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Efficiency of rLb6H recombinant protein from *Leishmania (Viannia) braziliensis* for the detection of canine visceral leishmaniasis

Eficiência da proteína recombinante rLb6H de *Leishmania (Viannia) braziliensis* na detecção da leishmaniose visceral canina

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ABSTRACT

Introduction: Visceral leishmaniasis (VL) is a serious endemic disease in many tropical and subtropical countries, with a strong incidence in Brazil. The disease is transmitted by the bite of infected female phlebotomine sandflies, with dogs being the main urban reservoirs of the parasite. The diverse clinical profile and the long incubation period are challenges for the diagnosis of canine visceral leishmaniasis (CVL). Recombinant proteins from *Leishmania spp*. have been studied as antigens that can increase the accuracy of serological tests. **Objective:** To evaluate the diagnostic performance of the recombinant protein rLb6H, from *Leishmania braziliensis*, in comparison to the reference antigens rK39 and rK28, from L. donovani, prioritizing the identification of subclinical infected dogs. **Material and Methods:** Serum IgG reactivity to rLb6H, rK28, and rK39 recombinant proteins was assessed in dogs with previously parasitological confirmation of CVL, subdivided according to their clinical status, using immunoenzymatic assay (ELISA). Diagnostic accuracy of each ELISA was evaluated by receiver operating characteristic (ROC) curve analysis. **Results:** While all antigens showed a better performance in detecting CVL in symptomatic dogs (SD), detection of CVL in the oligosymptomatic (OD) and asymptomatic (AD) groups was lower, but rLb6H achieved high sensitivity for asymptomatic CVL. Interestingly, the most reactive CVL samples to rK28 were barely detected by rLb6H, while the less reactive to rK28, mostly from the AD group, presented higher reactivity to rLb6H. **Conclusion:** The recombinant protein rLb6H showed utility in the detection of asymptomatic CVL, displaying a complementary reactivity to rK39 and rK28. Thus, these results suggest that rLb6H could be incorporated into multi-antigen strategies, to increase diagnostic accuracy of CVL.

Palavras-chave: Canine Leishmaniosis; Diagnosis; Recombinant Proteins; ELISA; rLb6H.

RESUMO

Introdução: A leishmaniose visceral (LV) é uma doença endêmica grave em muitos países tropicais e subtropicais, tendo forte incidência no Brasil. A doença é transmitida pela picada de flebotomíneos fêmeas infectadas, sendo os cães os principais reservatórios urbanos do parasito. O perfil clínico diversificado e o longo período de incubação são desafios para o diagnóstico da leishmaniose visceral canina (LVC). Proteínas recombinantes de *Leishmania spp*. têm sido estudadas como antígenos que podem aumentar a precisão de testes sorológicos. **Objetivo:** Avaliar o desempenho diagnóstico da proteína recombinante rLb6H, de *Leishmania braziliensis*, em comparação com os antígenos de referência rK39 e rK28, de L. donovani, priorizando a identificação de cães com infecção subclínica. **Material e Métodos:** A reatividade de anticorpos IgG séricos às proteínas recombinantes rLb6H, rK28 e rK39 foi avaliada em cães com confirmação parasitológica prévia de LVC, subdivididos de acordo com seu quadro clínico, utilizando ensaio imunoenzimático (ELISA). A precisão diagnóstica de cada ELISA foi avaliada pela análise da curva ROC (receiver operating characteristic curve). **Resultados:** Enquanto todos os antígenos mostraram um melhor desempenho na detecção de CVL em cães sintomáticos (SD), a detecção de CVL nos grupos oligossintomáticos (OD) e assintomáticos (AD) foi menor, mas rLb6H alcançou alta sensibilidade para CVL assintomática. Curiosamente, as amostras de CVL mais reativas a rK28 foram pouco detectadas por rLb6H, enquanto as menos reativas a rK28, principalmente do grupo AD, apresentaram maior reatividade a rLb6H. **Conclusão:** A proteína recombinante rLb6H mostrou utilidade na detecção de CVL assintomática, apresentando uma reatividade complementar a rK39 e rK28. Assim, estes resultados sugerem que o rLb6H pode ser incorporado em estratégias multi-antígeno para aumentar a acurácia diagnóstica da leishmaniose visceral.

