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

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25 KEY FINDINGS

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

29

30 **SUMMARY**

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

45

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

47

48 **Introduction**

49 Canine visceral leishmaniosis (CVL) is a neglected tropical disease caused by protozoa of the
50 genus *Leishmania* Ross, 1903, which affects dogs from all continents, except Oceania (Colwell et al. 2011,
51 Dantas-Torres et al. 2012, Otranto et al. 2019). More than 20 *Leishmania* species are known to exist, and
52 in dogs *Leishmania infantum* is the etiological agent of CVL. Visceral leishmaniosis (VL) also affects
53 humans in Europe, the Middle East, South Asia, Africa, and in Central and South America, where
54 transmission may be anthroponotic, but where dogs with CVL can also act as reservoir hosts for human
55 infection, transmitted by the bites of sand fly vectors (Chappuis et al. 2007, WHO, 2010). In the Americas,
56 human VL is mainly if not exclusively zoonotic and found in at least 12 countries, however, 90% of the
57 cases are reported in Brazil, causing approximately 4,200-6,300 infections per year, with a 7% mortality
58 rate (Romero et al. 2010, Alvar et al. 2012; WHO, 2017). The increasing spread of *L. infantum* into urban
59 areas in the Americas is of great concern, and is associated with migration, population growth and poor
60 living conditions (Werneck et al. 2008, Romero et al. 2010, Harhay et al. 2011). CVL is a multisystemic
61 disease with clinical signs that may be apparent, such as dermatitis, lymphadenomegaly, general muscular
62 atrophy, and renal disease (Baneth et al. 2008). However, control programmes for both the disease and
63 infection are essential, because many dogs remain asymptomatic, which makes it difficult to diagnose
64 infection and control transmission to humans (Gavgani et al. 2002; WHO, 2010). Current methods to
65 prevent the spread of CVL include the use of topical insecticides on dogs and canine vaccination, which
66 provides only a certain level of protection for dogs (Solano-Gallego et al. 2009; Sousa-Paula et al. 2019).
67 In addition, many countries have also used euthanasia of seropositive dogs (Dantas-Torres et al. 2012,
68 BRASIL, 2014). However, scientific studies have not yet shown that this euthanasia strategy decreases the
69 infection rate of VL (Romero et al. 2010, Sosa-Paula et al. 2019; Dantas-Torres et al. 2019). Moreover, the
70 method is not well-received by the population for ethical reasons (Costa et al. 2011, Pereira-da-Silva et al.
71 2017). The treatment of CVL-positive dogs is permitted in some countries, however, euthanasia may still
72 be the only option for owners who cannot afford to have their dogs treated. Therefore, the development of
73 more precise diagnostic methods for both rapid treatment of humans and control of CVL in dogs is urgently
74 required (Foglia Manzillo 2013, Fonseca et al. 2014, Coelho et al. 2016).

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

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

90 In Brazil the Ministry of Health has determined that the lateral flow strip test Dual-Path Platform
91 assay (TR-DPP® LVC) produced by Bio-Manguinhos/Fiocruz, Brazil (BRASIL 2014) should be used for
92 serological screening of dogs, followed by an ELISA test (EIE-LVC) for confirmation of *L. infantum*
93 infection (Grimaldi et al. 2012, Faria et al. 2015). Some purified recombinant antigens, such as the rK39
94 antigen, of various *Leishmania* species have been produced and used in serological assays of VL (Badaró
95 et al. 1996). Even though the TR-DPP® test uses a combination of recombinant proteins (rK26 and rK39)
96 which should improve sensitivity (Bhatia et al. 1999; Pattabhi et al. 2010), it can perform with low
97 sensitivity, which compromises the efficacy of the diagnosis of infection in Brazil (Grimaldi et al. 2012,
98 De Santis et al. 2013). Further, the crude parasite extract used in the EIE-LVC test has also shown false
99 positive results due to cross-reactivity with other pathogens, particularly *Trypanosoma cruzi*, in North,
100 Central and South America (Ferreira et al. 2007, Solano-Gallego et al. 2009). Cross-reactions are less likely
101 to occur when using recombinant proteins in ELISA, such as rA2, rK9, rK26 and rK39 (Solano-Gallego et
102 al. 2009). Considering the need for new antigens for improved serodiagnosis of CVL that show both high
103 specificity and sensibility, and no cross-reaction with other diseases, we undertook this study, in which we
104 investigated the use of the recently described Lbk39 recombinant protein (Souza et al. 2019) as an antigen
105 in CVL diagnosis by the ELISA method.
106
107

