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Improving the serodiagnosis of canine *Leishmania infantum* infection in geographical areas of Brazil with different disease prevalence

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ABSTRACT

Serodiagnosis of *Leishmania infantum* infection in dogs relies on the detection of antibodies against leishmanial crude extracts or parasitic defined antigens. The expansion of canine leishmaniasis from geographical areas of Brazil in which the infection is endemic to regions in which the disease is emerging is occurring. This fact makes necessary the analysis of the serodiagnostic capabilities of different leishmanial preparations in distinct geographical locations. In this article sera from dogs infected with *Leishmania* and showing the clinical form of the disease, were collected in three distinct Brazilian States and were tested against soluble leishmanial antigens or seven parasite individual antigens produced as recombinant proteins. We show that the recognition of soluble leishmanial antigens by sera from these animals was influenced by the geographical location of the infected dogs. Efficacy of the diagnosis based on this crude parasite preparation was higher in newly endemic regions when compared with areas of high disease endemicity. We also show that the use of three of the recombinant proteins, namely parasite surface kinetoplastid membrane protein of 11 kDa (KMP-11), and two members of the P protein family (P2a and P0), can improve the degree of sensitivity without adversely affecting the specificity of the diagnostic assays for canine leishmaniasis, independently of the geographical area of residence. In addition, sera from dogs clinically healthy but infected were also assayed with some of the antigen preparations. We demonstrate that the use of these proteins can help to the serodiagnosis of *Leishmania* infected animals with sub-

Abbreviations: BB, blocking buffer; CanL, Canine visceral leishmaniasis; EDCB, ELISA denaturant coating buffer; ELISA, enzyme-linked immunosorbent assay; HSP, Heat shock protein; KMP-11, Kinetoplastid-membrane protein of 11 kDa; LR, Likelihood ratio; MS, Mato Grosso do Sul State (Brazil); PBS, phosphate saline buffer; PI, Piaui State (Brazil); ROC, Receiver Operating Characteristic; RR, Relative reactivity; RT, Room temperature; SC, Santa Catarina State (Brazil); SLA, Soluble leishmanial antigen; VL, Visceral leishmaniosis; WB, Washing buffer.

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clinical infections. Finally, we propose a diagnostic protocol using a combination of KMP-11, P2a y P0, together with total leishmanial extracts.

1. Introduction

Canine leishmaniasis (CanL) is a potentially fatal zoonotic disease caused by infection with *Leishmania infantum* (syn. *L. chagasi* (Mauricio et al., 2000)). Infected dogs can develop different forms of the disease ranging from clinically healthy animals (subclinical infection) to animals showing the clinical form of the disease (clinically ill). Depending on the number and severity of the disease manifestations and the pathological abnormalities there are different stages of the clinical disease, ranging from mild to very severe CanL (Solano-Gallego et al., 2009; Solano-Gallego et al., 2011). Infected animals typically develop a specific humoral response against crude preparation of parasite proteins (SLA; soluble leishmanial antigens). The titer of anti-*Leishmania* antibodies are usually higher in canine patients showing the most severe forms of the disease (Maia and Campino, 2008; Noli and Saridomichelakis, 2014; Solano-Gallego et al., 2017). Subclinically infected dogs include animals at the initial stage of the disease that will evolve towards the clinical form, showing a concomitant increase in the magnitude of the humoral response against parasite antigens (Nieto et al., 1999; Leandro et al., 2001; Fernandez-Cotrina et al., 2013). In addition, dogs that are subclinically infected will remain healthy for many years, showing a limited humoral response against parasite antigens after mounting an effective cell-mediated immunity that prevent parasite proliferation, (Baneth et al., 2008; Noli and Saridomichelakis, 2014; Abbehusen et al., 2017; Hosein et al., 2017).

The correct diagnosis of CanL continues to be an unresolved question, since there is not a current gold standard method to detect the 100% of *Leishmania* infected individuals. Some approaches are based on detection of the parasite in biological samples by cytological assays (cell or tissue staining, immunochemistry or parasite culture) and molecular techniques for detection of the parasite DNA (Solano-Gallego et al., 2017). The presence of circulating anti-*Leishmania* specific antibodies in the blood of infected dogs has allowed the development of serologic assays for diagnosis of CanL. They include, among others, direct agglutination test, indirect fluorescent antibody test or enzyme-linked immunosorbent assays (ELISA). ELISA is an immunological test that uses simple methodologies and can therefore be used in the field diagnosis of the disease. In addition, it can be also employed for characterizing the diagnostic capacities of different antigens to further develop qualitative immune-chromatographic rapid tests that do not require laboratory equipment for their use (Travi et al., 2018). Different antigenic sources are employed in these diagnostic methods, including SLA, as well as different parasite antigenic fractions, individual recombinant proteins or small peptides containing defined antigenic determinants (Travi et al., 2001; Coelho et al., 2009; Solano-Gallego et al., 2009; Ker et al., 2013; Rodriguez-Cortes et al., 2013; Solano-Gallego et al., 2017). Some of these antigens are parasite-specific proteins like the kinetoplastid-membrane protein of 11 kDa (KMP-11) (Berberich et al., 1997), or members of intracellular protein families such as histones (Soto et al., 1999), heat shock proteins (HSP) (Angel et al., 1996; Quijada et al., 1996a; Oliveira et al., 2011), ribosome related factors including the acidic ribosomal protein family (Soto et al., 2009) or the recombinant K39 protein that contains an extensive repetitive domain located in the C-terminal region of the leishmanial kinesin protein (Scalone et al., 2002). Although these antigens are proteins commonly conserved in different organisms, in leishmaniasis patients the immune response is elicited specifically towards the parasite proteins. This is due to the localization of the main epitopes in protein regions that contain specific amino acids for the parasite (Requena et al., 2000).