Key-words: Leishmaniose Canina; Diagnóstico; Proteínas Recombinantes; ELISA; rLb6H.

INTRODUCTION

Leishmaniasis is a severe endemic disease in over 102 countries, including Brazil, and it is presented in three clinical forms: cutaneous (CL), visceral (VL), and mucocutaneous (MCL).[1] The disease is caused by the obligate intracellular protozoan *Leishmania*, which is transmitted by the bite of female infected phlebotomine sandflies *Lutzomyia longipalpis*.[2] In Brazil, the main etiologic agent of VL is *Leishmania infantum*, while the species responsible for CL and MCL are mainly *Leishmania braziliensis* and *Leishmania amazonensis*.[3] On average, 3.500 cases of VL are registered annually in Brazil, with an incidence rate of two cases per 100.000 inhabitants and a lethality of 7.1%.[4]

As a zoonotic disease, VL also involves mammals, with domestic dogs (*Canis familiaris*) considered the main rural and urban reservoir of *L. infantum*.[5] Canine visceral leishmaniasis (CVL) shows a diverse clinical spectrum, ranging from asymptomatic, with no suggestive signs of the disease, to symptomatic cases, with different pathological conditions.[6] Dogs are potential sources of infection for phlebotomine sandflies, even when not showing symptoms.[5,7] Among the measures of both human and canine VL control stand out the early diagnosis and appropriate treatment of human cases, control of insect vectors, and identification and culling of seropositive infected dogs.[5]

The gold standard for CVL definitive diagnosis is positive parasitological test. However, its sensitivity depends on parasite load, the type of immune response developed by the dog, and detection of the amastigote forms by microscopical examination of smears from tissues of different organs.[8] As an alternative, serodiagnosis have been performed, based on the characteristic hypergammaglobulinemia of CVL. Hence, infected animals are serologically detected through the demonstration of anti-*Leishmania* antibodies, mainly of IgG.[9] IgG titers have a strong correlation with clinical status, since symptomatic disease usually correlates with high levels of IgG and parasite load in different compartments when compared to asymptomatic disease.[10]

The antigen-specific enzyme-linked immunosorbent assay (ELISA) is largely used in CVL diagnosis due to its sensitivity and specificity, which are highly improved when recombinant proteins are used instead of crude antigens.[11,12] Among recombinant proteins, the most successful antigen moving from academic research to clinical settings is rK39, derived from *L. donovani* and *L. infantum*.[13] Although rK39 has good sensitivity to detect active CVL, it is less accurate for detecting asymptomatic disease.[13] rK28, a fusion protein of rK39, rK9, and rK26, derived from *L. donovani*, has a similar sensitivity and specificity as rK39, and both are routinely used for CVL diagnosis.[7,8]

Although VL in Brazil is caused by the *Leishmania infantum* species, the antigens used in the diagnosis of VL can have different origins,[15] which is explained by the fact that *Leishmania* species present genetic homology that varies from 69 to 90%. Consequently, heterologous antigens, from species other than the one which cause the disease in the region, are properly used in the diagnosis of infection and in vaccine development.[16] In this sense, recently, a recombinant version of a protein from *Leishmania (Viannia) braziliensis*, rLb6H, was generated, which showed 90% identity at the amino acid level with homologs in *L. infantum* and *L. major*, indicating the high conservation of rLb6H in *Leishmania* species.[17]

Despite all efforts, even with recombinant proteins, it is still difficult to detect asymptomatic disease or even oligosymptomatic CVL forms by using serodiagnosis, which restrains the control of VL in Brazil.[13,18] To overcome these barriers, in this present work, we assessed serum IgG reactivity to rLb6H, rK28, and rK39, in dogs with previously parasitological confirmation of CVL, subdivided according to their clinical status, using ELISA. Diagnostic accuracy of each ELISA was evaluated by receiver operating characteristic (ROC) curve analysis. By comparing the performance of this new recombinant *Leishmania* antigen, rLb6H, to the reference antigens, rK28 and rK39, we aimed to optimize CVL serological diagnosis, prioritizing the identification of subclinical infected dogs, represented by asymptomatic and oligosymptomatic groups.

