Production of a kinesin-related recombinant protein (Lbk39) from *Leishmania braziliensis* by *Leishmania tarentolae* promastigotes and its application in the serodiagnosis of leishmaniasis

Lígia Moraes Barizon de Souza[1]; Joyce Carvalho[1]; Michelle D. Bates[2]; Ricardo Rasmussen Petterle[3]; Vanete Thomaz-Soccol[1]; Paul Andrew Bates[2]*

[1]Laboratory of Molecular Biology, Department of Bioprocess Engineering and Biotechnology, Universidade Federal do Paraná, Avenida Coronel Francisco Heráclito dos Santos, 210 – Usina Piloto B, 81531-970, Curitiba, Paraná, Brazil.

[2]Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, LA1 4YQ, UK.

[3]Nucleus of Medical Education, Department of Community Health, Universidade Federal do Paraná, Rua Padre Camargo, 280 – 7th floor, 80060-240, Curitiba, Paraná, Brazil.

*Corresponding author at: Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, UK.

E-mail address: p.bates@lancaster.ac.uk (P.A. Bates).

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Abstract

The leishmaniases are multifactorial zoonotic diseases requiring a multidisciplinary One Health approach for diagnosis and control. For leishmaniasis diagnosis, here we describe production of a new recombinant protein based on a kinesin-related gene of *Leishmania braziliensis* (Lbk39), which shows 59% amino acid identity to the *L. infantum* homologue. The Lbk39 gene was synthesised, inserted into the pLEXSY-sat2 vector and transfected into *L. tarentolae* cells by electroporation. Culturing was carried out, and the secreted recombinant protein with a C-terminal histidine tag purified using nickel affinity chromatography on the culture supernatant, yielding a final product at 0.4 mg/mL. An indirect enzyme linked immunosorbent assay (ELISA) was standardised using sera from 74 Brazilian patients with cutaneous leishmaniasis and 11 with visceral leishmaniasis. Optimal ELISA conditions were established for the Lbk39 antigen in comparison with a crude extract from *L. braziliensis*. The sensitivity, specificity analysis and receiver operating characteristic (ROC) curve were determined with a significance level of 5%. The ROC curve showed a good accuracy with an area under curve (AUC) = 0.967, $p < 0.001$ (0.941-0.993) for CL patients and an AUC = 100 (100-100) for VL patients. The values of sensitivity and specificity were 88 and 98% for CL and 100 and 100% for VL, respectively. The study showed good production and expression of the target protein and has generated a potential new antigen for the diagnosis of leishmaniasis.

Key words: Diagnosis; cutaneous leishmaniasis; visceral leishmaniasis; recombinant protein; kinesin; rK39; *Leishmania braziliensis*.

1. Introduction

The leishmaniases are a group of largely zoonotic, vector-borne diseases transmitted by sand flies and caused by heteroxenous parasites of the genus *Leishmania* Ross, 1903. The complexity of their epidemiology means they require a One Health approach for their diagnosis and control, and they present a significant ongoing global public health challenge (Alvar et al., 2012; Vilas et al., 2014). Three varieties of epidemiological cycles are known: a sylvatic cycle in which human infection is accidental and transmission occurs in wild foci; a peridomestic cycle in which the reservoirs of infection are domestic animals and transmission to humans occurs around or within human dwellings; and an anthroponotic cycle in which transmission is human to human via anthropophilic sand flies (Burza et al., 2018). Depending on the parasite species and disease focus various combinations of these cycles may co-exist. Another layer of complexity is provided by the environmental factors that influence mammalian reservoir and sand fly vector distributions. For example, the emergence of new foci or re-emergence of leishmaniases has occurred due to invasion of the sand flies into urban areas (Thomaz-Soccol et al. 2018).

Upon infection these parasites interact with the host immune system in a variety of ways to enhance their survival (Kaye and Scott, 2011). A common feature of infection is that the ability of the host to control the parasite depends on the production of cell-mediated immune responses, which in turn are able to activate macrophages to eliminate the intracellular parasites (Taslimi et al. 2018). Although the resolution of the infection is largely mediated by Th1 cells secreting IFN-γ in response to an increase of IL-12, there is also development of a Th2 cell response. The resulting increase of IL-4 can result in progression of lesions and lead to systemic disease, but in addition, some *Leishmania* antigens can drive the differentiation of T-cells that can activate B-lymphocytes to

produce immunoglobulins. It is also known that antibodies are produced through neutrophil stimulation at the very beginning of the infection. Although various studies suggest that such antibodies play no role in host protection, they can be useful in diagnosis for determining the presence of the parasite (Al-Qadhi et al., 2015; Martins et al., 2016).

