THE EFFICACY OF RECOMBINANT PROTEIN LBK39 FOR THE DIAGNOSIS OF LEISHMANIOSIS IN DOGS

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KEY FINDINGS

- LbK39 is an excellent antigen for diagnosing canine visceral leishmaniosis;
- The sensitivity of the method was 100%;
- The specificity was 96.1%.

SUMMARY

Visceral leishmaniosis is one of the most important zoonotic diseases on the planet and dogs are the main reservoir of canine visceral leishmaniosis (CVL) in endemic areas. They play an important role in human infection because in dogs the disease appears long time after infection, and they can move uncontrollably, contributing to disperse the parasite. To take the decision to treat the animals or for euthanasia, in an elimination program, in order to reduce the parasitic load, it is necessary to diagnose correctly, having more effective tools. Our group has developed a new recombinant antigen-based kinesin-related gene of *Leishmania braziliensis* (Lbk39), which shows 59% amino acid identity to the *L. infantum* homologue. The Lbk39 gene was synthesized, inserted into the pLEXSY-sat2 vector and transfected into *L. tarentolae* cells by electroporation. The recombinant protein was secreted in the culture with a C-terminal histidine marker, purified, generating a product at 337.68 μg / mL**.** A total of 152 sera from dog's endemic and non-endemic areas were used, being 78 positives and 75 negatives. The antigen Lbk39 showed 100% sensitivity and 96.1% specificity. We compared this antigen with other antigens such as total extract of the parasite, TRDPP, and our data indicate that Lbk39 has potential application in the diagnosis of CVL through antibody detection.

Keywords: Recombinant antigen; ELISA; visceral leishmaniosis; dogs; *Canis familiaris*

Introduction

Canine visceral leishmaniosis (CVL) is a neglected tropical disease caused by protozoa of the genus *Leishmania* Ross, 1903, which affects dogs from all continents, except Oceania (Colwell et al. 2011, Dantas-Torres et al. 2012, Otranto et al. 2019). More than 20 *Leishmania* species are known to exist, and in dogs *Leishmania infantum* is the etiological agent of CVL. Visceral leishmaniosis (VL) also affects humans in Europe, the Middle East, South Asia, Africa, and in Central and South America, where transmission may be anthroponotic, but where dogs with CVL can also act as reservoir hosts for human infection, transmitted by the bites of sand fly vectors (Chappuis et al. 2007, WHO, 2010). In the Americas, human VL is mainly if not exclusively zoonotic and found in at least 12 countries, however, 90% of the cases are reported in Brazil, causing approximately 4,200-6,300 infections per year, with a 7% mortality rate (Romero et al. 2010, Alvar et al. 2012; WHO, 2017). The increasing spread of *L. infantum* into urban areas in the Americas is of great concern, and is associated with migration, population growth and poor living conditions (Werneck et al. 2008, Romero et al. 2010, Harhay et al. 2011). CVL is a multisystemic disease with clinical signs that may be apparent, such as dermatitis, lymphadenomegaly, general muscular atrophy, and renal disease (Baneth et al. 2008). However, control programmes for both the disease and infection are essential, because many dogs remain asymptomatic, which makes it difficult to diagnose infection and control transmission to humans (Gavgani et al. 2002; WHO, 2010). Current methods to prevent the spread of CVL include the use of topical insecticides on dogs and canine vaccination, which provides only a certain level of protection for dogs (Solano-Gallego et al. 2009; Sousa-Paula et al. 2019). In addition, many countries have also used euthanasia of seropositive dogs (Dantas-Torres et al. 2012, BRASIL, 2014). However, scientific studies have not yet shown that this euthanasia strategy decreases the infection rate of VL (Romero et al. 2010, Sosa-Paula et al. 2019; Dantas-Torres et al. 2019). Moreover, the method is not well-received by the population for ethical reasons (Costa et al. 2011, Pereira-da-Silva et al. 2017). The treatment of CVL-positive dogs is permitted in some countries, however, euthanasia may still be the only option for owners who cannot afford to have their dogs treated. Therefore, the development of more precise diagnostic methods for both rapid treatment of humans and control of CVL in dogs is urgently required (Foglia Manzillo 2013, Fonseca et al. 2014, Coelho et al. 2016).

For accurate diagnosis of CVL several approaches are available. The parasitological method of biopsy followed by detecting the amastigote forms of *L. infantum* by microscopy is still considered the gold standard (BRASIL, 2014). However, it is an invasive method and there are complications with its use due

to the need for a trained professional capable of recognising the parasites. Culture methods to supplement microscopy give improved sensitivity but are also highly susceptible to contamination (Boelaert et al. 2007, Goto et al. 2010, de Vries et al. 2015). Polymerase chain reaction (PCR) methods to detect *Leishmania* DNA show variable sensitivity, even though it is a species-specific method (Gomes et al. 2017, Rampazzo et al. 2017). Various serological methods can be used including enzyme-linked immunosorbent assay (ELISA), indirect fluorescence antibody test (IFAT), direct agglutination test, Western blot and immunochromatographic tests to detect specific antibodies present in serum samples; however, these can present problems in sensitivity and specificity (Georgiadou et al. 2015, Magalhães et al. 2017). Nonetheless, such serological techniques can be highly efficient in the detection of VL infection in humans and dogs. To standardise and optimise their use many recombinant proteins have been studied in recent years with the aim of increasing the sensitivity and specificity of such tests (Wolf et al. 2014, Celeste et al. 2004, Mniouil et al. 2018).

In Brazil the Ministry of Health has determined that the lateral flow strip test Dual-Path Platform assay (TR-DPP® LVC) produced by Bio-Manguinhos/Fiocruz, Brazil (BRASIL 2014) should be used for serological screening of dogs, followed by an ELISA test (EIE-LVC) for confirmation of *L. infantum* infection (Grimaldi et al. 2012, Faria et al. 2015). Some purified recombinant antigens, such as the rK39 antigen, of various *Leishmania* species have been produced and used in serological assays of VL (Badaró et al. 1996). Even though the TR-DPP® test uses a combination of recombinant proteins (rK26 and rK39) which should improve sensitivity (Bhatia et al. 1999; Pattabhi et al. 2010), it can perform with low sensitivity, which compromises the efficacy of the diagnosis of infection in Brazil (Grimaldi et al. 2012, De Santis et al. 2013). Further, the crude parasite extract used in the EIE-LVC test has also shown false positive results due to cross-reactivity with other pathogens, particularly *Trypanosoma cruzi*, in North, Central and South America (Ferreira et al. 2007, Solano-Gallego et al. 2009). Cross-reactions are less likely to occur when using recombinant proteins in ELISA, such as rA2, rK9, rK26 and rK39 (Solano-Gallego et al. 2009). Considering the need for new antigens for improved serodiagnosis of CVL that show both high specificity and sensibility, and no cross-reaction with other diseases, we undertook this study, in which we investigated the use of the recently described Lbk39 recombinant protein (Souza et al. 2019) as an antigen in CVL diagnosis by the ELISA method.

Materials and Methods

Production of Lbk39 recombinant protein

Lbk39 recombinant protein was produced essentially as previously described (Souza et al. 2019). Briefly, the Lbk39 fragment of a kinesin-related gene of *L. braziliensis* is composed of 843 base pairs and encodes repetitive immunological amino acids. The protein is related to the kinesin-related gene of *L. infantum* (Burns et al. 1993), which has 6.5 repetitions of 39 amino acids. The synthetic gene Lbk39 was cloned with a 6xHis-tag in the recombinant vector pLEXSY-sat2 and inserted into the eukaryotic host *L. tarentolae* (Souza et al. 2019).

An aliquot of transfected *L. tarentolae* was thawed and placed in 100 mL of culture medium. As soon as the cultures became slightly turbid after 24 h, the specific Streptothricin-class of aminoglycoside antibiotic Nourseothricin (LEXSY NTC, Jena Bioscience) for the pLEXSY-sat2 vector was added, and the culture maintained under the same conditions by subpassage every four days. After that the pre-inoculum was inoculated in BHI medium (2 L) supplemented with 2 mL of porcine hemin (Jena Bisocience), 5 mL of penicillin and streptomycin (Pen-Strep, Jena Bioscience) and 1 mL of NTC antibiotic (Jena Bioscience) and incubated at 26°C in the dark under aerated conditions. The culture was then centrifuged at 5000 g, 4°C, for 10 min, the supernatant medium removed and stored frozen at -80°C.