MATERIAL AND METHODS

Serum samples and classification of animals

Serum samples from adult dogs of both sexes, aged between 2 and 6 years, from Belo Horizonte, Minas Gerais, an endemic area for human and canine visceral leishmaniasis, uninfected (n= 14) or naturally infected with *Leishmania infantum* (n= 35) with parasitological confirmation were obtained from a serum repository of the Laboratory of Immunopathology at the Federal University of Ouro Preto, Minas Gerais. Parasitological diagnosis in tissue smears (ear skin, spleen, liver and lymph nodes) was performed after necropsy of the animals. Tissue samples were randomly collected irrespective of the presence or absence of lesions. Imprints were performed on two microscopic slides and after air-drying, samples were fixed in methanol, stained with Giemsa, and examined under optical microscopy, for the identification of *Leishmania* amastigote forms. The dogs were classified based on serological results of the indirect immunofluorescence reaction (IFAT), using promastigote antigens of *L. amazonensis* (MHOM/BR/1960/BH6) and *L. infantum* (MHOM/BR/1972/BH46) and determination of clinical and laboratorial signs.

Animals with titers <1:40 and without clinical and laboratory signs of CVL were included in the healthy endemic control group (EC). Dogs with titers ≥ 1:40 and *L. infantum* infection confirmed by parasitological examination were included in the infected group (INF) and then classified according to the presence of clinical symptoms into three groups: 1) asymptomatic (AD) (n= 11), without clinical signs suggestive of the disease; 2) oligosymptomatic (OD) (n= 11), with a maximum of three symptoms suggestive of CVL, including dull hair and/or localized alopecia and/or moderate weight loss; and 3) symptomatic (SD) (n= 13), with characteristic clinical signs of CVL, such as dull hair, severe weight loss, onychogryphosis, skin lesions, apathy and keratoconjunctivitis. This study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Juiz de Fora (protocol number 009/2017).

Recombinant antigens

The recombinant proteins rK28 and rK39 from *L. donovani*, and rLb6H from *L. braziliensis* were obtained from the Infectious Disease Research Institute (IDRI), Seattle, WA, USA. Recombinant antigens were freeze dried and stored at -80ºC until evaluation.

rLb6H was identified by scanning a genomic expression library of *L. (V.) braziliensis* with serum from a patient with mucocutaneous leishmaniasis. Recombinant rLb6H, rK28, and rK39 antigens were produced into *Escherichia coli* BL-21 or HMS-174 (DE3) host cells for expression. Recombinant proteins purified from either the soluble fraction or supernatant by affinity chromatography using nickle-nitrilotriacetic acid (Ni-NTA) agarose columns. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified using the BCA protein assay (Pierce, Rockford, IL).

Antigen-specific enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with 500ng/ml of either rK28, rK39 or rLb6H in 100ul/well of 0.1 M NaCO3 buffer, pH 9.6, at 4ºC overnight. Wells were than washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBSt) and blocked with 2% BSA-PBSt for 1 hour at room temperature. After washes with PBSt, 100µl of serum samples (diluted 1:2.000) were added and the plates were incubated at room temperature for 1 hour. After washing with PBSt, 50µl of peroxidase-conjugated rabbit anti-dog IgG (1:5.000 dilution) (Sigma, cod. A6792, St. Louis, MO, USA) were added and incubated at room temperature for 1 hour. The plates were then washed again and a substrate solution containing o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide (H2O2) was added. After color development, the reaction was stopped by the addition of 2N H2SO4 and the color-reaction was read at 492nm with a Spectramax-190 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as absorbance, which corresponds to the mean values of the optical density (OD) of each sample.

Statical analysis

Statistical analysis of mean optical densities (ODs) from control and infected groups was performed by Mann-Whitney U test. Comparisons of the mean ODs from EC, INF, AD, OD and SD groups were performed using Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test (GraphPad Software, San Diego, CA, USA). ROC areas of the tested recombinant proteins were evaluated by the nonparametric Wilcoxon Test, using MEDCALC 14.8.1 (MedCalc Sofware, Oostende, Belgium). The values of sensitivity and specificity were extracted at the best cutoff point given by each curve. Correlation of the three antigen-specific ELISAs were tested using the Spearman nonparametric method. *P* values of <0.05 was considered statistically significant.