The correct diagnosis of the leishmaniases is performed through a combination of clinical, epidemiological and laboratory findings (Szargiki et al., 2009). A range of diagnostic tools is available, but none of them are perfect and new more reliable diagnostic tests must still be developed, which should be easy to handle, cheap to produce, and perform with high sensitivity and specificity (Maia et al., 2012). Some purified recombinant antigens of various *Leishmania* species have been produced and used in serological assays, such as the rK39 antigen for the serodiagnosis of visceral leishmaniasis (VL) (Badaro et al., 1996; De Vries et al., 2015). The rK39 antigen is a recombinant protein derived from *Leishmania infantum* that contains 6.5 tandem copies of a B-cell antigenic epitope composed of 39 amino acids. This antigen is related to a kinesin motor protein, which is well conserved between *L. infantum* and *L. donovani*, and the corresponding gene reveals a single open-reading frame that encodes a total of 298 amino acids with a predicted molecular mass of 32.7 kDa (Burns et al., 1993). The *Leishmania* motor protein is involved in various intracellular processes and is present in the amastigote forms of many species.

Production of an antigenic protein of *Leishmania*, by heterologous expression of its specific epitopes in a prokaryotic system such as *Escherichia coli,* is a relatively straightforward technique that is both inexpensive for culturing and quick for processing the target recombinant protein. However, such systems lack eukaryotic post-translational activity, which is a significant disadvantage in producing many eukaryotic proteins. Further, high concentrations of the unfolded protein can occur, leading to a decline in

effective yield, and culturing at a temperature optimal for *E. coli* can also reduce yields of recombinant protein and increase protein degradation (Khow and Suntrarachun, 2012). The protozoan *Leishmania tarentolae,* which is not pathogenic to mammals*,* has been explored as a general eukaryotic host to develop a platform that allows complex eukaryotic protein expression at high levels, and which also has the ability to produce proteins with appropriate post-translational processing (Basile and Peticca, 2009). Moreover, the host is easy to manipulate and can be cultivated on a cheap medium with a 6 to 8 h doubling time. The maintenance of a transfected culture of *L. tarentolae* is performed under specific antibiotic selection and maintains the same level of protein expression after several months of culturing (Breitling et al., 2002; Kushnir et al., 2011; Klatt and Konthur, 2012). Finally, specifically with respect to this study, when the desired recombinant antigen itself is derived from a species of *Leishmania* use of this system maximises the probability of successful expression.

Based on what has been described above, the aim of this study was to explore the use of *L. tarentolae* as a host for the expression and secretion of a *L. braziliensis* kinesin-related recombinant protein, which was identified based on the reference kinesin-related rK39 gene of *L. infantum*. The diagnostic efficiency of this new antigen was evaluated by developing an indirect ELISA for leishmaniasis detection. Until now, no studies have reported on the levels of antibodies against *L. braziliensis* kinesin in cutaneous leishmaniasis (CL) patients.

2. Materials and Methods

2.1 Serum sample collection

123 The patients enrolled in the study were divided into four groups, according to clinical
124 classification (Table 1). In Group 0, 50 healthy individuals from a non-endemic area and
125 medically examined to eliminate any previous CL infection, were used to determine the
126 cut off for the ELISA test and the specificity. Patients with *L. braziliensis*, diagnosed with
127 infection by parasite isolation and clinical examination, were classified in Group 1
128 (n=74). Patients with *L. infantum* diagnosed by serology and PCR were classified in the
129 Group 2 (n=11). Patients with a positive leishmaniasis diagnosis were treated, by local
130 service staff, in accordance with the guidelines of the Brazilian Ministry of Health, as
131 described in the Manual of surveillance and control of American Integumentary
132 Leishmaniasis (2010). Patients with Chagas disease (n=13), confirmed by serology, were
133 also studied to assess the possibility of cross-reactivity. Patient serum samples were
134 stored frozen (-20°C) before use.

135 This study was conducted in accordance with the International Ethical Guidelines for
136 Biomedical Research in Human Beings. In addition, ethical approval was obtained from
137 the Universidade Federal do Paraná Ethical Committee under number 684.244, and in
138 accordance with the law of the Southern Common Market Treaty (Mercosur), Resolution
139 No. 129/96.