Lbk39 recombinant protein (250 mL) was purified from the supernatant medium using HisTrap HP column chromatography (GE HealthCare) by loading the thawed culture medium supernatant into the column and eluting the purified protein, according to the manufacturer's instructions. To remove salts and imidazole present in the purified protein, dialysis in PBS buffer 7.2 was performed at 4°C, once overnight, and twice for 2 h. The purified product (25 mL) was lyophilised to concentrate the Lbk39 recombinant protein. To determine the concentration of Lbk39 recombinant protein, the Micro BCA™ Protein Assay Kit (Thermo Fischer Scientific) was used, following the manufacturer's protocol. To verify that the protein was indeed purified, 15% SDS-PAGE SDS-polyacrylamide gel electrophoresis was performed on recombinant Lbk39 produced after the protein purification process, dialysis and lyophilisation (for details see De Souza et al. 2019). The gel was stained with nitrate silver (Fig. 1).

Collection and sample processing

For this study, 152 canine serum samples were used to evaluate the diagnostic potential of Lbk39 in the main part of the study. Of these, 73 sera were from healthy animals from a non-endemic area of Brazil

(Araucária, Parana), which can be assumed to be almost exclusively negative for CVL, with possible exception of rare imported cases. For the positive sera, we used dog samples from the region of the outbreak Foz do Iguaçu (for more details, see Thomaz Soccol et al. 2017). To assess the clinical diagnosis of CLV, clinical signals were recorded in an epidemiological questionnaire. Owned dogs were examined by veterinarians for clinical signs of the disease and each dog was given an individual data file including identification, traits, behaviour, migration history and health issues. Signals investigated were weight loss, adenomegaly, alopecia, skin lesions, mucosal lesions, hipkeratosis, and muscle atrophy. Blood samples were collected by venipuncture of the jugular vein, transferred to 10 mL polypropylene tubes and processed 3-4 hours after collection. In the laboratory, the blood was centrifuged at 1000 xg for 5-10 min and the sera were separated and stored at -20°C until analysed by serological methods. The TR-DPP (Bio-Manguinhos, Fiocruz, Brazil) as recommendation from the Brazilian Ministry of Health and used according to manufacturer's instructions. The indirect ELISA using total extract of *L. infantum* was performed as described by Maziero et al. (2014). Each sample was tested in triplicate intra- and inter-plate. Three positive and negative dog sera were included in each plate as controls, when testing individual sera.

Dogs that tested seropositive were examined by puncture of popliteal lymph nodes. The collected material was used for culture in NNN media and polymerase chain reaction (PCR) specific for *Leishmania*. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit in accordance with the manufacturer's recommendations. Cytochrome B1/B2 was used as an internal control to verify DNA amplification (Oshaghi et al. 2006). The PCR was performed using Internal Transcribed Spacer (ITS) primers (Schonian et al. 2003). The positive PCR products were sequenced. The sequencing was performed commercially by Macrogen Inc. (Seoul, South Korea). The 79 positives sera were from dogs in an area highly endemic for CVL (Foz do Iguaçu), and included 52 sera from clinical cases for CVL and 27 sera from no clinical dogs. In addition to these sera, to evaluate potential cross-reaction, 23 sera were used from dogs positive for *Toxoplasma gondii* infection.

Ethics Statement

For collection of dog's samples all procedures were carried out in strict compliance with the rules defined by the National Council for the Control of Animal Experiments (CONCEA), and every effort was made to minimize suffering. The work was approved by the Ethics Committee of the Federal University of Paraná, under protocol no. 044/2014. The owners have signed a consent form for the use of the samples.

Lbk39 antigen - Enzyme-linked immunosorbent assay (ELISA) standardization

The ELISA test was developed and standardised by analysing results using antigens at different concentrations (0.031, 0.062, 0.125, 0.250, 0.500 and 1,000 µg/well), two serum dilutions (1:100 and 1:200) and various dilutions of HRP-conjugated secondary antibodies (1:3500, 1:5000, 1:7500 and 1:10000). The Lbk39 antigen was diluted in carbonate-bicarbonate buffer, pH 9.6, and polystyrene microtiter plates (96-well EIA/RIA 12x8 well plates, Costar, USA) were coated with 100 µL/well of dilutions at all of the concentrations of antigens given above, and incubated overnight at 4°C. The next day, the plates were washed with 200 µL/well of washing solution (0.9% NaCl + 0.05% Tween 20), twice. Next, the wells were blocked with 120 µL of blocking solution composed of PBS + 0.1% casein at 37°C, for 1 h, and were then washed again, twice, with 200 µL/well of washing solution. After these steps, the serum samples were added and incubated at 37°C, for 1 h. The plates were then washed with 200 µL/well of washing solution four times. 100 µL of various dilutions of a polyclonal goat anti-dog IgG HRP (2 mg/mL) conjugate was plated at the concentrations given above at 37°C, for 1 h. Lastly, the reaction was developed by adding 100 µL/well of a solution prepared by combining 10.0 mL of citrate buffer (4.5% Na_2PO_4 + 3.25% citric acid + distilled water, pH 5.0), 2 mg of o-phenylenediamine dihydrochloride (2 mg/tablet, Sigma, USA) and 2 µL of 30% H_2O_2, and incubating at room temperature for 30 min, avoiding light. The reaction was stopped by adding 20 µL/well of 1:20 H_2SO_4 solution. The plates were read in a PowerWave HT reader (BioTek) at 492 nm and the values were expressed in absorbance. The cut-off was calculated from the mean value of the negative controls.

Tests used for comparison with the Lbk 39 antigen

To confirm the CVL status of dogs within the study the following three standard tests were used: parasitological diagnosis by culture of aspirates in Novy, Neal and Nicolle medium; serological diagnosis using the TR-DPP test; and serological diagnosis using an ELISA test using total crude extract of *L. infantum* promastigotes (equivalent to the EIE-LVC test). These results were compared with those obtained using ELISA with Lbk39 recombinant protein.

Parasitological diagnosis by culture was done as recommended by Szargiki et al. (2009). In brief, bone marrow aspirate, lymph node aspirate or the leukocyte layer of dogs were inoculated in Neal, Novy and Nicole (NNN) culture medium with 0.9% saline solution with weekly sub-passage, for four weeks at 24ºC.

The cultures were inspected every week for the presence of promastigotes. Positive cultures were identified as described by Thomaz Soccol et al. (2017) and all found to be *L. infantum.*

Statistical analysis

The receiver operating characteristics (ROC) curve, sensitivity, specificity analysis and comparison between tests were analysed by MedCalc, version 18.9 (R Core Team, 2018), Graphpad prism 5.0 was used to plot the graphs and a significance level of $p<0.05$ was adopted.

Results

Antigen production

A pre-inoculum culture was produced in 100 mL of medium, followed by the inoculation into one litre of supplemented BHI medium for antigen production. After four days the culture was centrifuged and separated into pellet and supernatant medium. The total protein concentration in the supernatant medium was 8,286.52 μg/mL. The supernatant medium was processed in 250 mL aliquots, and each was used for protein purification using chromatography by loading the culture medium on to a 1 mL HisTrap HP column (GE HealthCare), according to the manufacturer's instructions. After purification by elution from the column, dialysis was performed to remove salts and imidazole from the eluate. Finally, the product was lyophilised and the Lkb39 protein was resuspended in ultrapure water and concentration was determined to be 337.68 μg/mL measured by the Micro BCA™ method.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA test was evaluated for its ability to determine the level of anti-*Leishmania* antibodies in dog sera from *L. infantum*-infected and non-infected animals using as antigen the recombinant protein Lbk39. The ELISA was optimised by performing tests at six antigen concentrations and was able to differentiate sera positive for CVL from negative sera. The concentration chosen as standard was 125 μg protein per well, since this produced the best response for the most reasonable cost. In the assays performed, we observed a 34-fold increase in the absorbance values of positive controls compared with negative controls.

The cut-off value was calculated using ROC curve analysis. For serum dilution optimisation, no significant differences were observed amongst the dilutions tested, thus the 200-fold dilution was chosen because it uses a small quantity of serum and the background was lower. The dilution of the goat anti-dog IgG HRP conjugate that gave the best results was 1: 7500, so this was selected for use as standard. To determine the cut-off level of the reaction of positive and negative sera, the mean and standard deviation absorbances of the canine samples negative for CVL (73 sera samples) were determined. The absorbance cut-off was set at 0.156.