Human visceral leishmaniases (VL) and CanL are endemic in European and African countries of the Mediterranean basin, Middle-East, Asia and in Latin America (Dantas-Torres et al., 2012; Pigott et al., 2014) being domestic dogs the main reservoir for the infection to human (Pennisi, 2015). During the last years several reports indicate that there has been an expansion of human VL and CanL from its historical endemic regions to traditionally non-endemic areas such North Europe and North America countries (Dujardin et al., 2008; Petersen, 2009; Ready, 2010, Mattin et al., 2014). Evolution of human VL cases in Brazil perfectly reflects the disease expansion from regions with high number of cases (Northern States) to recently colonized areas (Southern States) (Harhay et al., 2011; Reis et al., 2017). In this work we have first evaluated the diagnostic properties of the SLA and different *Leishmania* antigenic proteins using sera collections of clinically ill dogs obtained in three geographical distant regions of Brazil with differences in the prevalence of CanL. Secondly, we have determined the diagnostic properties of some of the protein preparations for the detection of subclinically infected animals. On the basis of our results we propose the combination of three recombinant proteins besides SLA for serodiagnosis of CanL.

2. Materials and methods

2.1. Parasites and dogs

Leishmania infantum (MHOM/BR/2000/MER-STRAIN2) promastigotes were cultured in Schneider's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin, at pH 7.4.

Serum samples were collected in the city of Teresina, state of Piaui (PI) in the Northeast region; in the cities of Camapuã and Campo Grande, state of Mato Grosso do Sul (MS) in the Central-West region; and in the city of Florianopolis, state of Santa Catarina (SC) in the South region of Brazil (Supplementary Fig. 1). For the first objective, sera from clinically ill CanL (PI, n = 46; MS, n = 57; SC, n = 52) dogs were collected at the three State locations. For the second objective, sera from *Leishmania* subclinically infected animals were collected in PI (n = 46) and MS (n = 35) and analyzed as a single group. For animal classification, the clinical profile of the animals included in the study was evaluated taking into account different clinical sign as previously reported (Silva et al., 2017). Absence of the clinical signs was scored as 0. According to the severity of the presented clinical signal, 1 point was assigned to the milder signal and 2 points for the most severe clinical signs. Subclinically infected animals were classified as having a total score of up to 3 points, being the group of clinically ill dogs those showing a clinical score higher than 3 points. For all the animals, the presence of amastigote forms was confirmed by direct observation after Giemsa staining of lymph nodes or bone marrow aspirates. Sera from healthy animals (clinical score = 0 and *Leishmania* negative) were collected in PI (n = 45), MS (n = 39) and SC (n = 82). Finally, sera from *Leishmania* negative animals affected by canine monocytic ehrlichiosis were collected in PI (n = 46) and MS (n = 30). *Ehrlichia canis* infection was monitored with the ALERE ERLIQUIOSE Ac TEST KIT (Bionote Inc., Gyeonggi-do, Korea) following manufacturer instructions.

This project was approved by the Animal Experimentation Ethics Committee of the Federal University of Piauí under protocol number 092/15, as well as the consent of the owners of the dogs to carry out the samples for analysis.

2.2. Antigen preparation

Freezed-thaw SLA was prepared from stationary phase promastigotes of *L. infantum* as previously described (Souza et al., 2013). Recombinant proteins were expressed in bacteria (*Escherichia coli*) transformed with pQE plasmids (Qiagen, Hilden, Germany) recombinant for the next *L. infantum* coding regions: KMP-11 (Fuertes et al., 2001); H2A (Iborra et al., 2004); HSP83 (Angel et al., 1996); HSP70 (Souza et al., 2013); LiP2a and LiP2b (Iborra et al., 2007); LiP0 (Iborra et al., 2003). Gene expression and protein purification of the different his-tagged recombinant proteins were performed by affinity chromatography using Ni-NTA resin (Qiagen), under denaturant conditions as described (Garde et al., 2018). Proteins were stored at −20 °C in ELISA denaturant coating buffer (EDCB: 3 M urea, 0.5 M NaCl, 5 mM imidazol, 1 mM 2-mercaptoethanol in 20 mM Tris HCl pH 8).