140

141 *2.2 Lbk39 plasmid construction, cloning, and propagation in Escherichia coli*

142 A homology search was performed by means of BLAST similarity (Altschul et al., 1997)
143 in the TritrypDB database website. Sequences derived from a kinesin-related gene of *L.*
144 *braziliensis*, henceforth called Lbk39, and comprised of 828 nucleotides (nt) were used
145 for initial plasmid construction (Suppl. Fig. 1A). These were identified by homology with
146 the kinesin-related gene of *L. infantum* - Genebank: L07879, described by Burns et al.
147 (1993), and containing 39 amino acid repeats. Lbk39 also contains a related 39 amino

148 acid sequence (Suppl. Fig. 1B) and is also predicted to comprise immunologically
149 dominant B-cell epitopes (BepiPred; http://www.cbs.dtu.dk/services/BepiPred/).
150 For expression of the target recombinant protein, the synthetic gene Lbk39 was
151 assembled from synthetic oligonucleotides by Invitrogen (Germany), and the fragment
152 was inserted into the pLEXSY-sat2 recombinant vector, developed by Jena Bioscience
153 (Germany), and cloned with a 6×His-tag into the corresponding site of the above-
154 mentioned recombinant vector. The expression vector was designed for integration into
155 the chromosomal 18SrRNA (*ssu*) locus of the parasite (Breitling et al., 2002), allowing
156 for the true expression of the eukaryotic protein; also, for this specific study, the target
157 protein was selected to be secreted into the culture medium.
158 Following the construction of the Lbk39 plasmid, the One Shot™TOP10
159 Chemically Competent *Escherichia coli* strain (Invitrogen) was chosen for the plasmid
160 cloning and propagation, and the procedure for culturing was followed according to the
161 manufacturer's instructions, except for the incubation temperature, which was 30 °C for
162 plasmid stability reasons. After that, the plasmid was purified from the *E. coli* strain using
163 the Geneflow Q-Spin Plasmid DNA Purification Kit and was sent for sequencing. The
164 forward P1442 (5'-CCGACTGCAACAAGGTGTAG-3') and reverse A264 (5'-
165 CATCTATAGAGAAGTACACGTAAAAG-3') sequencing primers, included in the
166 LEXSY kit, were used to confirm the plasmid identity and sequence.
167
168 *2.3 Lkb39 plasmid transfection into LEXSY culture and Lbk39 LEXSY culturing*
169 The propagated and purified Lbk39 plasmid from the *E. coli* strain was linearised through
170 digestion with the *Swa*I (*Smi*I) enzyme, from *Streptococcus milleri* S - 10U/μL (Thermo
171 Fischer Scientific), to prepare for plasmid transfection into the LEXSY host *L. tarentolae*,
172 according to the manufacturer's protocol. To confirm the correct procedure for

linearization and to isolate the fragment corresponding to the plasmid, 1% agarose gel-isolation of the expression cassette with an Agarose Gel Extraction Kit (Jena Bioscience) was performed according to the manufacturer's instruction. The LEXSY strain was previously prepared for transfection according to the LEXSYcon2 Expression Kit manual (for detail see https://www.jenabioscience.com /images/ ae3a4f50f1/EGE-1310.pdf). When ready for transfection through electroporation, the cultured cells were handled according to the same manual mentioned above. Other aliquots of LEXSY cells were electroporated without DNA under the same conditions as a negative control. Then the electroporated cells were transferred to tissue culture flasks containing 10-mL Brain Heart Infusion (BHI) medium supplemented with porcine hemin (Jena Bisocience) and penicillin and streptomycin (Pen-Strep, Jena Bioscience) at 26 °C in the dark under aerated conditions. As soon as the cultures became slightly turbid (24 h after electroporation), 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.

The genomic integration of the Lbk39 plasmid into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* strain was confirmed by PCR. Genomic DNA was extracted from 2 mL of a dense Lbk39 LEXSY culture by means of the DNeasy Blood and Tissue Quick-start kit (Qiagen) according to the manufacturer's recommendation. After that, 200 ng of genomic DNA was added to two 0.2-mL microtubes (100 ng in each), containing the mixed solution of ultra-pure RNAse-free water, 5xHotStar HiFidelity PCR Buffer (including dNTPs), HotStar HiFidelity DNA Polymerase from HotStar HiFidelity Polymerase Kit (Qiagen) and into the first tube the specific primers (Jena Bioscience) for the genomic integration diagnostic: the F3001 forward primer (5'-GATCTGGTTGATTCTGCCAGTAG-3'), responsible for the integration of all *ssu*