After standardisation, we used the optimised ELISA with Lbk39 antigen to analyse all 152 canine sera in the study. From this analysis, of the 79 dog sera from the CVL endemic region of Foz do Iguaçu, 76 were positive and 3 were negative in the Lbk39 ELISA assay. Of these 3 negative animals, two were in the group with no clinical signs, one of these was negative in all other assays used and one was positive with the TR-DPP assay. The negative result from the dog with clinical signs was positive with the crude antigen ELISA and TR-DPP assays. Therefore, we conclude that two of these negative results from Foz do Iguaçu were false negatives and one was a true negative. All the dog sera from Araucária showed negative results with the Lbk39 assay. The ROC curve analysis of absorbance distribution against the Lbk39 antigen for various groups studied (all positives from any assay, dogs with clinical signs, dogs without clinical signs) is shown in Figure 2. The difference in canine samples was significant between these groups ($P< 0.001$) and accurate. The positive group (78 samples) presented good accuracy (AUC, 0.998), similar to the no clinical signs group (AUC, 0.998), while the animals with clinical signs presented higher accuracy (AUC, 0.999).

Culture is used as a standard test for CVL diagnosis, because although it may sometimes lack sensitivity, it has very high specificity, as when it is positive it definitively means that the dog has the parasite. Therefore, we evaluated the subset of 56 dogs that presented with a positive culture. Table 2 shows the results for sensitivity and specificity of the Lbk39 assay with these sera, also subdivided into those with clinical signs or no clinical signs. As shown, the Lbk39 assay performed with very high sensitivity and specific against this subset of dog sera. ROC curve analysis of the absorbance distribution for the Lbk39 ELISA against the positive culture subset confirmed that the test worked very well ($P< 0.001$) and was accurate (Fig. 3). The total positives group presented the same accuracy (AUC, 0.999) as the clinical signs subgroup (AUC, 0.999), while the no clinical signs subgroup presented with slightly lower accuracy (AUC, 0.997).

Analysis of the distribution of ELISA readings from the Lbk39 assay in various subgroups of dog sera is shown in Figure 4. Comparisons are shown between negative controls, positives, those positives with clinical signs and those positives with no clinical signs, either for all sera (Fig. 4a) or for the subset of Lbk39 ELISA positives with positive culture (Fig. 4b). All groups and subgroups of positives were significantly different to the negative controls. When the analysis was confined to sera from dogs that also had a positive culture, as expected the discrimination was greater (Fig. 4b).

Comparison of Lbk39 with other diagnostic tests

In addition to evaluating the performance of the Lbk39 ELISA against clinical and parasitological diagnostic tests, we also we compared our standardised assay with other commonly used serological tests, ELISA with a crude antigen preparation and the TR-DPP assay. For these comparisons we considered a true positive to be any animal that was positive in any of the assays used. This comparison showed that the Lbk39 assay outperformed all the other tests in terms of sensitivity (Table 3). The Lbk39 assay also performed well in terms of specificity (96.1%), with either TR-DPP being slightly better (97.2%). As mentioned previously, culture is highly specific, but this has a relative poor sensitivity (77%). Operationally, dogs with CVL are first identified by clinical signs and then the TR-DPP test is applied, sometimes this being the only testing performed. Therefore, we divided the positive group into cases with clinical signs and without clinical signs and evaluated the sensitivity and specificity of Lbk39 comparing with the TR-DPP assay (Table 4). When the individual groups were analysed, this showed that the group of sera from dogs with clinical signs were detected with similar and high sensitivity and specificity for both tests, the TR-DPP being slightly more specific. However, in dogs without clinical signs the Lbk39 ELISA was significantly more sensitive, and slightly less specific than TR-DPP.

Cross-reaction with Lbk39

To check for potential cross-reactivity of the Lbk39 antigen with another intracellular parasite, we performed ELISA with Lbk39 against sera from toxoplasmosis-positive canine samples. No cross-reaction was observed (Fig.5).

DISCUSSION

Leishmania braziliensis is endemic in the study area and, *L. infantum* has been recognized since 2012 (Dias et al., 2013; Thomaz Soccol et al., 2017), therefore to work with specific species of CVL dogs

were subjected to clinical evaluation and identification of the parasite present in the popliteal ganglion. In endemic areas of *L. braziliensis*, diseased dogs generally have typical well-limited ulcerated lesions with raised edges. The main locations of ulcers are in the scrotal sac and on top of the ears (Dantas-Torres et al. 2010). Dogs with CVL generally exhibit lymphadenomegaly, exfoliative dermatitis and weight loss as the most relevant signals on which veterinarians have based their suspicion of infection (Gálvez et al. 2011). In the present study, no animals showed specific signs of CL. In addition, all animals that tested serologically positive on initial testing the parasite isolated was identified as *L. infantum*.

Accurate diagnosis of CVL is important in efforts to interrupt the life cycle of the parasite and prevent the spread of the disease in endemic areas (Fraga et al. 2014; Singh et al. 2015). The recombinant protein Lbk39 previously described by our group (de Souza et al. 2019) has been validated here for use in the diagnosis of CVL. Antigen production was not performed in *E. coli*, but in *L. tarentolae*. As the desired recombinant antigen is derived from a *Leishmania* species, the use of this system maximizes the probability of successful expression. The antigenic protein produced by heterologous expression of specific *Leishmania* epitopes in a prokaryotic system such as *E. coli* is inexpensive to culture and allows rapid processing of the target recombinant protein. However, this system has drawbacks, e.g. it requires post-translational eukaryotic activity, may have high levels of unfolded proteins, resulting in reduced efficacy. In addition, the ideal culture temperature for *E. coli* may reduce recombinant protein yield and increase protein degradation (Khow and Suntrarachun. 2012). *Leishmania tarentolae*, which is a non-mammalian pathogenic species, has been explored as a general eukaryotic host with the aim of developing a platform within it that allows high levels of expression of complex eukaryotic proteins and has the ability to produce proteins with appropriate post-translational processing (Basile and Peticca, 2009). This type of host is also easy to manipulate and has an inexpensive culture medium with an average doubling time of 6-8 hours. The recombinant protein, produced by our group, has been tested for LC and VLH with excellent results (de Souza et al. 2019).

Here, we used this protein for CVL and an ELISA test format was chosen because it is a preferred choice for serodiagnosis screening of the disease in the laboratory, being generally more reliable and sensitive than other laboratory tests (Srividya et al. 2012; Abass et al. 2015). The Lbk39 antigen showed higher sensitivity (100%) when compared with TR-DPP (93.8%), ELISA using as antigen the parasite total extract (98.1%), or parasite culture (77%). The specificity was similar to that of TR-DPP (96.1 and 97.2%, respectively), and better than crude parasite extract (86.9%). These findings showed that Lbk39 has the

highest probability of a true positive result in the presence of the disease, and it has the lowest probability of a false negative result in the absence of the disease compared with other tests. To increase specificity and minimise the possibility of a false positive, we used a cut-off value based on the standard deviation of the results of negative controls from healthy dogs, and as a consequence the Lbk39 assay did not produce many false negative results. However, when we evaluated only the canine samples from dogs with a positive culture with or without clinical signs, we observed that our protein showed 100% sensitivity in both groups, and a small variation in specificity between the groups with clinical signs (98.7%) and without clinical signs (96.0%). While evaluating TR-DPP, we observed that the group with clinical signs showed higher sensitivity (98%) than the group without (88%). As for the specificity, in positive group, dogs with either no clinical signs or clinical signs for TR-DPP presented 100%, whereas our protein presented specificity from 96.1 to 98.7%.

In the last 10 years several CVL diagnostic methods have been used. Their specificity and sensitivity can vary. For example the specificity of molecular tests varies from 95 to 100%, where conventional PCR (cPCR) has a sensitivity of 89% to 100% in polysymptomatic dogs (Lachaud et al. 2002; Manna et al. 2004; Carson et al. 2010). However, sensitivity decreases when the method is applied to asymptomatic dogs (Francino et al. 2006; Gao et al. 2015). Currently, quantitative PCR (qPCR) is considered the most reliable test because it has a sensitivity of 91% compared to cPCR (72%), ITS-1 PCR (54%), and PCR hybridization (61%) (Carson et al. 2010). The molecular test with loop-mediated amplification (LAMP) is capable to amplify DNA in constant temperature in blood of VL patients. It has a sensitivities and specificities of 90.7 to 96.4% and 98.5 to 100%, respectively. In China, the LAMP identified 61%-infected dogs, however, not amplify strains from other countries (Gao et al. 2015). The direct agglutination test (DAT) present sensitivities of 91 to 100% and specificities of 72 to 100% but yet subjective reading of end-point titles leads to interrupt server discrepancy (Adams et al. 2012; Oliveira et al. 2016). Although some serological tests for canine leishmanosis have high specificity and sensitivity, the presence of cross-reactivity remains controversial. The immunofluorescence antibody test is reference qualitative in CVL diagnosis (Paltrinieri et al. 2016), with sensitivity and specificity close to 100%, in symptomatic animals, though show some limitations as, cross-reactivity with trypanosomes pathogens (Solano-Gallego et al. 2014; Paltrinieri et al. 2016) and the significantly lower sensitivity in identifying asymptomatic dogs compared with ELISA (Mettler et al. 2005).