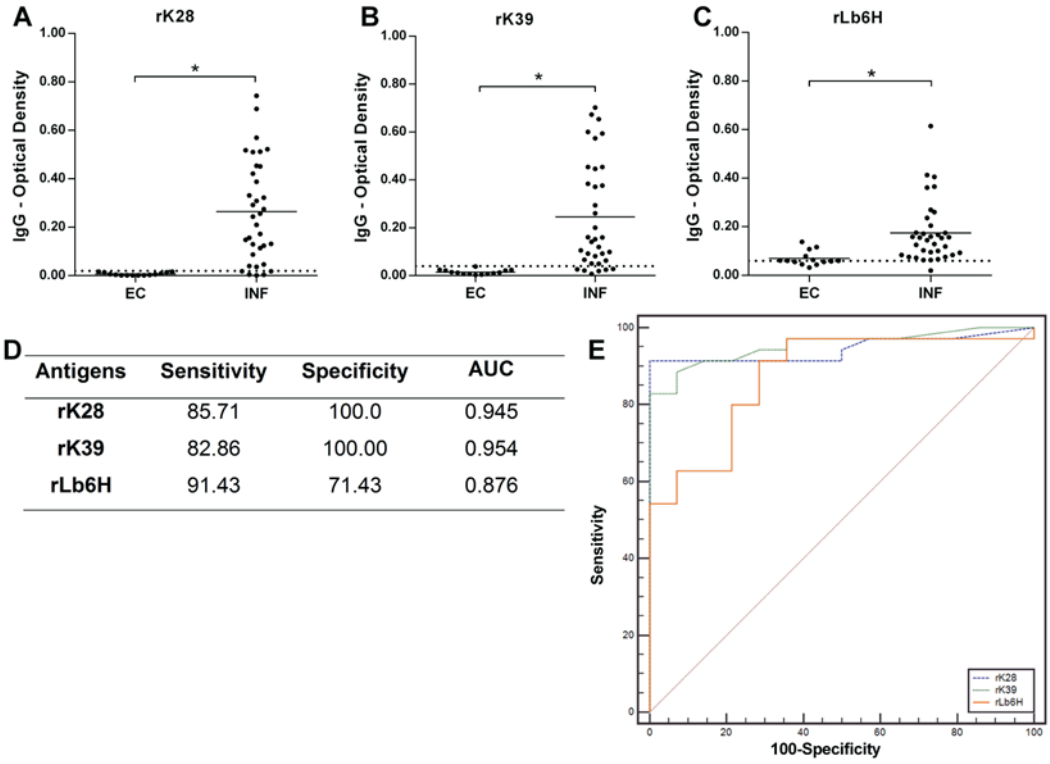
RESULTS

IgG antibody reactivity to different recombinant proteins from *Leishmania* is higher in VL infected dogs

Variable sensitivity of the serological tests continues to be a major problem for the diagnosis of visceral leishmaniasis.[9,11,17,18] To study the utility of rLb6H, a recombinant protein from *L. braziliensis*, in the diagnosis of CVL, serum IgG antibody reactivity against rLb6H was measured and compared with the reactivity against two reference antigens obtained from *L. infantum*, rK39 and rK28, using ELISA. Figure 1 (A, B and C) shows that the three antigens were able to discriminate the infected (INF) and endemic control (EC) groups. Interestingly, rLb6H sensitivity (91.43%) was higher than rK39 (82.85%) and rK28 (85.71%) sensitivities (Figure 1D and E). However, while the reference antigens presented 100% of specificity, specificity was reduced to 71.43% for rLb6H antigen (Figure 1D). Accordingly, ROC curve analysis of the antigen-specific ELISAs showed higher AUC values for both rK39 (0.954) and rK28 (0.945) than that of the rLb6H-ELISA (0.876), confirming the high diagnostic performance of the rK28 and rK39 antigens (Figure 1D and E).

