNED, Belo Horizonte, pp. 15.
Marcondes, M., Biondo, A.W., Gomes, A.A.D., Silva, A.R.S., Vieira, R.F.C., Camacho, A.A., Quinn, J., Chandrashekar, R., 2011. Validation of a *Leishmania infantum* ELISA rapid test for serological diagnosis of *Leishmania chagasi* in dogs. Vet. Parasitol. 175, 15–19.
Mendonça, I.L., Batista, J.F., Schallig, H., Pires E Cruz, M.S., Alonso, D.P., Ribolla, P.E.M., Costa, D.L., Costa, C.H.N., 2017. The performance of serological tests for *Leishmania infantum* infection screening in dogs depends on the prevalence of the disease. Rev. Inst. Med. Trop. São Paulo. 59, e39.
Oliveira, E., Saliba, J.W., Oliveira, D., Dias, E.S., Paz, G.F., 2016. A prototype of the direct agglutination test kit (DAT-Canis) for the serological diagnosis of canine visceral leishmaniasis. Vet. Parasitol. 221, 9–13.
Paz, G.F., Rugani, J.M.N., Marcelino, A.P., Gontijo, C.M.F., 2018. Implications of the use of serological and molecular methods to detect infection by *Leishmania* spp. in urban pet dogs. Acta Trop. 182, 198–201.
Peixoto, M.H., Oliveira, M.R.F., Romero, G.A.S., 2015. Serological diagnosis of canine visceral leishmaniasis in Brazil: systematic review and meta-analysis. Trop. Med. Int. Health 20, 334–352.
Quinnell, R.J., Carson, C., Reithinger, R., Garcez, L.M., Courtenay, O., 2013. Evaluation of rk39 rapid diagnostic tests for canine visceral leishmaniasis: longitudinal study and meta-analysis. PLoS Negl. Trop. Dis. 7, e1992.
Ribeiro, V.M., Lima, M.C.C.D., Nogueira, F.S., Michalick, M.S.M., 2009. Serological diagnosis of canine visceral leishmaniasis in Brazil: a comparison of results from private laboratories and from one reference laboratory. 4th World Congress on Leishmaniasis 2009, WorldLeish4. 3rd to 7rth February 2009 Abstract Book.
Schubach, E.Y., Figueiredo, F.B., Romero, G.A., 2014. Accuracy and reproducibility of a rapid chromatographic immunoassay for the diagnosis of canine visceral leishmaniasis in Brazil. Trans. R. Soc. Trop. Med. Hyg. 108, 568–574.
Solano-Gallego, L., Miro, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit. Vectors 4, 86.
Souza Filho, J.A., Barbosa, J.R., Figueiredo, F.B., Mendes Júnior, A.A., Silva, S.R., Coelho, G.L., Marcelino, A.P., 2016. Performance of ALERE™ immunochromathographic test for the diagnosis of canine visceral leishmaniasis. Vet. Parasitol. 225, 114–116.
WHO, 2010. Control of the Leishmaniasis: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases. World Health Organization (WHO), Geneva, pp. 22–26.
Woyames Pinto, A.J., Ribeiro, V.M., Tafuri, W.L., 2016. The immunochromatography use in canine visceral leishmaniasis in Brazil: a “quick solution” of a complex diagnostic? Rapid test in dogs with leishmaniasis. Ann. Clin. Cytol. Pathol. 2.