348 The main sera used to verify cross-reactivity against the antigen of choice would be *T. cruzi*,
349 *Babesia canis*, *Dirofilaria immitis* and *T. gondii*, however, in the present study, it was possible to test only
350 the cross-reactivity against *T. gondii* serum available for use. In previous studies, cross reactivity between
351 *Leishmania* species and *T. gondii* were tested by ELISA and IFAT (Boelaert et al. 2007; Ferreira et al.
352 2007) and showed no cross-reactivity. However, other studies showed that half of dogs (5/10; 50%) with
353 anti-*T. gondii* antibodies were erroneously considered serologically positive for visceral leishmaniasis
354 (Távora et al. 2007). In evaluating the validity of diagnostic methods, such problems need to be taken fully
355 into account in order to obtain reliable and accurate results. The use of Lbk39 against dog sera from animals
356 positive for *T. gondii* no showed no cross-reactivity, such that the results of these samples were very similar
357 to negative CVL samples.
358 Our data corroborate the work of Grimaldi et al. (2012), who showed that TR-DPP has good
359 detection rates in symptomatic dogs (98%) when screening is performed in endemic areas, but detection is
360 lower when performed on asymptomatic dogs (47%). In the same way, Laurenti et al. (2014) showed that
361 TR-DPP is better at detecting symptomatic dogs (92.1%) than asymptomatic dogs (89.4%). The Lbk39
362 antigen studied here presented excellent accuracy (0.998), showing a potential for use in diagnostic tests.
363 The literature shows that the best antigens are composed of repetitive motifs, which are present in
364 various organisms and are characterised by the presence of two or more copies of amino acid sequences.
365 This is because these sequences stimulate B cells by binding to these repetitive antigens, independent of T
366 lymphocyte stimulation (Rosati et al. 2003; Goto et al. 2008, 2010; Valiente-Gabioud et al. 2011). Thus,
367 proteins that exhibit these characteristics are strong candidates for use as diagnostic and vaccine targets.
368 Our results corroborate these previously published data in which repetitive proteins showed higher
369 sensitivity than non-repetitive proteins with canine samples. An important difference in sensitivity has also
370 been shown between human and dog sera (Vos et al. 2000; Laurenti et al. 2014). This may be related to
371 host-parasite reactions in relation to the recognition and presentation of the antigens studied to the immune
372 system or to the different survival mechanisms of the parasite in different hosts (Laurenti et al. 2014). In
373 fact, the test results may vary depending on the stage of the disease during which serum was collected
374 (Mettler et al. 2005). This indicates that dogs with clinical signs show a better response to the serological
375 tests, as also observed here. However, the recombinant protein evaluated here showed a CI min of 88.9 and
376 a CI max of 99.2% for specificity, when the dogs showed no clinical signs and positive culture. When
377 testing all the positive samples, the recombinant protein Lbk39 presented a sensitivity of 100% and a

specificity of 96.1%, which is effective for diagnosing *L. infantum* in infected dogs. In addition, the protein was produced on a scale of two litres and purified without affecting its functionality. Therefore, this protein is a good candidate for use as a diagnostic target.

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REFERENCES

Abass, E, Kang, C, Martinkovic, F, Semiao-Santos, SJ, Sundar, S, Walden, P, Piarroux, R, Harith, A, Lohoff, M, and Ulrich Steinhoff, U (2015) Heterogeneity of *Leishmania donovani* parasites complicates diagnosis of visceral leishmaniasis: comparison of different serological tests in three endemic regions. *PLoS One* **10**, e0116408. doi: 10.1371/journal.pone.0116408.

Adams, ER, Jacquet, D, Schoone, G, Gidwani, K, Boelaert, M and Cunningham, J (2012). Leishmaniasis Direct Agglutination Test: Using Pictorials as Training Materials to Reduce Inter-Reader Variability and Improve Accuracy. *PLoS Neglected Tropical Diseases* **6**, 1–6. doi: 10.1371/journal.pntd.0001946.

Alvar, J, Vélez, ID, Bern, C, Herrero, M, Desjeux, P, Cano, J, Jannin, J, and den Boer, M (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. 2012; **7**, e3567. doi: 10.1371/journal.pone.0035671.

Alves, WA, and Bevilacqua, PD (2004) Quality of diagnosis of canine visceral leishmaniasis in epidemiological surveys: an epidemic in Belo Horizonte, Minas Gerais, Brazil, 1993–1997. *Caderno de Saúde Publica* **20**, 259–265. doi:10.1590/S0102-311X2004000100043.

Badaró R, Benson D, Eulálio MC, Freire M, Cunha S, Netto EM, Pedral SD, Madureira C, Burns JM, Houghton RL, David JR and Reed, SG (1996). rK39: A Cloned Antigen of *Leishmania Chagasi* That Predicts Active Visceral Leishmaniasis. The Journal of Infectious Disease 173, 758-761. doi: 10.1093/infdis/173.3.758.

Basile, G and Peticca, M (2009) Recombinant protein expression in *Leishmania tarentolae*. *Molecular Biotechnology* **43**, 273–278. doi: 10.1007/s12033-009-9213-5.

Baneth, G, Koutinas, A, Solano-Gallego, L, Bourdeau, P, and Ferrer, L (2008) Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part one. *Trends in Parasitology* **24**, 324–330. doi: 10.1016/j.pt.2008.04.001.

Bhatia A, Daiffalla NS, Jen S, Badaró R, Reed SG and Skeiky, YAW (1999). Cloning, characterization and serological evaluation of K39 and K26: two related hydrophilic antigens of *Leishmania chagasi*. Molecular and Biochemical Parasitology **102**, 249-261. doi: 10.1016/S0166-6851(99)00098-5

Boelaert, M, Bhattacharya, S, Chappuis, F, El Safi, SH, Hailu, A, and Mondal, D (2007) Evaluation of rapid diagnostic tests: visceral leishmaniasis. *Nature Reviews Microbiology* **5**, S30–S39. doi: 10.1038/nrmicro1766.

Brasil, Ministério da Saúde. Secretaria de Vigilância em Saúde. Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília, D.M.d.S. (2014) Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília, DF: Ministério da Saúde, 2014, Secretaria de Vigilância em Saúde, 2014, p. 121.

Burns, JM Jr, Shreffler, WG, Benson, DR, Ghalib, HW, Badaro, R, and Reed SG (1993). Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci U S A.* **90,** 775-779. doi:10.1073/pnas.90.2.775.

Carson, C, Quinnell, RJ, Holden, J, Garcez, LM, Deborggraeve, S and Courtenay, O (2010). Comparison of *Leishmania* OligoC-TesT PCR with conventional and real-time PCR for diagnosis of canine *Leishmania* infection. *Journal of Clinical Microbiology* **48**, 3325–3330. doi: 10.1128/JCM.02331-09.

Celeste, BJ, Angel, SO, Castro, LG, Gidlund, M and Goto H (2004) *Leishmania infantum* heat shock protein 83 for the serodiagnosis of tegumentary leishmaniasis. *Braz J Med Biol Res* **37**, 1591– 1593. doi: oi.org/10.1590/S0100-879X2004001100001.

Chappuis, F, Sundar S, Hailu, A, Ghalib, H, Rijal, S, Peeling, RW, Alvar, J, and Boelaert, M (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology* **5**, 873–882. PMID:17938629.

Coelho, EAF, Costa, LE, Lage, DP, Martins, VT, Garde, E, and Jesus-Pereira, NC (2016) Evaluation of two recombinant *Leishmania* proteins identified by an immunoproteomic approach as tools for the serodiagnosis of canine visceral and human tegumentary leishmaniasis. *Veterinary Parasitology* **215**, 63–71. doi: 10.1016/j.vetpar.2015.11.006.