Utility of the rLb6H recombinant protein in diagnosis of asymtomatic CVL

Detection of CVL in asymptomatic dogs



Antigens	Sensitivity	Specificity	AUC
rK28	85.71	100.0	0.945
rK39	82.86	100.00	0.954
rLb6H	91.43	71.43	0.876

Figure 1: Evaluation of the recombinant proteins in the serodiagnosis of canine visceral leishmaniasis (CVL). Levels of IgG antibodies against rK28 (A), rK39 (B) and rLb6H (C) in serum of dogs with confirmed CVL (CVL; n= 35) and in endemic controls (EC; n= 14) were measured by ELISA. The cutoff points (dashed bars) were established by the ROC curve. Horizontal bars represent mean optical densities. (D) The sensitivity and specificity were determined by receiver operating characteristic (ROC) curve. (E) ROC curve analysis of ELISA data was performed, comparing the areas under the curve (AUC). *= P <0.05.

represents a great challenge to the development of more efficient serological tests, in part because of reduced titers of specific antibodies, resulting in a smaller sensitivity.[13,18] The diagnostic performance of the rLb6H, rK39, and rK28 antigens was evaluated in groups of dogs with different clinical conditions: asymptomatic (AD), oligosymptomatic (OD), and symptomatic (SD). Serum IgG titers to the three antigens were higher in the infected groups, when compared with the endemic controls (EC), regardless of either the presence or the intensity of symptoms (Figure 2). However, while for the reference antigens the amount of specific IgG (determined by optical density) was higher in the SD group (Figure 2A and B), rLb6H-specific IgG levels did not differ among the different clinical forms (Figure 2C). Despite showing low specificity (71.43%), the rLb6H antigen demonstrated high sensitivity (100%) in the AD group, while the reference antigens showed a lower sensitivity (72.72%, Figure 2). Thus, our data indicate an important role of rLb6H in the detection of asymptomatic CVL.

rLb6H diverges from the reference antigens, improving diagnosis in less reactive samples

A strong linear correlation was observed in the ELISA results between rK28 and rK39 antigens (95.01%) (Figure 3A). In contrast, each of these reference antigens revealed poor correlation with rLb6H (rK28, r= 0.3586; rK39, r= 0.4324) (Figure 3B and C). To extend the analysis, we reassess IgG antibody reactivity to rLb6H using the most and the least reactive samples for the rK28 (Figure 3D, E and F). Interestingly, the least rK28-reactive samples displayed a greater reactivity for rLb6H, while the most reactive samples for rK28 presented lower reactivity to rLb6H (Figure 3D, E and F). These results demonstrate the importance of combining different *Leishmania* antigens to improve the diagnosis of CVL, such as the rK39, rK28 and rLb6H antigens, thus enabling a better detection of asymptomatic cases. Furthermore, it suggests that the rLb6H antigen may be important in the diagnosis of CVL cases that are difficult to detect with the reference antigens, and which may be related to visceralization of cutaneous or mucocutaneous forms of leishmaniasis.[19]

DISCUSSION

In the present study, serum IgG reactivity to rLb6H, rK28, and rK39 recombinant proteins was