2.3. Qualitative ELISA

Microtiter immunoassay plates MaxiSorp™ (Nunc, Roskilde, Denmark) were coated with *L. chagasi* SLA (0.2 μg per well; 100 μl total volume) or each one of the recombinant proteins (0.1 μg per well) in EDCB buffer for 12 h at 4 °C. After coating, four washes were performed in 200 μl of washing buffer (WB: phosphate saline buffer [PBS] + 0.5% Tween 20). Free binding well sites were blocked with WB supplemented with 5% (w/v) non-fat milk (blocking buffer: BB) for 1 h at room temperature (RT). After, plates were incubated with 100 μl of canine sera (1:400 dilution in BB) for 2 h at RT. Then, wells were washed with WB as indicated above and incubated with 100 μl of secondary antibody (anti-dog IgG antibody (Sigma, St. Louis, USA) horseradish peroxidase conjugated; 1:6000 dilution in BB) for 1 h at RT. After 4 washes, reaction was developed with 100 μl of H_2O_2-ortophenylenediamine solution (Sigma) for 20 min in the dark, and stopped by addition of 50 μl of H_2SO_4 2 N. Absorbance values were determined at 490 nm in an ELISA microplate reader.

In all the plates the same negative control (canine sera from a healthy dog living in a non-endemic region for *Leishmania*) was always included to calculate the relative reactivity (RR) of each sample. RR was defined as the ratio between the absorbance of a given sample and the negative control sample taken from a selected healthy dog. As another technical control all plates also included a positive sera obtained from a CanL dog selected because of its reactivity ($O.D._{450} > 0.5$) to all antigens assayed.

2.4. Statistical analysis

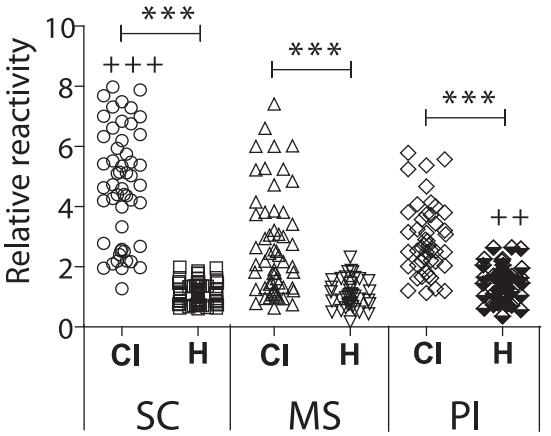
The statistical analysis was made using the GraphPad Prism software. The ELISAs cut-off values were calculated by comparison of the RR from the CanL sera (clinically ill or subclinically infected) and healthy (or *E. canis* infected dogs when indicated) using the Receiver Operating Characteristic (ROC) analysis. This test also allowed the determination of sensitivity, specificity and the Likelihood ratio (LR) defined as the ratio of expected ELISA result in dogs with CanL to the dogs without the disease (Simundic, 2009). D'Agostino and Pearson test was employed to analyze the Gaussian distribution of the samples. The Mann-Whitney non-parametric test was employed to assess the existence of significant differences between two groups. *P*-values lower than 0.05, 0.01 or 0.001 were represented as *, ** or ***, respectively. The Kruskal-Wallis non-parametric test was employed to analyze more than two groups. *P*-values lower than 0.01 or 0.001 were represented as $^{++}$ or $^{+++}$.

3. Results

3.1. Anti-leishmanial humoral response in clinically ill dogs

Our first objective was to analyze the reactivity against SLA of sera collections taken in three different States of Brazil: SC, MS, PI. The RR against SLA of the sera from clinically ill CanL animals, independently of the sampling place, was significantly higher than the RR calculated using sera from healthy animals residing in the same equivalent geographical regions (Fig. 1A).

A



B

	SC	MS	PI
Cut-off value	1.963	1.894	2.679
Sensitivity (%)	94.23	56.14	50.00
Specificity (%)	98.77	97.44	97.78
Likelihood ratio	76.33	21.89	22.50

Fig. 1. Sera from clinically ill CanL patients collected in different geographical locations show differences in the recognition of *Leishmania* soluble leishmanial antigens (SLA). Sera from CanL clinically ill dogs (CI) or healthy dogs (H) collected in Santa Catarina (SC), Mato Grosso do Sul (MS) or Piaui (PI) were assayed by ELISA against soluble leishmanial antigen (SLA). In the graph it is shown the relative reactivity (RR) defined as the absorbance of a given sera divided by the absorbance of a control healthy sera included in all plates. The symbol *** indicates significant differences ($P < 0.001$) between clinically ill CanL and healthy sera from the same location (Mann Whitney test). The symbol +++ indicates a significant increase ($P < 0.001$) between the clinically ill CanL from SC with regard the equivalent sera from the other two locations and the symbol ++ indicates a significant increase in the RR values of healthy sera taken in PI with regard the equivalent sera from SC and MS (Kruskal-Wallis test) (A). Table showing the diagnostic parameters calculated by a ROC analysis (B).

Interestingly, the RR found for SC sera was significantly higher than that found for MS and PI. On the contrary, the RR value for PI healthy samples was significantly higher than the equivalent sera from the other regions. These differences are reflected in the sensitivity value of the SLA-based assay, being the highest value for the SC samples and the lowest for the PI sera (Fig. 1B). Thus, diagnostic accuracy for SLA determined by the LR was very low, especially in MS and PI (Fig. 1B).