Colwell, DD, Dantas-Torres, F, and Otranto, D (2011) Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. *Veterinary Parasitology* **182**, 14–21. doi: 10.1016/j.vetpar.2011.07.012.

Costa, CH (2011) How effective is dog culling in controlling zoonotic visceral leishmaniasis? A critical evaluation of the science, politics and ethics behind this public health policy. *Revista da Sociedade Brasileira de Medicina Tropical* **44**, 232–242. doi: 10.1590/S0037-86822011005000014.

Cota, GF, Sousa, MR, Demarqui, FN and Rabello, A (2012) The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. *PLoS Neglect Tropical Disease* **6**, e1665. doi: 10.1371/journal.pntd.0001665.

Dantas-Torres, F, de Paiva-Cavalcanti, M, Figueredo, LA, Melo, MF, da Silva, FJ, da Silva, AL, Almeida, EL and Brandão-Filho, SP (2010). Cutaneous and visceral leishmaniosis in dogs from a rural community in northeastern Brazil. *Veterinary Parasitology* **170**, 313–317. doi: 10.1016/j.vetpar.2010.02.019.

Dantas-Torres, F, Solano-Gallego, L, Baneth, G, Ribeiro, VM, Paiva-Cavalcanti, M, and Otranto, D (2012) Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. *Trends Parasitology* **28**, 531–538. doi:10.1016/j.pt.2012.08.007.

Dantas-Torres F, Miro´ G, Bowman DD, Gradoni L, Otranto D (2019) Culling dogs for zoonotic visceral leishmaniasis control: the wind of change. *Trends in Parasitology* 35(2):97–101. doi:10.1016/j.pt.2018.11.005

De Souza, LMB, Carvalho, J, Bates, MD, Petterle, RR, Thomaz-Soccol, V, and Bates, PA (2019). Production of a kinesin-related recombinant protein (Lbk39) from Leishmania braziliensis by Leishmania tarentolae promastigotes and its application in the serodiagnosis of leishmaniasis. *One Health* **8**, 100111. doi:10.1016/j.onehlt.2019.100111.

De Santis B, Santos EG, Souza CS, and Chaves AS (2013). Performance of DPPTM immunochromathographic rapid test (IRT) for canine visceral leishmaniasis: comparison with other serological methods in suspected dogs from Cuiabá, Mato Grosso State, Brazil. *Braz J Vet Res Anim Sci*. **50**, 198-205. doi:10.1017/CBO9781107415324.004.

De Vries, HJ, Reedijk, SH, and Schallig, HD (2015) Cutaneous leishmaniasis: recent developments in diagnosis and management. *American Journal of Clinical Dermatology* **16**(2), 99–109. doi: 10.1007/s40257-015-0114-z.

Dias, DS, Martins, VT, Ribeiro, PAF, Ramos, FF, Lage, DP, Tavares, GSV, Mendonça, DVC, Chávez-Fumagalli. MA, Oliveira, JS, Silva, ES, Gomes, DA, Rodrigues, MA, Duarte, MC, Galdino, AS, Menezes-Souza, D, and Coelho, EAF (2017) Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis. *Parasitology* **8**, 1–12. doi: 10.1017/S0031182017001731.

Dias, RCF, Thomaz-Soccol, V, Bisetto, JrA, Pozzolo, EM, Chiyo, L, Freire, RL (2013) Occurrence of anti-*Leishmania* spp. antibodies in domiciled dogs from the city of Foz do Iguacu, State of Parana, Brazil. In: World Congress on Leishmaniasis, 5, 2013, Porto de Galinhas. Abstract. Porto Galinhas: Sociedade Brasileira de Medicina Tropical 875–876.

Elmahallawy, EK, Sampedro, MA, Rodriguez-Granger, J, Hoyos-Mallecot, Y, Agil, A, Navarro - Mari, JM, and Gutierrez, FJ (2014) Diagnosis of leishmaniasis. *The Journal of Infection in Developing Countries* **8**, 961–972. doi:10.3855/jidc.4310.

Faria, AR de Castro Veloso L, Coura-Vital W, Reis AB, and Damasceno LM (2015) Novel recombinant multiepitope proteins for the diagnosis of asymptomatic *Leishmania infantum*-infected dogs. *PLoS Neglected Tropical Disease* **9**(1): e3429. doi:10.1371/journal.pntd.0003429.

Ferreira, EC, de Lana, M, Carneiro, M, Reis, AB, Paes, DV, da Silva, ES, Schallig, H, and Gontijo, CM (2007) Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. *Veterinary Parasitology* **146**, 235–241. doi:10.1016/j.vetpar.2007.02.015.

Foglia Manzillo, V, Di Muccio, T, Cappiello, S, Scalone, A, Paparcone, R, Fiorentino, E, Gizzrelli, M, Gramiccia, M, Gradoni, L, and Oliva, G (2013) Prospective Study on the Incidence and Progression of Clinical Signs in Naïve Dogs Naturally Infected by Leishmania infantum. *PLOS Neglected Tropical Diseases* **7**, e2225. doi:10.1371/journal.pntd.0002225.

Fonseca, AM, Faria, AR, Rodrigues, FTG, Nagem, RAP, Magalhães, RDM, Cunha, JLR, Bartholomeu, DC, and Andrade HM (2014) Evaluation of three recombinant *Leishmania infantum*

antigens in human and canine visceral leishmaniasis diagnosis. *Acta Tropica* **137**, 25–30. doi: 10.1016/j.actatropica.2014.04.028.

Fraga, DB, Da Silva, ED, Pacheco, LV, Borja, LS, Coura-Vital, W, Monteiro GR, Oliveira GG, Jerônimo SM, Reis AB, and Veras PS (2014) A multicentric evaluation of the recombinant *Leishmania infantum* antigen-based immunochromatographic assay for the serodiagnosis of canine visceral leishmaniasis. *Parasites and Vectors* **7**, 136. doi: 10.1186/1756-3305-7-136.

Francino, O, Altet, L, Sánchez-Robert, E, Rodriguez, A, Solano-Gallego, L, Alberola, J, Ferrer, L, Sánchez, A and Roura, X (2006). Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Veterinary Parasitology* **137**, 214—221. doi: 10.1016/j.vetpar.2006.01.011.

Gálvez R, Miró G, Descalzo MA and Molina, R (2011) Questionnaire-based survey on the clinical management of canine leishmaniosis in the Madrid region (central Spain). *Preventive Veterinary Medicine* **102**, 59–65. doi: 10.1016/j.prevetmed.2011.07.002.

Gao, CH, Ding, D, Wang, JY, Steverding, D, Wang, X, Yang, YT and Shi, F (2015). Development of a LAMP assay for detection of *Leishmania infantum* infection in dogs using conjunctival swab samples. *Parasites and Vectors* **8**, 1–8. doi: 10.1186/s13071-015-0991-2.

Gavgani, AS, Hodjati MH, Mohite, H, and Davies, CR (2002) Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. *The Lancet* **360**, 374–379. doi:10.1016/S0140-6736(02)09609-5.

Georgiadou, SP, Makaritsis, KP, and Dalekos, GN (2015) Leishmaniasis revisited: current aspects on epidemiology, diagnosis and treatment. *Journal of Translational Internal Medicine* **3**,43–50. doi: 10.1515/jtim-2015-0002.

Gomes, CM, Cesetti, MV, Paula, NA, Vernal, S, Gupta, G, Sampaio, RN and Roselino, AM (2017) Field validation of SYBR green- and TAQMAN-based real-time PCR using biopsy and swab samples to diagnose American tegumentary leishmaniasis in an area where *Leishmania (Viannia) braziliensis* is endemic. *Journal of Clinical Microbiology* **55**,526–534. doi: 10.1128/JCM.01954-16.

Goto, H and Lindoso, JAL (2010) Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Review of Anti-infective Therapy* **8**,419–433. doi: 10.1586/eri.10.19.

Goto, Y, Carter, D, Guderian, J, Inoue, N, Kawazu, S and Reed, SG (2010) Upregulated expression of B-cell antigen family tandem repeat proteins by *Leishmania* amastigotes. *Infection and Immunity* **78**, 2138–2145. doi: 10.1128/IAI.01102-09.

Goto, Y, Carter, D and Reed, SG (2008) Immunological dominance of *Trypanosoma cruzi* tandem repeat proteins. *Infection and Immunity* **76**, 3967–3974. doi: 10.1128/IAI.00604-08.