198 expression vectors; and the A1715 reverse primer (5'-
199 TATTCGTTGTCAGATGGCGCAC-3'), responsible for the integration of all "AP"
200 expression vectors with 5'UTR *aprt.* The second tube was prepared identically except for
201 the primers (Jena Bioscience): the F2999 forward primer (5'-
202 CCTAGTATGAAGATTTCGGTGATC-3'), responsible for the integration diagnostics
203 of all *sat* expression vectors; and the F3002 reverse primer (5'-
204 CTGCAGGTTCACCTACAGCTAC-3'), responsible for the integration diagnostics of
205 all *ssu* integration vectors. The PCR conditions for the F3001/A1715 pair of primers were
206 as follows: 1 cycle at 95 °C for 5 min for the initial denaturation, followed by 35 cycles
207 of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and 1 cycle at 72 °C for 10 min for
208 final extension; whereas the PCR conditions for the F2999/F3002 pair of primers were: 1
209 cycle of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 53°C for 30 s and 72
210 °C for 1 min, and 1 cycle at 72 °C for 10 min.

211 Lbk39 recombinant protein purification was carried out using a HisTrap HP 1-mL
212 column (GE HealthCare) by loading the culture media onto the column according to the
213 manufacturer's instructions. Afterwards, salts and imidazole were removed by dialysis in
214 a PBS buffer at 4 °C, twice for 2 h and once overnight. Then, lyophilisation was
215 performed to concentrate the purified recombinant protein. To analyse whether the
216 purification process had been successful, the purified and dialysed recombinant protein
217 was concentrated with trichloroacetic acid (TCA), as indicated in the LEXSYcon2
218 Expression kit manual, loaded on a 15% SDS-polyacrylamide gel electrophoresis (SDS-
219 PAGE), and stained with silver nitrate. The protein determination was performed using
220 the Micro BCA™ Protein Assay Kit (Thermo Fischer Scientific) following the
221 manufacturer's procedure.

222

223 *2.4 Enzyme-linked immunosorbent assay (ELISA)*

224 To determine whether levels of anti-*Leishmania* antibodies in human serum from
225 uninfected individuals and those infected with CL and other diseases can be detected
226 using Lbk39 epitopes, optimal ELISA conditions were established (De Souza et al.,
227 2018). A range of serum dilutions (1:100, 1:200, 1:400 and 1:800), antibody-conjugate
228 dilutions (1:5,000, 1:10,000 and 1:20,000) and antigen dilutions (0.1 µg, 0.5 µg and 0.85
229 µg/100 µL/well) were tested in various combinations.

230 High-binding polystyrene microtiter plates (96 well EIA/RIA 1x8 Stripwell Plate,
231 Costar, USA) were coated overnight at 4 °C with 100 µL/well solution of antigen diluted
232 in a carbonate–bicarbonate buffer (pH 9.6). On the following day, the plates were washed
233 twice with 200 µL/well of a washing solution (0.9% w/v NaCl, 0.05% v/v Tween 20),
234 and then the wells were blocked with 120 µL of a blocking solution (PBS + 0.1% w/v
235 casein) for 1 h at 37 °C. Afterwards, they were washed twice again with 200 µL/well of
236 the washing solution. Following the washing step, serum samples were diluted in an
237 incubation solution (PBS + 0.25% w/v casein) and were added in their respective wells
238 and incubated at 37 °C for 1 h. Then the plates were washed four times with 200 µL/well
239 of the washing solution, and a polyclonal goat anti-human IgG HRP conjugate (2 mg/mL,
240 SanBio Científica) was diluted and was added to each well for 1 h at 37 °C. Finally, the
241 reaction was developed by adding 100 µL of a 10.5-mL citrate buffer (4.5% w/v Na_2PO_4,
242 3.25% w/v citric acid, pH 5.0), with 2 mg of o-Phenylenediamine dihydrochloride (2
243 mg/tablet, Sigma, USA) and 2 µL of 30% (w/w) H_2O_2 to each well at room temperature
244 for 15 min, avoiding light, and then 20 µL of a solution 1:20 of H_2SO_4 was added to stop
245 the reaction. Plates were read in a Powerwave HT reader (BioTek) at 492 nm, and values
246 were expressed in absorbance.

Soluble proteins from the crude extract of *L. braziliensis* promastigotes (strain MHOM/BR/84/LTB300) were included in the study as a positive control. As additional controls, the pooled positive and negative serums were included in each plate when testing individual sera; each sample was measured in triplicate, and the whole assay described above was performed in duplicate.