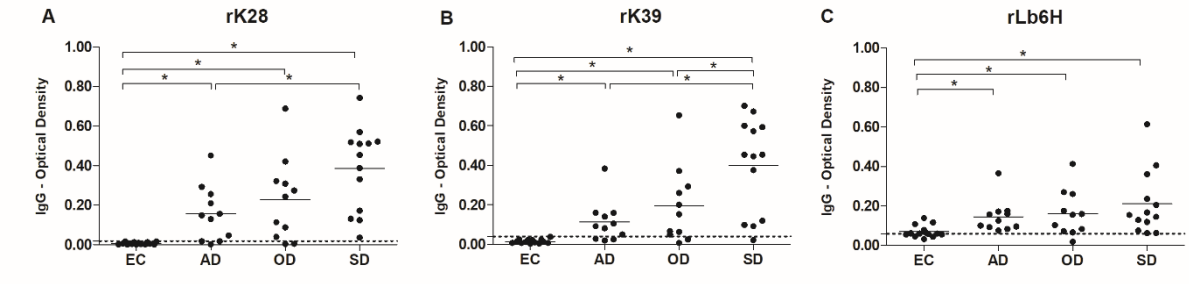


Figure 2: Antigen-specific antibody responses in dogs with different clinical forms of CLV. Levels of IgG antibodies against rK28 (A), rK39 (B), and rLb6H (C) in serum of dogs with asymptomatic (n= 11), oligosymptomatic (n= 11), and symptomatic (n= 13) CVL and in endemic controls (EC; n = 14) were measured by ELISA. The cut-off point (dashed line) was established by the ROC curve while the horizontal bars represent mean. *= p <0.05.

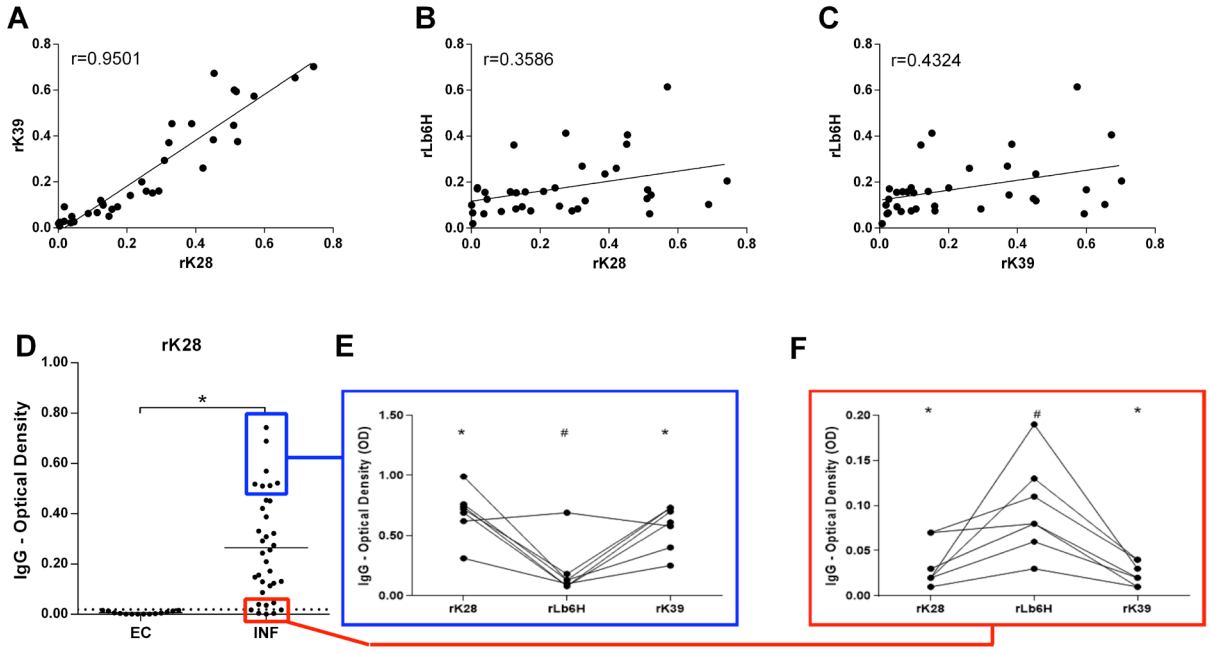


Figure 3: Antibody reactivities to rK28 and rK39 are similar but differ in comparison to rLb6H. Correlation assessment between antibody responses to (A) rK39 versus rK28, (B) rLb6H versus rK28, and (C) rLb6H versus rK39. (D) Serum IgG responses to rK28 in dogs with CVL (INF) and in endemic controls (EC). (E) Serum samples of infected dogs with high reactivity to rK28 show low reactivity to rLb6H, and (F) samples with low reactivity to rK28 showed high reactivity to rLb6H.

investigated in dogs with previously parasitological confirmation of CVL, subdivided according to their clinical status. Our results show that, regardless of the antigen used, IgG reactivity was higher in the symptomatic group (92.30% to 100%). It was also significantly increased in the asymptomatic and oligosymptomatic groups compared to the healthy controls. Interestingly, rLb6H, a protein derived from *L. braziliensis*, was the most effective in detecting asymptomatic and oligosymptomatic dogs. Therefore, our data not only corroborate studies that show high sensitivity of the reference antigens rK28 and rK39 in detecting symptomatic dogs,[13,18] but also indicate the applicability of rLb6H in serodiagnosis of asymptomatic CVL.