Goto, Y, Howard, RF, Bhatia, A, Trigo, J, Nakatani, M, Netto, EM, and Reed, SG (2009) Distinct antigen recognition pattern during zoonotic visceral leishmaniasis in humans and dogs. *Veterinary Parasitology* **160**, 215–220. doi: 10.1016/j.vetpar.2008.10.097.

Grimaldi, GJr, Teva, A, Ferreira, AL, dos Santos, CB, Pinto, I, de-Azevedo, CT, and Falqueto, A (2012) Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP(R) CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **106**, 54–59. doi: 10.1016/j.trstmh.2011.10.001.

Harhay, MO, Olliaro, PL, Costa, DL, and Costa, CH (2011) Urban parasitology: visceral leishmaniasis in Brazil. *Trends in Parasitology* **27**, 403–409. doi: 10.1016/j.pt.2011.04.001.

Jamal, F, Dikhit, MR, Singh, MK, Shivam, P, Kumari, S, Pushpanjali, S, Sardar, AH, Pushpanjali, Murugesan, S, Narayan, S, Gupta, AK, Pandey, K, Das, VNR, Ali, V, Bimal, S, Das, P, and Singh, SK (2016) Identification of Bcell epitope of *Leishmania donovani* and its application in diagnosis of visceral leishmaniasis. *Journal of Biomolecular Structure and Dynamics* **25**,1–2. doi: 10.1371/journal.pone.0182474.

Khow, O and Suntrarachun, S (2012) Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pacific Journal of Tropical Biomedicine 2, 159–162. doi: 10.1016/S2221-1691(11)60213-X.

Lachaud, L, Chabbert, E, Dubessay, P, Dereure, J, Lamothe, J, Dedet, JP and Bastien, P (2002). Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. *Parasitology* **125**, 197–207. doi: 10.1017/s0031182002002081.

Laurenti, MD, de Santana Leandro, MVJr, Tomokane, TY, De Lucca, HR, Aschar, M, Souza, CS, Silva, RM, Marcondes, M, and da Matta, VL (2014) Comparative evaluation of the DPP® CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. *Veterinary Parasitology* **205**, 444–450. doi: 10.1016/j.vetpar.2014.09.002.

Lopes, EG, Sevá, AP, Ferreira, F, and Nunes, CM (2017) Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: one out of five seronegative dogs are infected. *Epidemiology &. Infection* **145**, 2436–2444. doi: 10.1017/S0950268817001443.

Magalhães, FB, Castro-Neto, AL, Nascimento, MB, Santos, WJT, Medeiros, ZM, Neto, ASL, **Costa, LD, Costa, HNC, dos Santos, WLC, de Carvalho, LCP, Oliveira, GGS, and Melo Neto, OP** (2017) Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis. *PLoS One* **12**, e0184867. doi: 10.1371/journal.pone.0184867.

Manna, L, Vitale, F, Reale, S, Caracappa, S, Pavone, LM, Morte, R, Della Cringoli, G, Staiano, N and Gravino, AE (2004). Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis. *Veterinary Parasitology* **125**, 251–262. doi: 10.1016/j.vetpar.2004.07.019.

Maziero N, Thomaz-Soccol V, Steindel M, Link JS, Rossini D, Alban, SM, and Nascimento, AJ (2014). Rural-urban focus of canine visceral leishmaniasis in the far western region of Santa Catarina State, Brazil. *Vet Parasitol* 205, 92-5. doi: 10.1016/j.vetpar.2014.06.005.

MedCalc Statistical Software version 18.11.6 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2019).

Mettler, M, Grimm, F, Capelli, G, Camp, H, and Deplazes, P (2005) Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. *Journal of Clinical Microbiology* **43**, 5515–5519. doi: 10.1128/JCM.43.11.5515-5519.2005.

Mniouil, M, Fellah, H, Amarir, F, Sadak, A, Et-Touys, A, Bakri, Y, Moustachi, A, Tassou, FZ, Hida, M, Lyagoubi, M, Adlaoui, EB, Rhajaoui, M, and Sebti, F (2018) Comparative evaluation of immunochromatographic dipstick test (ICT) rk39, soluble antigen ELISA and IFAT for the sero-diagnosis

572 of visceral leishmaniasis in Morocco. *Acta Tropica Journal* **182**,185–189. doi:
573 10.1016/j.actatropica.2018.03.007.
574 **Oshaghi, MA, Chavshin, AR, Vatandoost, H, Yaaghoobi, F, Mohtarami, F, Noorjah, N** (2006) Effects
575 of postingestion and physical conditions on PCR amplification of host blood meal DNA in mosquitoes.
576 *Experimental Parasitology* **112**: 232–236.
577 **Oliveira, GG, Magalhaes, FB, Teixeira, MC, Pereira, AM, Pinheiro, CG, Santos, LR, Nascimento,**
578 **MB, Bedor, CNG, Albuquerque, AL, dos-Santos, WLC, Gomes, YM, Moreira, EDJr, Brito, MEF,**
579 **Pontes de Carvalho, LC, and Melo-Neto, OP** (2011) Characterization of novel *Leishmania infantum*
580 recombinant proteins encoded by genes from five families with distinct capacities for serodiagnosis of
581 canine and human visceral leishmaniasis. *The American Journal of Tropical Medicine and Hygiene* **85**,
582 1025–1034. doi: 10.4269/ajtmh.2011.11-0102.
583 **Oliveira, E, Saliba, JW, Oliveira, D, Dias, ES and Paz, GF** (2016). A prototype of the direct agglutination
584 test kit (DAT-Canis) for the serological diagnosis of canine visceral leishmaniasis. *Veterinary Parasitology*
585 **221,** 9–13. doi: 10.1016/j.vetpar.2016.02.006.
586 **Otranto, D, Dantas-Torres, F, and Breitschwerdt, EB** (2019) Managing canine vector-borne diseases
587 of zoonotic concern: part one. *Trends in Parasitlogy* **25**, 157–163. doi: 10.1016/j.pt.2009.01.003.
588 **Paltrinieri, S, Gradoni, L, Roura, X, Zatelli, A and Zini, E** (2016). Laboratory tests for diagnosing and
589 monitoring canine leishmaniasis. *Veterinary Clinical Pathology* **45**, 552–578. doi: 10.1111/vcp.12413.
590 **Pattabhi S, Whittle J, Mohamath R, El-Safi S, and Moulton GG** (2010) Design, Development and
591 Evaluation of rK28-Based Point-of-Care Tests for Improving Rapid Diagnosis of Visceral Leishmaniasis.
592 *PLoS Negl Trop Dis* 4(9): e822. doi:10.1371/journal.pntd.0000822
593 **Pereira-da-Silva, S, Marques, LFV, Lamounier, KC, de Castro, JM and Borja- Cabrera, G** (2017)
594 Leishmaniose visceral humana: reflexões éticas e jurídicas acerca do controle do reservatório canino no
595 Brasil. *Revista de Bioética y Derecho* **39**,135–151. doi: 10.1344/rbd2017.39.17719.
596 **Portela, ASB, Costa, LE, Salles, BCS, Lima, MP, Santos, TTO, Ramos, FF, Lage, DP, Martins, VT,**
597 **Caligiorne, RB, Lessa, DR, Silva, FR, Machado, AS, Nascimento, GF, Gama, IS, Chávez-Fumagalli,**
598 **MA, Teixeira, AL, Rocha, MOC, Rocha, RL, and Coelho, EAF** (2017) Identification of immune
599 biomarkers related to disease progression and treatment efficacy in human visceral leishmaniasis.
600 *Immunobiology* **223**, 303–309. doi: 10.1016/j.imbio.2017.10.043.
601 **R Development Core Team R** (2018) R: A language and environment for statistical computing. R Found.
602 Stat. Comput. 1:409. Available at https://www.gbif.org/tool/81287/r-a-language-and-environment-for-
603 statistical-computing.
604 **Rampazzo, RCP, Solcà, MDS, Santos, LCS, Pereira, LN, Guedes Jr, JO, Veras, PST, Fraga, DBM,**
605 **Kriegera, MA, and Costa, ADT** (2017) A ready-to- use duplex qPCR to detect *Leishmania infantum* DNA
606 in naturally infected dogs. *Veterinary Parasitology* **246**, 100–107. doi: 10.1016/j.vetpar.2017.09.009.
607 **Romero, GA and Boelaert, M** (2010) Control of visceral leishmaniasis in Latin America a systematic
608 review. *PLOS Neglected Tropical Diseases* **4**, e584. doi: 10.1371/journal.pntd.0000584.
609 **Rosati, S, Ortoffi, M, Profiti, M, Mannelli, A, Mignone, W, Bollo, E, and Gradoni, L** (2003)
610 Prokaryotic expression and antigenic characterization of three recombinant *Leishmania* antigens for

serological diagnosis of canine leishmaniasis. *Clinical and Diagnostic Laboratory Immunology* **10**, 1153–1156. doi: 10.1128/CDLI.10.6.1153-1156.2003.