We next use seven individual antigenic proteins of *Leishmania* produced as heterologous recombinant proteins in bacteria, to perform ELISAs using the same sera collections (sera from clinically ill CanL animals and healthy dogs, respectively). All the proteins were recognized as antigenic when analyzed with SC sera. The RR values for the sera of clinically ill dogs were significantly higher than the RR of the healthy sera (Fig. 2A). A similar behavior was found for the sera from MS (Fig. 2B) or PI (Fig. 2C) except for the histone H2A, which was not recognized. The highest percentages of sensitivity for each one of the antigens were observed for sera taken in SC, resulting also in the highest value of the LR when compared to data from MS and PI sera data (Table 1). None of the assays performed with the recombinant proteins and sera from SC of MS (Table 1) reach the LR found for the SLA (Fig. 1B). This situation changes when data of the sera collected in PI were analyzed. For this sera collection, the KMP-11 surface protein and the LiP0 ribosomal protein showed slightly higher sensitivity percentages (60% and 54.35%, respectively) or LR values (27 and 24.46, respectively) (Table 1) than the SLA (50% of sensitivity and 22.5 of LR, respectively) (Fig. 1B). We conclude that the use of SLA-based methods can compromise the results of the diagnosis and that the individual antigens diagnostic performances can be of interest to improve these obtained with SLA.

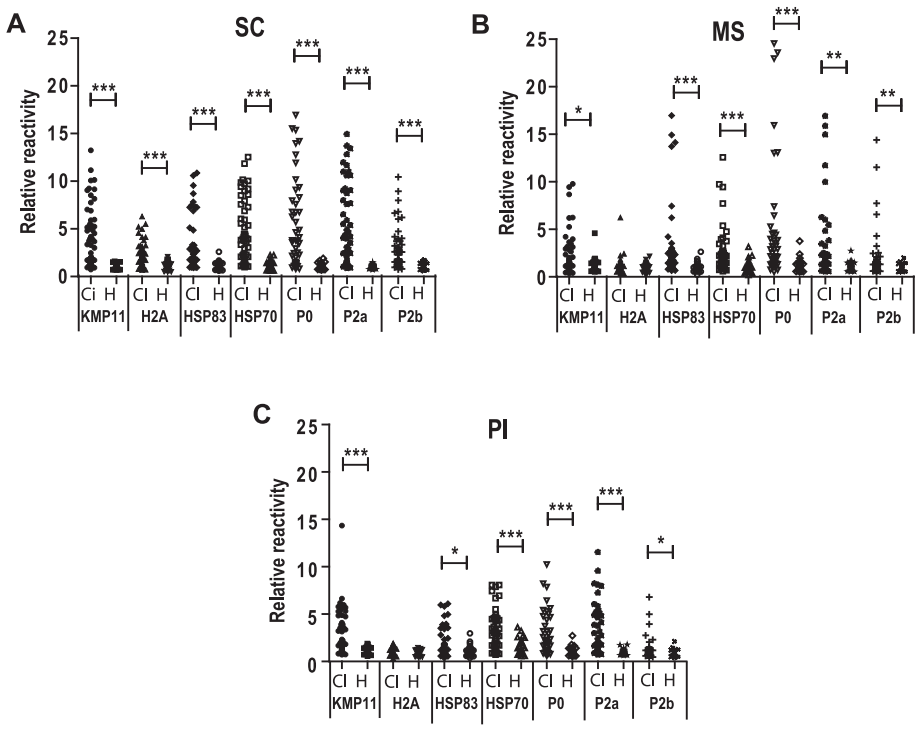


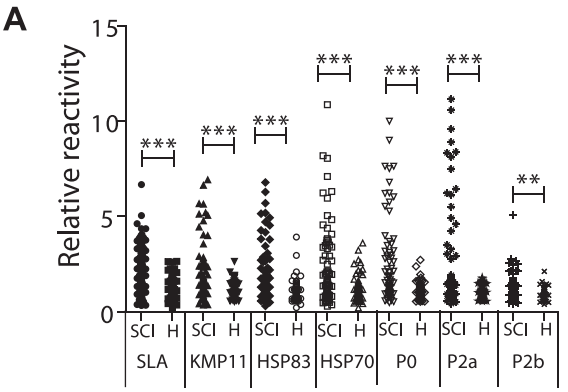
Fig. 2. Reactivity of sera from clinically ill CanL patients against single *Leishmania* recombinant antigenic proteins. Different *Leishmania* antigens purified as recombinant proteins after the expression of their coding regions in *E. coli* were employed for coating ELISA plates: Kinetoplastid membrane protein of 11 kDa (KMP11), histone H2A, the heat shock proteins of 70 kDa (HSP70) or 83 kDa (HSP83) and the acidic ribosomal proteins P2a, P2b and P0. Sera from CanL clinically ill dogs (Cl) or healthy dogs (H) collected in Santa Catarina (SC) (A), Mato Grosso do Sul (MS) (B) or Piaui (PI) (C) were assayed by ELISA. The scatter plots show the relative reactivity of the sera represented individually. Symbols * ($P < 0.05$), ** ($P < 0.05$) and *** ($P < 0.001$) indicate significant differences between clinically infected CanL and healthy sera from the same geographical location (Mann Whitney test).

