Parasite Epidemiology and Control
Volume 8, February 2020, e00126

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

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Received 25 July 2019, Accepted 19 November 2019, Available online 21 November 2019, Version of Record 27 November 2019.



https://doi.org/10.1016/j.parepi.2019.e00126



**Referred to by** Erratum regarding missing Declaration of Competing Interest statements in previously published articles
Parasite Epidemiology and Control, Volume 11, November 2020, Pages e00196



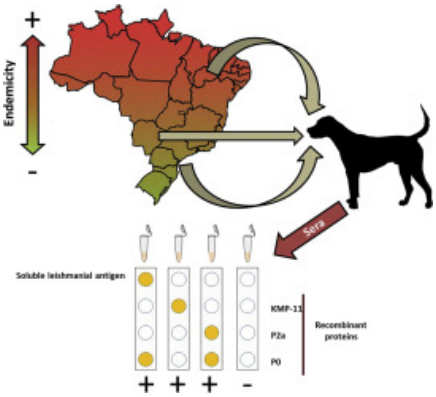
## Highlights

- Serodiagnosis of canine leishmaniasis based on SLA is compromised in regions of high endemicity.
- The use of individual antigens improves serodiagnosis of canine leishmaniasis.
- Sera from clinically ill and subclinically infected dogs possess specific antibodies for KMP-11, LiP2a or LiP0 proteins.
- The best criterion of positivity is to have reactivity for at least one of the next antigens: SLA, KMP-11, LiP2a, LiP0.

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


## Graphical abstract

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## 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


## Keywords

*Leishmania*; Canine leishmaniasis; Serodiagnosis; Antibodies; Recombinant proteins

## 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

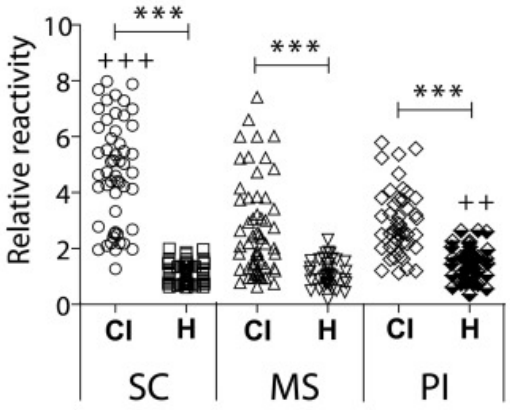
The statistical analysis was made using the GraphPad Prism software. The ELISAs cut-off values were calculated by comparison of the RR from the CanL sera (clinically ill or subclinically infected) and healthy (or *E. canis* infected dogs when indicated) using the Receiver Operating Characteristic (ROC) 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). 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).

**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 |

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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).

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.

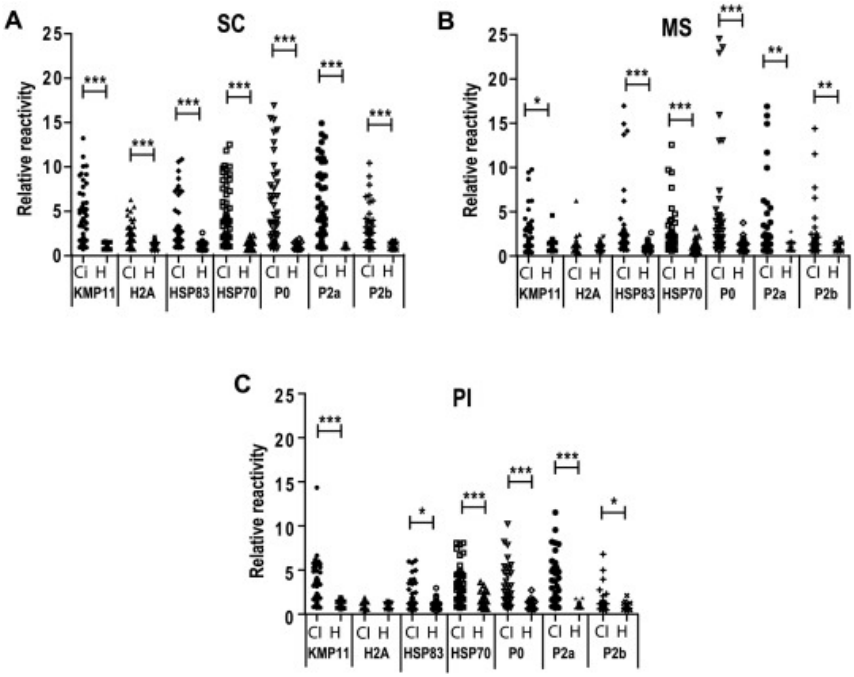


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Fig. 2. Reactivity of sera from clinically ill CanL patients against single *Leishmania* recombinant antigenic proteins. Different *Leishmania* antigens purified as recombinant proteins after the expression of their coding regions in *E. coli* were 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 (CI) 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).

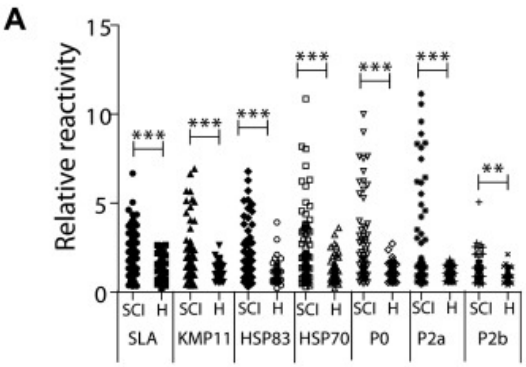
Table 1. Detailed information about diagnostic properties of the *Leishmania* antigens obtained from the ROC curve analysis.

| 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 |

### 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 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).

A




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 |

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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).

### 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).