108 **Materials and Methods**

109

110 *Production of Lbk39 recombinant protein*

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

117 An aliquot of transfected *L. tarentolae* was thawed and placed in 100 mL of culture medium. As
118 soon as the cultures became slightly turbid after 24 h, the specific Streptothricin-class of aminoglycoside
119 antibiotic Nourseothricin (LEXSY NTC, Jena Bioscience) for the pLEXSY-sat2 vector was added, and the
120 culture maintained under the same conditions by subpassage every four days. After that the pre-inoculum
121 was inoculated in BHI medium (2 L) supplemented with 2 mL of porcine hemin (Jena Bisocience), 5 mL
122 of penicillin and streptomycin (Pen-Strep, Jena Bioscience) and 1 mL of NTC antibiotic (Jena Bioscience)
123 and incubated at 26°C in the dark under aerated conditions. The culture was then centrifuged at 5000 g,
124 4°C, for 10 min, the supernatant medium removed and stored frozen at -80°C.

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

135

136 *Collection and sample processing*

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

139 (Araucária, Parana), which can be assumed to be almost exclusively negative for CVL, with possible
140 exception of rare imported cases. For the positive sera, we used dog samples from the region of the outbreak
141 Foz do Iguaçu (for more details, see Thomaz Soccol et al. 2017). To assess the clinical diagnosis of CLV,
142 clinical signals were recorded in an epidemiological questionnaire. Owned dogs were examined by
143 veterinarians for clinical signs of the disease and each dog was given an individual data file including
144 identification, traits, behaviour, migration history and health issues. Signals investigated were weight loss,
145 adenomegaly, alopecia, skin lesions, mucosal lesions, hipkeratosis, and muscle atrophy. Blood samples
146 were collected by venipuncture of the jugular vein, transferred to 10 mL polypropylene tubes and processed
147 3-4 hours after collection. In the laboratory, the blood was centrifuged at 1000 xg for 5-10 min and the sera
148 were separated and stored at -20°C until analysed by serological methods. The TR-DPP (Bio-Manguinhos,
149 Fiocruz, Brazil) as recommendation from the Brazilian Ministry of Health and used according to
150 manufacturer's instructions. The indirect ELISA using total extract of *L. infantum* was performed as
151 described by Maziero et al. (2014). Each sample was tested in triplicate intra- and inter-plate. Three positive
152 and negative dog sera were included in each plate as controls, when testing individual sera.

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

163

164 *Ethics Statement*

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

169

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

171 The ELISA test was developed and standardised by analysing results using antigens at different
172 concentrations (0.031, 0.062, 0.125, 0.250, 0.500 and 1,000 µg/well), two serum dilutions (1:100 and 1:200)
173 and various dilutions of HRP-conjugated secondary antibodies (1:3500, 1:5000, 1:7500 and 1:10000). The
174 Lbk39 antigen was diluted in carbonate-bicarbonate buffer, pH 9.6, and polystyrene microtiter plates (96-
175 well EIA/RIA 12x8 well plates, Costar, USA) were coated with 100 µL/well of dilutions at all of the
176 concentrations of antigens given above, and incubated overnight at 4°C. The next day, the plates were
177 washed with 200 µL/well of washing solution (0.9% NaCl + 0.05% Tween 20), twice. Next, the wells were
178 blocked with 120 µL of blocking solution composed of PBS + 0.1% casein at 37°C, for 1 h, and were then
179 washed again, twice, with 200 µL/well of washing solution. After these steps, the serum samples were
180 added and incubated at 37°C, for 1 h. The plates were then washed with 200 µL/well of washing solution
181 four times. 100 µL of various dilutions of a polyclonal goat anti-dog IgG HRP (2 mg/mL) conjugate was
182 plated at the concentrations given above at 37°C, for 1 h. Lastly, the reaction was developed by adding 100
183 µL/well of a solution prepared by combining 10.0 mL of citrate buffer (4.5% Na_2PO_4 + 3.25% citric acid +
184 distilled water, pH 5.0), 2 mg of o-phenylenediamine dihydrochloride (2 mg/tablet, Sigma, USA) and 2 µL
185 of 30% H_2O_2, and incubating at room temperature for 30 min, avoiding light. The reaction was stopped by
186 adding 20 µL/well of 1:20 H_2SO_4 solution. The plates were read in a PowerWave HT reader (BioTek) at
187 492 nm and the values were expressed in absorbance. The cut-off was calculated from the mean value of
188 the negative controls.