2.5 Statistical analysis

The receiver operating characteristic (ROC) curve was derived based on the logistic regression model, considering the classification of the samples (presence or absence of the disease) as a dependent variable and each antigen as an independent variable. Logistic regression model, ROC curve and sensitivity and specificity analyses were performed using R software (R CORE TEAM, Version 3.4.4, 2018) with an auxiliary pROC system (Robin et al., 2011). We used analysis of variance (ANOVA) and Tukey's test to compare the differences in absorbance between the groups. A significance level of $p<0.05$ was adopted.

3. Results

3.1 Lbk39 plasmid construction

Gene synthesis using pLEXSY *E.coli*/*L.tarentolae* shuttle vectors was used to construct the Lbk39 plasmid, encoding *L. braziliensis* sequences homologous to the *L. infantum* rK39 antigen (Suppl. Fig. 1A). BLAST similarity sequence analysis of the cloned sequence confirmed it comprised a 843-bp product (828 plus 15 bp vector flanking sequences) homologous to the locus LBRM_14_1110 of *L. braziliensis* (strain MHOM/BR/75/M2904), and which exhibited 84% nucleotide sequence identity and 59% amino acid identity with the equivalent kinesin-related gene of *L. infantum* (Suppl. Fig.

1C). The predicted protein encoded a protein of 281 amino acids with a predicted molecular mass of 30 kDa, with six copies of 39 AA repeats. The cloned sequence was then inserted into the pLEXSY-sat2 vector for secretion and addition of a C-terminal 6×His-tag (Suppl. Fig. 1B). The purification of the Lbk39 plasmid yielded 1.5 µg/µL of DNA, and plasmid identity was confirmed by sequencing the purified product, with 100% identity for both forward P1442 and reverse A264 sequencing primers.

3.2 Lbk39 plasmid transfection into Leishmania tarentolae

Linearization of the plasmid through digestion with the *SwaI* (*SmiI*) enzyme generated a 2.9-kbp fragment related to the *E. coli* part and a larger fragment (approximately 5 kbp) related to the Lbk39 plasmid (Fig. 1A). The linearized plasmid was used to transfect *L. tarentolae* under antibiotic selection, and after approximately 10 days of Lbk39 LEXSY culturing, the cultures became turbid (10^7 cells/mL), and there was no noticeable growth of the parasites in the negative control flasks. On the 12th day of culturing, another passage was made, and 2 mL of that dense culture were withdrawn to perform the confirmation of Lbk39 genomic integration through PCR. For this objective, two pairs of primers were used: one of them amplifying from within the expression cassette and the other amplifying to a chromosomal *ssu*-flanking sequence that was not present on the plasmid. The PCR reactions resulted in two DNA fragments of different sizes, one for each pair of primers, as expected and indicated by the manufacturer: a 1.1 kbp fragment size for F3001/A1715 primers and a 2.3 kbp fragment size for F2999/F3002 (Fig. 1B).

3.3 Purification of Lbk39 protein

The Lbk39 protein was expressed as a 6xHis-tagged recombinant protein in the pLEXSY-sat2 vector, inserted into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* and designed to be secreted into the culturing media with predicted molecular mass of 31.2

kDa (Suppl. Fig. 1B). The purification procedure and the protein expression were confirmed by SDS-PAGE, with the purified recombinant protein exhibiting a molecular mass of approximately 35 kDa (Fig. 2). The purified Lbk39 recombinant protein was obtained at a final concentration of 0.4 mg/mL.

3.4 Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was developed and standardised using the recombinant protein as an antigen for detection of specific anti-*Leishmania* antibodies in the sera of leishmaniasis patients. The optimum combination of conditions were found to be as follows: antigen concentration of 0.1 µg/100 µL/well; serum samples diluted to 1:200 in incubation solution (PBS + 0.25% w/v casein); and polyclonal goat anti-human IgG HRP conjugate (2 mg/mL) diluted to 1:10,000.

The presence of anti-*Leishmania* antibodies was determined by comparing antibody levels in patients infected with CL and VL with healthy individuals living in the same endemic area, as well as individuals with Chagas disease, using the Lbk39 recombinant protein as the antigen. The results obtained were compared using the same parameters and the same sample groups using as a positive control antigen a crude extract of *L. braziliensis* promastigotes. Comparing the two types of *Leishmania* antigen for patients known to have leishmaniasis, the Lbk39 antigen showed sensitivity of 88% for CL and of 100% for VL patients. The specificity was 98% and 100%, respectively (Table 2 and Fig. 3). Based on the percentage positivity for all serum samples and sensitivity and specificity values, the Lbk39 antigen was able to detect antibodies from both CL and VL patients. The results obtained with the Lbk39 antigen were equivalent to or better than those using crude promastigote extract, except for a small reduction in specificity for CL patients (98% versus 100%).