An early and efficient diagnosis of leishmaniasis is crucial for controlling the disease. The accuracy of diagnostic methods depends on the stage of infection, type of immune response, technique employed as well as geographical variations, and, regarding serological evaluation, the nature of the antigen is also critical.[20,21] In this regard, kinesin-related antigens have proved to be highly effective in CVL diagnosis. Two of those can be highlighted, rK39, a repeated sequence of 39 amino

acids highly preserved in *L. infantum* and *L. donovani*; and fusion antigen rK28 (K39, K26 and K9), both presenting high sensitivity and specificity rates, indeed our results show 100% specificity for these antigens, yet sensitivity values were not optimal.[7,20,22]

Several factors can impact sensitivity values; however, it is common to observe reduced sensitivity rates among asymptomatic dogs,[21] usually attributed to an initial phase of CVL, before seroconversion, in which serological methods are less sensitive.[6,18] Moreover, the agreement between serological analysis and other strategies is lower for asymptomatic dogs.[23] In fact, sensitivity of the reference antigens (rK39 and rK28) in the asymptomatic group (72.72%) was lower than in the symptomatic group. On the other hand, the antigen rLb6H, from *L. braziliensis*, presented optimal sensitivity, detecting all the tested asymptomatic dogs. Therefore, the use of the rLb6H antigen seems to be a good alternative to overcome the usual low detection of asymptomatic dogs, as it presents reactivity regardless of the clinical form.

Sato et al.[17] tested rLb6H against sera from American tegumentary leishmaniasis (ATL) patients and compared with samples from patients with VL and other infectious diseases, using ELISA. rLb6H had better performance and reacted with 100.0% of the ATL and 89.4% of the VL samples. Only a minority of samples from Chagas disease patients possessed antibodies against rLb6H, and all these responses were low. Taken together, these data support the potential of rLb6H to be used in routine serological diagnosis of both human VL and ATL, without, or with minimal, interference from other potentially confounding pathogens.[17]

The geographic distribution of *L. braziliensis,* the main species that causes cutaneous leishmaniasis in Latin America, can also impact CVL diagnosis.[18,24] Just like humans, dogs can be co-infected by *L. infantum* and *L. braziliensis*,[25] which could cause cross-reactions in serological tests in regions where there is overlapping of endemicities,[26] a condition in which fits the region of origin of the animals evaluated here.[27,28] Due to its reduced specificity in relation to its sensitivity, rLb6H does not seem to be a good diagnostic option as a single antigen. Not testing samples from dogs affected with other diseases represents a limitation of the current study, and cross reactivity cannot therefore be ruled out as an influence on the low specificity of rLb6H. Our data support further studies with this consideration.

The homology of the antigens used relates to the reactivity pattern, as highly reactive samples to rK28, display low reactivity to rLb6H, and vice versa, while rK39 presents a strong correlation with rK28 reactivity. This indicates that rLb6H can complement the standard diagnosis, by increasing detection of asymptomatic dogs. The use of combined antigens in the improvement of *Leishmania* diagnosis has already been proven to increase performance, either by creating multi-epitope proteins or making a mixed coat [29,30] or even a score strategy using multiple antigens.[31] In that sense, rLb6H seems to be a good candidate to be incorporated on any of the listed strategies.

CONCLUSION

Our results suggest that rLb6H, a recombinant protein from *Leishmania braziliensis*, has utility in the detection of asymptomatic CVL. The reactivity pattern of rLb6H was different from the standard antigens rK39 and rK28, displaying a complementary reactivity to them. These features suggest that rLb6H could be incorporated into multi-antigen strategies, to increase diagnostic accuracy of visceral leishmaniasis.

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CONFLIT OF INTEREST

The authors certify that there is no conflict of interest.

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