189

190 *Tests used for comparison with the Lbk 39 antigen*

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

196 Parasitological diagnosis by culture was done as recommended by Szargiki et al. (2009). In brief, bone
197 marrow aspirate, lymph node aspirate or the leukocyte layer of dogs were inoculated in Neal, Novy and
198 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.

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

235 After standardisation, we used the optimised ELISA with Lbk39 antigen to analyse all 152 canine
236 sera in the study. From this analysis, of the 79 dog sera from the CVL endemic region of Foz do Iguaçu, 76
237 were positive and 3 were negative in the Lbk39 ELISA assay. Of these 3 negative animals, two were in the
238 group with no clinical signs, one of these was negative in all other assays used and one was positive with
239 the TR-DPP assay. The negative result from the dog with clinical signs was positive with the crude antigen
240 ELISA and TR-DPP assays. Therefore, we conclude that two of these negative results from Foz do Iguaçu
241 were false negatives and one was a true negative. All the dog sera from Araucária showed negative results
242 with the Lbk39 assay. The ROC curve analysis of absorbance distribution against the Lbk39 antigen for
243 various groups studied (all positives from any assay, dogs with clinical signs, dogs without clinical signs)
244 is shown in Figure 2. The difference in canine samples was significant between these groups ($P < 0.001$)
245 and accurate. The positive group (78 samples) presented good accuracy (AUC, 0.998), similar to the no
246 clinical signs group (AUC, 0.998), while the animals with clinical signs presented higher accuracy (AUC,
247 0.999).

248 Culture is used as a standard test for CVL diagnosis, because although it may sometimes lack
249 sensitivity, it has very high specificity, as when it is positive it definitively means that the dog has the
250 parasite. Therefore, we evaluated the subset of 56 dogs that presented with a positive culture. Table 2 shows
251 the results for sensitivity and specificity of the Lbk39 assay with these sera, also subdivided into those with
252 clinical signs or no clinical signs. As shown, the Lbk39 assay performed with very high sensitivity and
253 specific against this subset of dog sera. ROC curve analysis of the absorbance distribution for the Lbk39
254 ELISA against the positive culture subset confirmed that the test worked very well ($P < 0.001$) and was
255 accurate (Fig. 3). The total positives group presented the same accuracy (AUC, 0.999) as the clinical signs
256 subgroup (AUC, 0.999), while the no clinical signs subgroup presented with slightly lower accuracy (AUC,
257 0.997).