3.2. Reactivity of subclinically infected CanL sera against the antigenic preparations

The second objective of this work was to assay the reactivity of the SLA or the recombinant proteins except H2A (due to the lack of antigenicity when sera from MS and PI were assayed) with the sera obtained from clinically healthy animals infected with *Leishmania*. With this purpose, sera were collected from subclinically infected animals in MS and PI and analyzed as a single

Table 1
Detailed information about diagnostic properties of the *Leishmania* antigens obtained from the ROC curve analysis.

Antigen	Sera origin	Cut-off	Sensitivity (%)	Specificity (%)	Likelihood ratio
KMP-11	SC	1.56	78.85	98.78	64.65
	MS	1.97	39.13	97.22	14.09
	PI	1.81	60.00	97.78	27.00
H2A	SC	1.94	57.69	98.78	50.46
	MS	1.83	7.02	97.44	2.74
	PI	1.38	19.57	97.83	9.00
HSP83	SC	1.69	59.62	98.78	48.88
	MS	1.94	45.61	97.44	17.79
	PI	2.21	28.26	97.73	12.4345
HSP70	SC	1.94	75.00	97.56	30.75
	MS	2.24	33.33	97.94	13.00
	PI	3.31	47.83	97.78	21.52
P0	SC	1.78	61.54	98.78	50.46
	MS	2.42	40.35	97.44	15.74
	PI	1.81	54.35	97.78	24.46
P2a	SC	1.43	69.23	98.78	58.35
	MS	1.69	38.60	97.44	15.05
	PI	1.48	60.87	95.35	13.09
P2b	SC	1.58	59.62	98.78	48.88
	MS	1.62	35.42	96.67	10.63
	PI	1.49	15.22	97.83	7.00



B

	SLA	KMP11	HSP83	HSP70	P0	P2a	P2b
Cut-off value	2.64	2.10	3.02	3.27	2.50	1.75	1.60
Sensitivity (%)	38.27	32.10	17.28	25.93	32.10	28.40	22.22
Specificity (%)	98.81	98.75	98.78	98.78	98.78	98.78	98.63
Likelihood ratio	32.15	25.68	14.17	21.26	26.32	23.28	13.22

Fig. 3. Reactivity of sera from CanL subclinically infected animals against SLA and the antigenic recombinant proteins. Sera from CanL dogs that are subclinically infected (SCI) or healthy dogs (H) were assayed by ELISA against SLA or against the indicated *Leishmania* antigens. A scatter plot indicating the relative reactivity values is shown. Symbols ** ($P < 0.05$) and *** ($P < 0.001$) indicate significant differences between subclinically infected CanL and healthy sera (Mann Whitney test) (A). Table showing the diagnostic parameters calculated by a ROC analysis (B).

group. The median of the RR values of the subclinically infected sera were significantly higher than the median value of the RR of the sera collected from healthy animals living in both locations for all antigenic samples tested, i.e. SLA and recombinant proteins (Fig. 3A). Sensitivity percentages range from the 38.27% for the SLA to the 17.28% for the HSP83 recombinant protein. The LR for the SLA was higher than those found for the individual antigens, being the HSP83 and the LiP2b the protein preparations that obtained the lowest LR values (Fig. 3B).

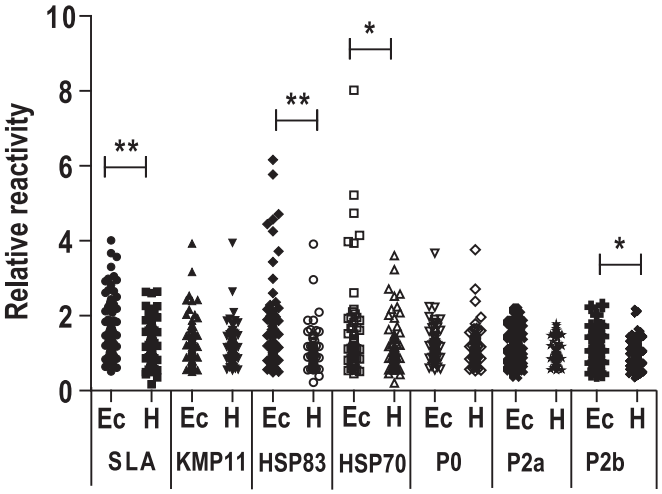


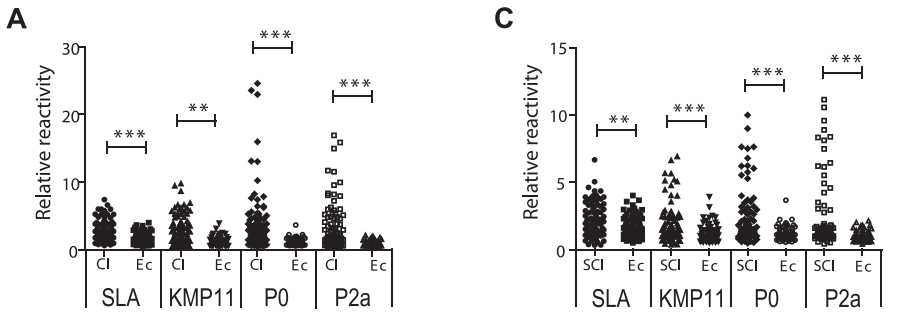
Fig. 4. Serologic cross-reactivity of *Ehrlichia canis* infected dogs' sera and *Leishmania* antigens. Sera from *E. canis* infected dogs (Ec) and healthy animals (H) were assayed by ELISA against SLA or against the indicated *Leishmania* antigens. A scatter plot indicating the relative reactivity values is shown. Symbols * ($P < 0.001$) or ** ($P < 0.05$) indicate significant differences between samples from dogs suffering ehrlichiosis and healthy animals (Mann Whitney test).