Satragno, D, Faral-Tello, P, Canneva, B, Verger, L, Lozano, A, Vitale, E, Greif, G, Soto, C, Robello, C, and Basmadjián, Y (2017) Autochthonous outbreak and expansion of canine visceral leishmaniasis, Uruguay. *Emerging Infectious Diseases journal* **23**, 536–538. doi: 10.3201/eid2303.160377.

Schonian, G, Naserddin, A, Dinse, N, Scheweynoch, C, Schallig, HDFH, Presber, W, Jaffe, CL (2003). PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagnostic Microbiology Infectious Disease* **47**, 349–358.

https://www.ncbi.nlm.nih.gov/pubmed/12967749

Singh, OP, and Sundar, S (2015) Developments in diagnosis of visceral leishmaniasis in the elimination era. *Journal of Parasitology Research* **239469**, 1-10. doi: 10.1155/2015/239469.

Solano-Gallego, L, Koutinas, A, Miro, G, Cardoso, L, Pennisi, MG, Ferrer, L, Bourdeau, P, Oliva, G and Baneth, G (2009) Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Veterinary Parasitology* **165**, 1–18. doi: 10.1016/j.vetpar.2009.05.022.

Solano-Gallego, L, Villanueva-Saz, S, Carbonell, M, Trotta, M, Furlanello, T and Natale, A (2014). Serological diagnosis of canine leishmaniosis: Comparison of three commercial ELISA tests (Leiscan®, ID Screen® and *Leishmania* 96®), a rapid test (Speed Leish K®) and an in-house IFAT. *Parasites and Vectors* **7**, 1–10. doi: 10.1186/1756-3305-7-111.

Sousa-Paula LCd, Silva LGd, Sales KGdS, and Dantas-Torres F (2019) Failure of the dog culling strategy in controlling human visceral leishmaniasis in Brazil: A screening coverage issue? *PLoS Neglected Tropical Diseases* 13(6): e0007553. doi.org/10.1371/journal.pntd.0007553.

Souza, LMB, Carvalho, J, Bates, MD, Petterle, R, Thomaz-Soccol, V, and Bates, PA (2019) Production of a kinesin-related recombinant protein (Lbk39) from *Leishmania braziliensis* by *Leishmania tarentolae* promastigotes and its application in the serodiagnosis of leishmaniasis, *One Health* 8: doi.org/10.1016/j.onehlt.2019.100111.

Szargiki, R, Castro, EA, Luz, E, Kowalthuk, W, Machado, AM, and Thomaz-Soccol, V (2009). Comparison of serological and parasitological methods for cutaneous leishmaniasis diagnosis in the state of Paraná, Brazil, Braz. *J. Infect. Dis*. **13**, 47–52. doi:10.1590/S1413-86702009000100011.

Távora MPF, Pereira MAVC, Silva VL, and Vita GF (2007) Estudo de validação comparativo entre as técnicas Elisa e RIFI para diagnosticar *Leishmania* sp. em cães errantes apreendidos no município de Campos dos Goytacazes, Estado do Rio de Janeiro. *Revista da Sociedade Brasileira de Medicina Tropical* **40**:482-483.

Thomaz Soccol, V, Pasquali, AKS, Pozzolo, EM, Leandro, ADS, Chiyo, L, and Baggio, RA (2017) More than the eyes can see: The worrying scenario of canine leishmaniasis in the Brazilian side of the triple border. *PLoS ONE* **12**, e0189182. doi.org/10.1371/journal.pone.0189182.

Srividya, G, Kulshrestha, A, Singh, R, and Salotra, P (2012) Diagnosis of visceral leishmaniasis: developments over the last decade. *Parasitology Research* **110**,1065–1078. doi: 10.1007/s00436-011-2680-1.

649 **Valiente-Gabioud, AA, Veaute, C, Perrig, M, Galan-Romano, FS, Sferco, SJ, and Marcipar, IS**
650 (2011) Effect of repetitiveness on the immunogenicity and antigenicity of *Trypanosoma cruzi* FRA protein.
651 *Experimental Parasitology* **127**, 672–679. doi: 10.1016/j.exppara.2010.11.011.
652 **Vos, Q, Lees, A, Wu, ZQ, Snapper, CM, and Mond, JJ** (2000) B-cell activation by T-cell-
653 independent type 2 antigens as an integral part of the humoral immune response to pathogenic
654 microorganisms. *Immunology Review* **176**, 154–170. PMID: 11043775.
655 **Werneck, GL** (2008) Forum: geographic spread and urbanization of visceral leishmaniasis in Brazil.
656 Introduction. *Cadernos de Saúde Pública* **24**, 2937–2940. doi:10.1590/S0102-311X2010000400001 .
657 **Wolf, D, Failing, K, Taubert, A, and Pantchev, N** (2014) Serological diagnosis of canine leishmaniosis:
658 comparison of three commercially available tests. *Parasitology Research* **113**,1997–2002. doi:
659 10.1007/s00436-014-3865-1.
660 **World Health Organization** (2010) Leishmaniasis: country profiles. Available at
661 http://www.who.int/leishmaniasis/resources/BRAZIL.pdf (Accessed 12 February 2019).
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663 *Table 1.* Characteristics and location of canine blood samples.

Characteristics	Locality	N	Diagnostic tests
Dogs with clinical signs* from endemic area	Foz do Iguaçu - PR	52	Culture, ELISA, TR-DPP
No clinical signs from endemic area	Foz do Iguaçu - PR	27	Culture, ELISA, TR-DPP
Negatives controls	CCZ - Araucária - PR	73	Culture, ELISA, TR-DPP

664 * Clinical signs: the main clinical features observed for this classification were alopecia, peeling dry skin, brittle skin,
665 nodules on the skin, ulcers, weakness, low weight, and ocular lesions.
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668 *Table 2.* Lbk39 test sensitivity and specificity for the sample of 56 positive canine sample cultures,
669 according to clinical classification (with or without clinical signs).

Positive culture	Sensitivity (%)			Specificity (%)		
Lbk39	Estimate	CI min	CI max,	Estimate	CI min	CI max
Clinical signs	100	90.3	100	98.7	92.8	100
No clinical signs	100	80.5	100	96	88.8	99.2

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673 *Table 3.* Comparision between Lbk39 antigen and other methods (TR DPP®, iELISA and culture in
674 artificial media) analysing 78 positive dogs and 75 negative controls.

Test	Sensitivity (%)			Specificity (%)		
	Estimate	CI min	CI max	Estimate	CI min	CI max
ELISA-Lbk39 antigen	100	95.3	100	96.1	89	99.2
TR DPP	93.8	90	96.3	97.2	95.7	98.2
ELISA crude extract	98.1	95.5	99.2	86.9	84.5	89
Culture	77	69.8	82.9	100	-	-

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677 *Table 4*. Comparision between Lbk39 antigen and TR DPP for the sample of 78 positive controls, according

678 to clinical classification (with or without clinical signs).