258 Analysis of the distribution of ELISA readings from the Lbk39 assay in various subgroups of dog
259 sera is shown in Figure 4. Comparisons are shown between negative controls, positives, those positives
260 with clinical signs and those positives with no clinical signs, either for all sera (Fig. 4a) or for the subset of
261 Lbk39 ELISA positives with positive culture (Fig. 4b). All groups and subgroups of positives were
262 significantly different to the negative controls. When the analysis was confined to sera from dogs that also
263 had a positive culture, as expected the discrimination was greater (Fig. 4b).
264
265 *Comparison of Lbk39 with other diagnostic tests*
266 In addition to evaluating the performance of the Lbk39 ELISA against clinical and parasitological
267 diagnostic tests, we also we compared our standardised assay with other commonly used serological tests,
268 ELISA with a crude antigen preparation and the TR-DPP assay. For these comparisons we considered a
269 true positive to be any animal that was positive in any of the assays used. This comparison showed that the
270 Lbk39 assay outperformed all the other tests in terms of sensitivity (Table 3). The Lbk39 assay also
271 performed well in terms of specificity (96.1%), with either TR-DPP being slightly better (97.2%). As
272 mentioned previously, culture is highly specific, but this has a relative poor sensitivity (77%).
273 Operationally, dogs with CVL are first identified by clinical signs and then the TR-DPP test is applied,
274 sometimes this being the only testing performed. Therefore, we divided the positive group into cases with
275 clinical signs and without clinical signs and evaluated the sensitivity and specificity of Lbk39 comparing
276 with the TR-DPP assay (Table 4). When the individual groups were analysed, this showed that the group
277 of sera from dogs with clinical signs were detected with similar and high sensitivity and specificity for both
278 tests, the TR-DPP being slightly more specific. However, in dogs without clinical signs the Lbk39 ELISA
279 was significantly more sensitive, and slightly less specific than TR-DPP.
280
281 *Cross-reaction with Lbk39*
282 To check for potential cross-reactivity of the Lbk39 antigen with another intracellular parasite, we
283 performed ELISA with Lbk39 against sera from toxoplasmosis-positive canine samples. No cross-reaction
284 was observed (Fig.5).
285
286 **DISCUSSION**
287 *Leishmania braziliensis* is endemic in the study area and, *L. infantum* has been recognized since
288 2012 (Dias et al., 2013; Thomaz Soccol et al., 2017), therefore to work with specific species of CVL dogs

289 were subjected to clinical evaluation and identification of the parasite present in the popliteal ganglion. In
290 endemic areas of *L. braziliensis*, diseased dogs generally have typical well-limited ulcerated lesions with
291 raised edges. The main locations of ulcers are in the scrotal sac and on top of the ears (Dantas-Torres et al.
292 2010). Dogs with CVL generally exhibit lymphadenomegaly, exfoliative dermatitis and weight loss as the
293 most relevant signals on which veterinarians have based their suspicion of infection (Gálvez et al. 2011).
294 In the present study, no animals showed specific signs of CL. In addition, all animals that tested
295 serologically positive on initial testing the parasite isolated was identified as *L. infantum*.

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

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

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

330 In the last 10 years several CVL diagnostic methods have been used. Their specificity and
331 sensitivity can vary. For example the specificity of molecular tests varies from 95 to 100%, where
332 conventional PCR (cPCR) has a sensitivity of 89% to 100% in polysymptomatic dogs (Lachaud et al. 2002;
333 Manna et al. 2004; Carson et al. 2010). However, sensitivity decreases when the method is applied to
334 asymptomatic dogs (Francino et al. 2006; Gao et al. 2015). Currently, quantitative PCR (qPCR) is
335 considered the most reliable test because it has a sensitivity of 91% compared to cPCR (72%), ITS-1 PCR
336 (54%), and PCR hybridization (61%) (Carson et al. 2010). The molecular test with loop-mediated
337 amplification (LAMP) is capable to amplify DNA in constant temperature in blood of VL patients. It has a
338 sensitivities and specificities of 90.7 to 96.4% and 98.5 to 100%, respectively. In China, the LAMP
339 identified 61%-infected dogs, however, not amplify strains from other countries (Gao et al. 2015). The
340 direct agglutination test (DAT) present sensitivities of 91 to 100% and specificities of 72 to 100% but yet
341 subjective reading of end-point titles leads to interrupt server discrepancy (Adams et al. 2012; Oliveira et
342 al. 2016). Although some serological tests for canine leishmanosis have high specificity and sensitivity, the
343 presence of cross-reactivity remains controversial. The immunofluorescence antibody test is reference
344 qualitative in CVL diagnosis (Paltrinieri et al. 2016), with sensitivity and specificity close to 100%, in
345 symptomatic animals, though show some limitations as, cross-reactivity with trypanosomes pathogens
346 (Solano-Gallego et al. 2014; Paltrinieri et al. 2016) and the significantly lower sensitivity in identifying
347 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

378 specificity of 96.1%, which is effective for diagnosing *L. infantum* in infected dogs. In addition, the protein
379 was produced on a scale of two litres and purified without affecting its functionality. Therefore, this protein
380 is a good candidate for use as a diagnostic target.
381

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662

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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667