3.3. Differential diagnosis of CanL and ehrlichiosis

Next, we made a comparative analysis between sera from dogs infected with *E. canis* and the sera of healthy animals to test the specificity of the recognition of the parasite antigenic preparations (SLA and recombinant proteins, except H2A). Both sera groups were collected in PI and MS and grouped independently of the locations they were obtained. Four antigenic preparations including SLA, the HSP70 and HSP83, as well as the LiP2b protein presented higher RR values for sera of animals affected by ehrlichiosis than sera for co-residing healthy animals (Fig. 4). For SLA the RR values were homogeneously distributed along the ordinate axis, showing a RR median value significantly increased with regard the median of the RR of the healthy data. On the other hand, for the heat shock proteins, besides the incremented RR value for most of the sera population, the results showed high RR values for some individual sera (Fig. 4), suggesting the presence of highly cross-reactive antigenic determinants in these proteins, despite the evolutionary distance between *Leishmania* and *Ehrlichia* genus. On the other hand, no cross-reactivity was found for the surface located KMP-11 antigen or the acidic ribosomal proteins P0 and P2a. For these three proteins similar RR values were found in the sera from *E. canis* infected animals and the healthy ones (Fig. 4).

Thus, we decided to analyze the diagnostic value of these three individual proteins in comparison to SLA, using clinically ill (Fig. 5A and B) and subclinically infected (Fig. 5C and D) sera from PI and MS endemic areas. *Leishmania*-negative data were obtained from the sera of animals suffering mononuclear ehrlichiosis and collected in the same locations, simulating the worst conditions to perform a specific diagnosis of CanL. The median values of the RR were significantly higher in clinically ill (Fig. 5A) and subclinically infected (Fig. 5C) CanL serum groups when compared with sera from *E. canis* infected animals. The highest LR for diagnosis of clinically ill (Fig. 5B) or subclinically infected (Fig. 5D) CanL was found for the LiP2a and LiP0 acidic ribosomal proteins, being the lowest the LR of the KMP-11.

In spite of these statistical significant differences, the main problem was the low sensitivity. Thus, protein with the highest LR, LiP0, was only able to diagnose up to 38% of the CanL animals showing clinical signs and up to 34.57% of the subclinically infected animals. In order to solve the low sensitivity value of the diagnostic based on single proteins we propose to establish the next criterion of positivity: a CanL patient will be one who gives a positive response to at least one of the three candidates. This condition was based on the high degree of heterogeneity found in the recognition of the KMP-11, the LiP0 and the LiP2a antigens by each one of the individual sera from the highly endemic areas (Supplementary Fig. 2A for clinically ill CanL sera and Supplementary Fig. 2B for subclinically infected CanL sera). By the use of the cut-off values shown in Fig. 5 to classify each serum as positive or negative, a percentage of 56.28% (59/103) of the clinically ill and 39.5% (32/81) of the subclinically infected CanL sera were diagnosed as positive. Including the SLA beside the recombinant proteins we were able to rescue an additional sera in the clinically ill group 58.25% (60/103) (Supplementary Fig. 2A) and two sera for subclinically infected group CanL 41.97% (34/81) (Supplementary Fig. 2B). Finally we determined the number of sera diagnosed as positive from non-endemic area (SC) using the same criterion and the cut-off value obtained using the *E. canis* infected dogs simulating the worst diagnostic conditions. As it is shown in the Supplementary Fig. 2C, recombinant proteins were able to diagnose the 75% (39/52) of the positive sera and the SLA alone rescued 76.92% (40/52). The best results were obtained when the diagnostic criterion is based on the reactivity towards any of the four antigen preparations: 82.69% (43/52). Thus, even in non-endemic regions the inclusion of the recombinant proteins improves



B

	SLA	KMP11	P0	P2a
Cut-off value	3.70	3.17	2.25	2.071
Sensitivity (%)	26.21	23.19	38.41	30.43
Specificity (%)	98.67	98.67	98.65	98.67
Likelihood ratio	19.66	17.39	28.42	22.83

D

	SLA	KMP11	P0	P2a
Cut-off value	3.67	3.36	2.25	2.081
Sensitivity (%)	17.28	14.81	34.57	24.69
Specificity (%)	98.67	98.67	98.65	98.67
Likelihood ratio	12.96	11.11	25.58	18.52

Fig. 5. Comparative diagnostic performance of SLA and the selected leishmanial antigenic preparations. Sera from clinically ill (CI) or subclinically infected (SCI) dogs affected by CanL and dogs infected by *E. canis* (Ec) were assayed by ELISA against SLA or against *Leishmania* KMP-11, P0 and P2a antigens. A scatter plot showing the relative reactivity values (A) or a table showing the diagnostic parameters (B) from CanL clinically ill animals are shown. In (C) and (D) equivalent information for the CanL subclinically infected collection is included. Symbols ** ($P < 0.05$) and *** ($P < 0.001$) indicate significant differences between the sera from CanL animals and sera from *E. canis* infected dogs (Mann Whitney test).

the diagnostic sensitivity of the SLA. We propose to use the three selected antigens in combination with SLA for obtaining the best results for a high sensitive serodiagnosis.