Test	Sensitivity (%)			Specificity (%)		
	Estimate	CI min	CI max	Estimate	CI min	CI max
Lbk39 ELISA						
Clinical signs	98	89.6	100	98.7	92.9	100
No clinical signs	100	86.3	100	96.1	88.9	99.2
TR-DPP test						
Clinical signs	98	90	100	100	95	100
No clinical signs	88	70	98	100	95	100

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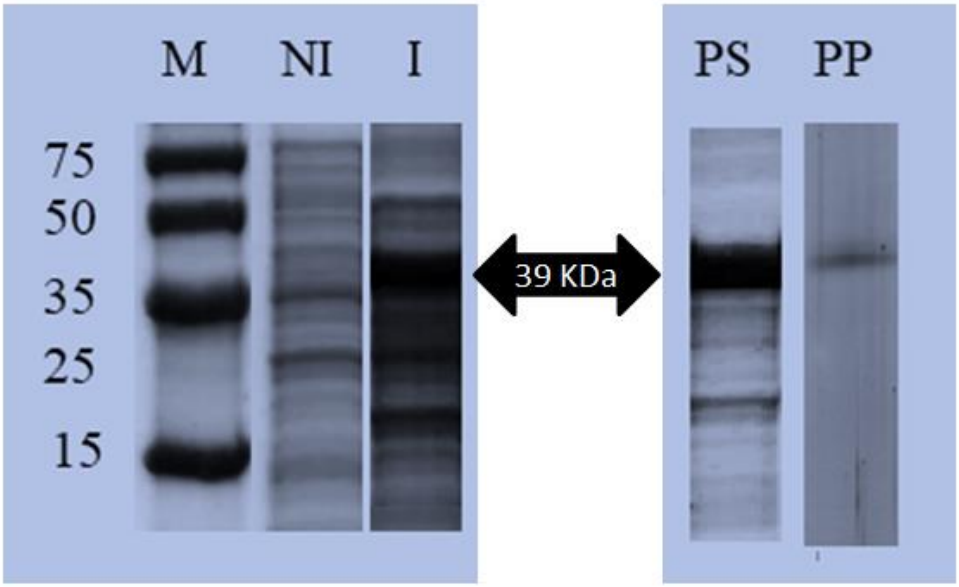

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682 *Figure 1*. SDS PAGE of induction and purification of LKb39.

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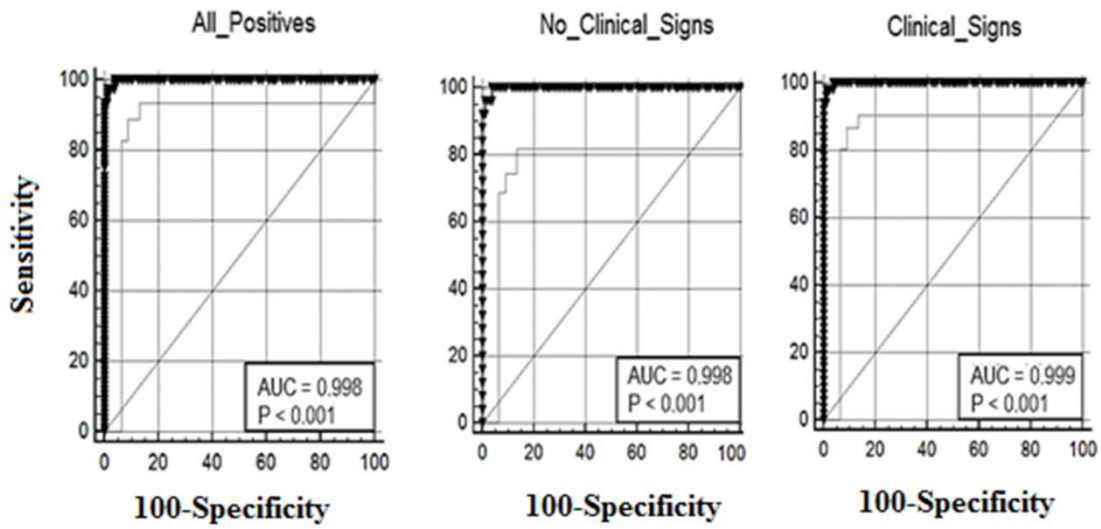


Figure 2. Comparison of ELISA reactivity of canine sera against Lbk39 recombinant proteins. AUC: accuracy.

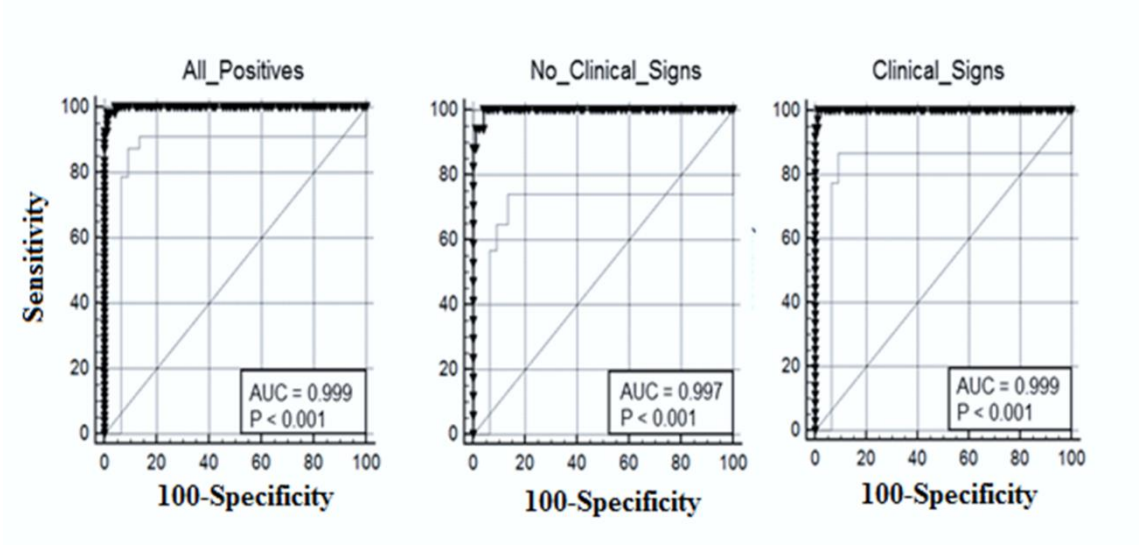
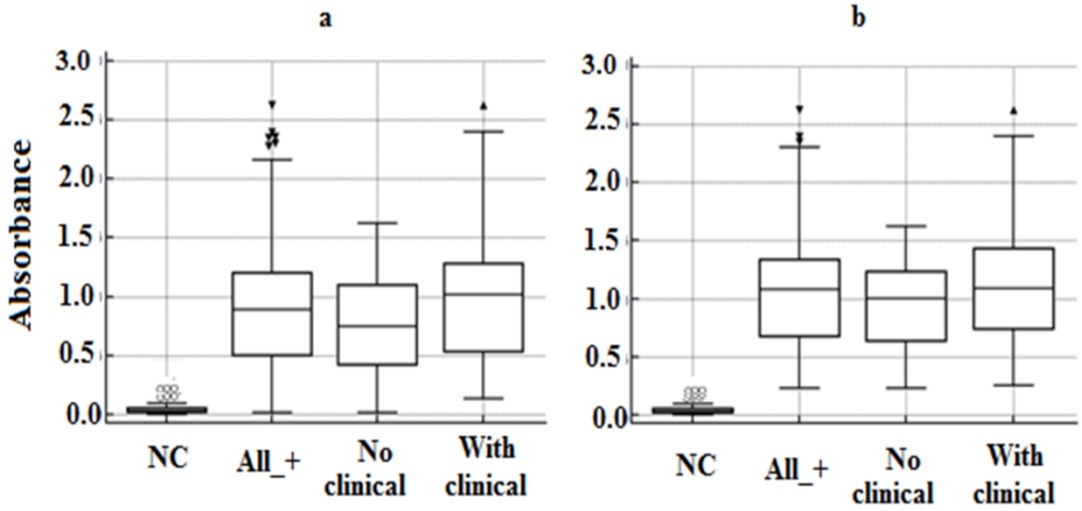


Figure 3. Comparison of ELISA reactivity of canine sera against Lbk39 recombinant proteins in true positive cultures. AUC: accuracy.



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694 *Figure 4*. Box and whisker plot **c**omparisons of absorbance distributions in different groups of dog sera. a)
695 Analysis of all samples. b) Analysis of the subset of ELISA positives that also had a positive culture. The
696 symbols represent outliers, where each corresponds to the result obtained with an individual serum.

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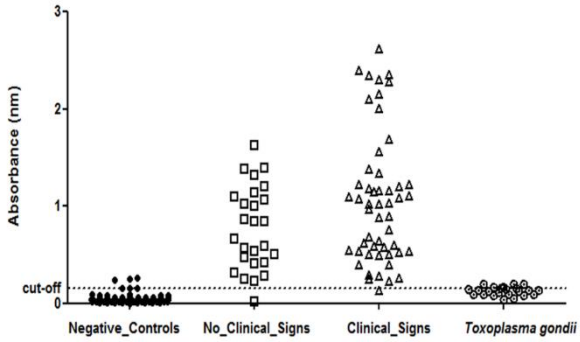

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700 *Figure 5*. Analyses of cross-reaction with Lbk39 against *Toxoplasma gondii* compared with negative
701 controls and dogs with and without clinical signs.

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