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	-	-

675

676

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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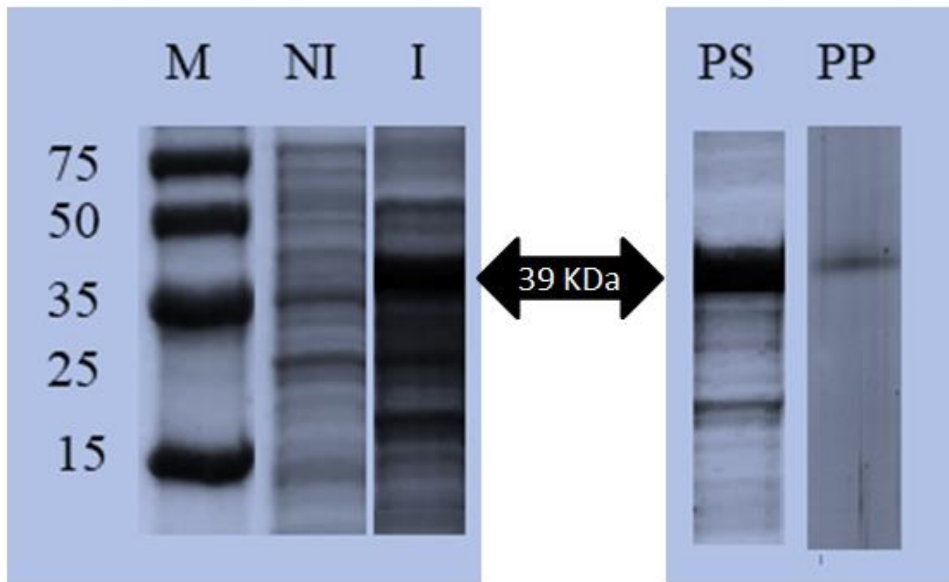

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682 *Figure 1*. SDS PAGE of induction and purification of LKb39.

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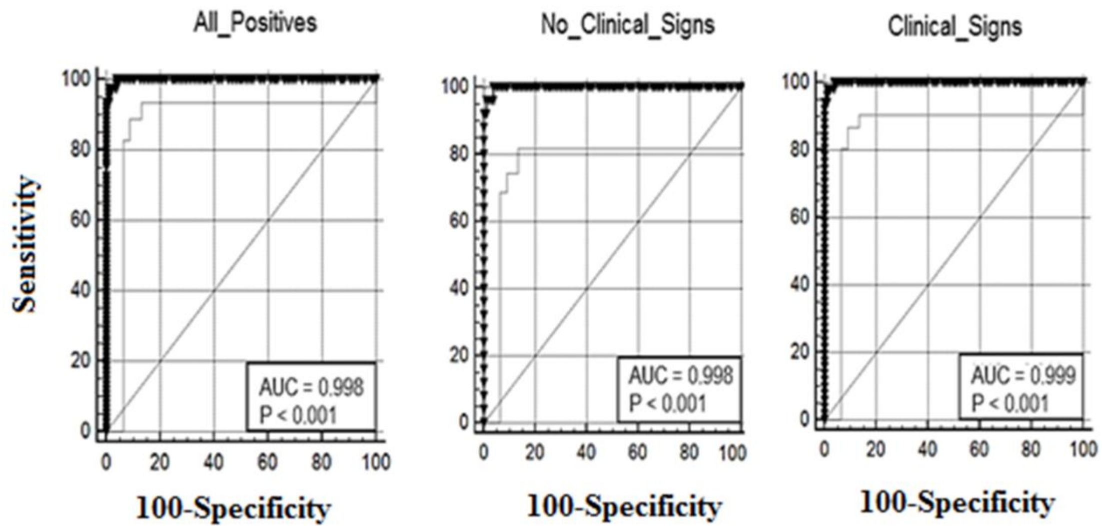

685 *Figure 2*. Comparison of ELISA reactivity of canine sera against Lbk39 recombinant proteins. AUC:
686 accuracy.