4. Discussion

In this work we have made a comparative analysis of two different antigenic sources, SLA (a soluble leishmanial total extract preparation) and a series of different individual parasite antigens, obtained by a biotechnological approach as heterologous recombinant protein produced in bacteria. Regarding the SLA preparation and in coincidence with other authors assaying sera collected in the New or the Old World, our results shown that most of the CanL clinically ill animals possess antibodies against total parasite antigen preparations (Boarino et al., 2005; Porrozzi et al., 2007; Coelho et al., 2009; Fraga et al., 2014; Laurenti et al., 2014). We found a 94% of assay sensitivity for SC sera collection when the results of the CanL clinically ill sera results are compared to these obtained for healthy dogs from the same geographical area (Fig. 1). Interestingly, the sensitivity values of our ELISA test based on in-house SLA preparation were significantly affected by the geographic location of canine patients. In this sense, the percentages of positive animals residing in MS or PI diminished to percentages of 56% or 50%, respectively. This decrease was related to the lower reactivity value of the sera from these CanL clinically ill animals (both in MS and PI) and to an increase in the non-specific signal of the sera of healthy animals living in the most Northern State (PI). The existence of differences in the diagnostic capacities of a given test in different geographical areas also occurred when a SLA-based commercial kit was assayed for sera samples from different Brazilian region, with sensitivity values ranging from 70% to 91% (Lira et al., 2006; Laurenti et al., 2014; Fraga et al., 2016; de Carvalho et al., 2018). This sensitivity differences may limit the usefulness of the SLA-based methods.

Another drawback for the use of SLA for the CanL serodiagnosis is the existence of cross reactions between the total proteins of *Leishmania* and the serum of animals harboring other pathogens, including protozoan parasites (dogs infected with *Babesia*, or *Trypanosoma*) or bacteria like *E. canis* (Lira et al., 2006; da Silva et al., 2013; Fraga et al., 2014; Laurenti et al., 2014). One of the limitations of our work is the lack of cross-reactivity data between the SLA and the sera of animals infected with the most common pathogens in South America (recently reviewed in (Maggi and Kramer, 2019)) except *Ehrichia*. Employing the sera from dogs affected by monocytic ehrlichiosis, a highly endemic disease in several regions of Brazil (Maia and Campino, 2008), we detected a positive cross-reactivity, being the median value of the reactivity against SLA of these sera significantly higher than the reactivity detected for the sera of healthy animal (Fig. 4). This finding results in an increase of the cut-off value for positive/negative discrimination, negatively affecting diagnostic tests sensitivity (Porrozzi et al., 2007; Alves et al., 2012). Our data demonstrate that the maintenance of a high value of specificity (98.7%) provoked a concomitant decrease in the sensitivity of the SLA-based assay to diagnose CanL clinically ill animals. Regarding serodiagnosis of CanL in dogs that are subclinically infected, and in accordance with our results, the diagnostic properties of SLA-based methods give raise to lower sensitivity values than these observed for clinically ill dogs, which decrease more markedly when cross-reactivity parameters are also taken into consideration (Ferreira Ede et al., 2007; Laurenti et al., 2014; Zhao et al., 2016; de Carvalho et al., 2018). In our case, the sensitivity value fell from 38.27% (Fig. 3) to 17.28% (Fig. 5D) when considering the cross-reactivity parameter to calculate the cut-off.

Molecular methods can be employed for detecting clinically ill and subclinically infected animals because of their high sensitivity (Albuquerque et al., 2017; Mendonca et al., 2017; de Carvalho et al., 2018; Riboldi et al., 2018). However, development of serological improved systems would allow an easier monitoring of large number of samples in endemic areas. In this sense it is likely that the characterization of individual antigens can help to improve the serodiagnosis of CanL cases, because of the improvement of the sensitivity values without increasing the specificity (Carvalho et al., 2017; Magalhaes et al., 2017; Dias et al., 2018). In this regard we found that all the recombinant proteins included in this work, except histone H2A, were recognized by the canid sera from the three different Brazilian States. The lack of sera recognition for H2A in MS or PI should be taken as an example regarding the variability in the quality of the humoral response of leishmaniasis patients in different parts of the world. These differences in antigen recognition may be influenced by differences in animal nutrition (Calder et al., 2006) or changes in the immune system capacities due to previous contact or co-infection with other pathogens (Lescano et al., 2012; Murphy et al., 2013), that could be more habitual in the most endemic regions for *Leishmania* (PI and MS). We have found the H2A histone is recognized by approximately the 58% of the *Leishmania*-infected dogs in SC (Table 1), in accordance with the positive reactivity found in the 78% of the CanL sera collected in Spain (Soto et al., 1995). Also, this protein is recognized by the sera from human patients suffering VL in Tunisia (Maalej et al., 2003) or tegumentary leishmaniasis in the New World (Souza et al., 2013). However, the data obtained in this work discourages its use in a serological diagnostic test. Interestingly, our results demonstrate that such differences in sensitivity of the clinically ill sera due to geographical reasons were not observed for certain individual antigens, especially KMP-11 and the acidic ribosomal proteins P0 and P2a (Table 1). In addition to this property, no cross-reactivity was found against these proteins when assayed with the sera form *E. canis* infected dogs. Our data also dampen the use of the HSP70 and HSP83 heat shock proteins for serodiagnosis. In spite of being recognized by infected animals in the three locations studied in this work, as well as by a high number of *Leishmania*-infected human or canine patients in different parts of the world (Celeste et al., 2004; Angel et al., 1996; Quijada et al., 1996a; Rafati et al., 2007; Abanades et al., 2012; Souza et al., 2013), a positive reactivity with sera from dogs affected with ehrlichiosis was found. An interesting alternative to not discard these proteins, would be to explore the location of their cross-reactive antigenic determinants to produce non cross-reactive recombinant proteins (or synthetic peptides) containing the specific epitopes (Quijada et al., 1996b). Finally, the use of the P2b for diagnosis was discarded because of its cross-reactivity with the sera from dogs affected by ehrlichiosis and the low percentages of sera recognizing this antigen especially in the most endemic areas (Fig. 4 and Table 1, respectively).