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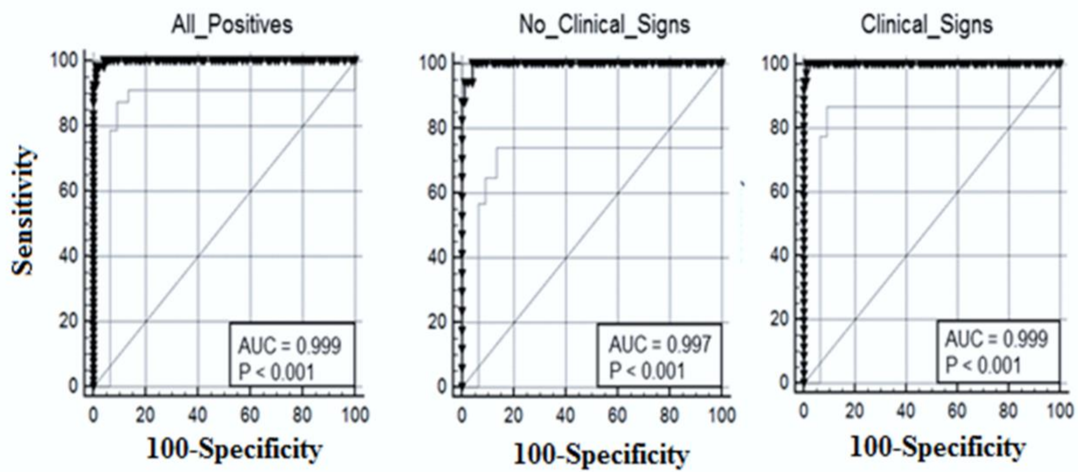

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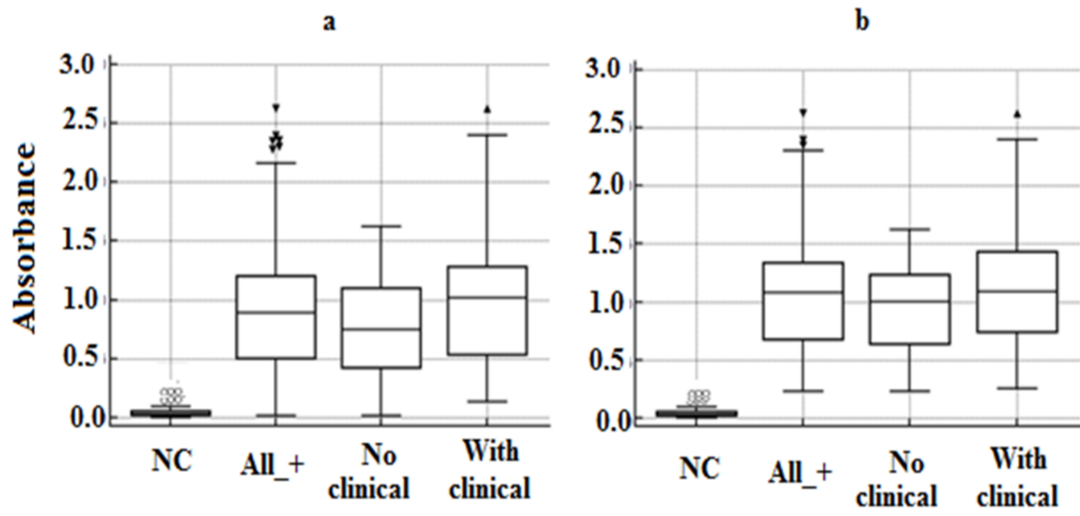
690 *Figure 3*. Comparison of ELISA reactivity of canine sera against Lbk39 recombinant proteins in true
691 positive cultures. AUC: accuracy.

692



693

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.

697

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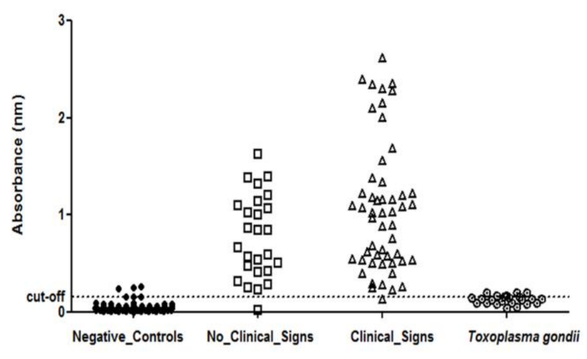

699

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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