One of the main findings of our work is the characterization of the diagnostic properties of the recombinant version of the KMP-11, the P2a and the P0 proteins. In this sense, as it is deduced from data shown in Fig. 5, P2a and P0 performance for the diagnosis of CanL for both, clinically ill or subclinically infected animals was higher than this detected for the SLA, when cut-off is calculated using as control group the sera from dogs affected by mononuclear ehrlichiosis. Diagnostic performance of the KMP-11 offered similar values than the SLA. However, the main drawback observed for the use of the three selected individual antigens for diagnosis (either in the clinically ill or in the subclinically infected forms) is related to the low value of sensitivity observed when the specificity of the assay is maintained up to 98%. Coinciding with other authors, it looks like the use of single recombinant antigen is insufficient to constructs serodiagnostic tests able to detect high percentages of the leishmaniasis patients (Maalej et al., 2003; Porrozzi et al., 2007; Oliveira et al., 2011). The high degree of variability in the humoral response of patients and infected dogs against individual parasite proteins (Porrozzi et al., 2007; Goto et al., 2009) offers the possibility of improving the sensitivity degree by using combination of recombinant proteins or chimeric recombinant proteins in which the antigenic determinants of different parasite proteins are fused (Soto et al., 1998; Faria et al., 2015). This is the case of a rapid test (DPP) based on recombinant fusion products containing the antigenic regions of different parasite antigen that have shown high sensibility for diagnosing clinically ill CanL cases (Grimaldi Jr et al., 2012; Morales-Yuste et al., 2012; da Silva et al., 2013; Rodriguez-Cortes et al., 2013; Marcondes et al., 2013; Laurenti et al., 2014; Fraga et al., 2016; Riboldi et al., 2018), although its sensitivity decreases in canine patients showing low clinical signs or in subclinically infected cases (Grimaldi Jr et al., 2012; Lopes et al., 2017). As it is deduced from data shown in the Supplementary Fig. 2, a high degree of heterogeneity was found for the recognition of the individual antigen by each of the sera assayed. Thus, complementarity found in the immunoreactivity of the sera against the three recombinant antigens and SLA allowed us the increase of the number of sera diagnosed as positive when the criterion of positivity is to have a positive value (over the most restricted cut-off) for any of the selected individual antigens. If SLA is also included (with cut-off values maintaining a high specificity degree to discriminate animals affected by ehrlichiosis), we were able to diagnose up to a 58.25% of the clinically ill dogs (approx. 42% of the subclinically infected canine samples) living in the most endemic regions, maintaining a high value of sensitivity for clinically ill dogs living in the new colonized regions (82.69% for SC, using restrictive cut-off values). It has to be highlighted that the seropositivity values obtained here are lower than those reported for other antigenic preparations (Laurenti et al., 2014; Faria et al., 2015; Fraga et al., 2016; de Carvalho et al., 2018). This is due to our proposal of maintaining a high value of specificity for not to diagnose false positive animals, a necessary property for a serodiagnostic test. Before moving to the field, the specificity in the recognition of recombinant proteins should be analyzed using sera from animals infected by other pathogens that coexist with *Leishmania* (Maggi and Kramer, 2019) including the study of cross-reactivity with dogs infected with *T. cruzi* (Roque et al., 2013; Freitas et al., 2018).

5. Conclusion

From the results obtained in this work it can be concluded that the combined use of different antigenic preparations improves the sensitivity and specificity of CanL diagnosis. This conclusion is valid to confirm the disease in animals that present clinical signs compatible with leishmaniasis. The proposed strategy has also been shown to be able to increase the number of subclinically infected animals diagnosed because of the presence of anti-*Leishmania* antibodies. Undoubtedly, the percentages of positivity must be improved before moving to the field. We plan to study new recombinant proteins that can act cooperatively to these studied in this work in order to achieve better diagnostic performances for confirming the disease in clinically infected animals, as well as identifying dogs subclinically infected.

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