323 The range of absorbance readings obtained in these assays are shown in Figure 4.
324 Using the crude antigen, the range of readings obtained was high with the CL patients
325 (Group 1), otherwise the readings were well grouped. However, the crude antigen also
326 gave high readings with patients diagnosed with Chagas disease (Group 3). With the
327 recombinant Lbk39 antigen the cross-reactivity with Chagas patient sera was much
328 reduced, and the readings with the CL patient group were more tightly grouped. Even so
329 the median value for the Chagas patients (Group 3) was still higher than that for CL
330 patients. However, a wider range was found in the VL group using the LbK39 antigen.
331 ANOVA indicated significant differences between the means of the various groups for
332 both antigens. Pairwise comparisons using Tukey's test showed these differences were
333 significant ($p < 0.05$) in all cases except between Groups 1 and 2 (CL and VL patients)
334 with *L. braziliensis* crude antigen, and between Groups 1 and 3 (CL and Chagas patients)
335 with LbK39. In all cases the control patients (Group 0) were significantly different to all
336 infection groups.
337
338 4. Discussion
339 Several recombinant proteins have been investigated for their anti-*Leishmania*
340 antibody responses in patients in attempts to develop the most suitable antigens for
341 diagnostic purposes, of which the best so far is the rK39 antigen for VL (Badaro et al.
342 1996). However, although recombinant proteins have been extensively used for specific
343 antibody detection, their use in diagnostic tests has revealed some problems. For example,
344 they can be less immunoreactive than the corresponding purified antigen due to the
345 absence of post-translational modifications, depending on the protein expression system.
346 Further, their production in high quality and quantity is almost always laborious and they
347 can be expensive to produce. Here we explored the use of the pLEXSY/*Leishmania*
348 *tarentolae* system for cloning, transfection and recombinant protein production for

leishmaniasis immunodiagnosis, in particular for the diagnosis of *L. braziliensis* infection.

We designed a sequence based on *L. braziliensis* to generate a product that we named Lbk39, which exhibited 59% amino acid identity to the kinesin-related gene of *L. infantum* (rK39) that is already used in the serodiagnosis of VL. The Lbk39 sequence was synthesized, inserted into pLEXSY-sat2 recombinant vector and cloned with a 6×His-tag, then inserted into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* and selected to be secreted into the culturing media. Successful expression of a ~35kDa protein was achieved, correlating with the predicted molecular mass of the 289 amino acid 31.2 kDa recombinant protein (Suppl. Fig. 1B). Therefore, we can now add Lbk39 to the wide range of proteins that can be expressed in the *L. tarentolae* system (Kushnir et al., 2011). Recombinant protein production via large-scale fermentation of *L. tarentolae* is not expensive and allows yields of 0.1 to 5 mg/L to be achieved (Basile and Peticca, 2009; Mureev et al., 2009; Kovtun et al. 2011). As used in our study, improved expression is also obtained if the target gene is followed by the 3'-UTR (intergenic untranslated regions) from a highly expressed gene, because in trypanosomatids the regulation of protein expression generally occurs by a post-transcriptional process involving the UTRs as in present work (Niimi, 2012; Sugino and Niimi, 2012; Fernández and Veja, 2013). Expression and purification of the target antigen in the current study yielded recombinant Lbk39 antigen at 0.400 mg/mL.

Lbk39 was evaluated for the serodiagnosis of CL due to *L. braziliensis* and showed 88% sensitivity and 98% specificity, compared to 98% sensitivity and 100% specificity in VL patients. Absorbance values ranged between 0.46 and 0.016 for CL and between 0.92 and 0.03 for VL patients. The difference between positive and negative sera was similar (30 and 28.4 times for VL and CL, respectively. These findings show that,

whilst the target protein was produced from a kinesin-related gene of *L. braziliensis* and performed well with *L. braziliensis* patients, antibodies from patients with VL (*L. infantum*) were also able to recognise Lbk39. The recombinant protein was also recognised by sera from Chagas patients. These are interesting results, however, they do not compromise the use of Lbk39 for serodiagnosis of CL as the other clinical features of infection in VL or Chagas disease are quite different. In fact, given the difficulty in making a positive diagnosis for CL, it may be an advantage and facilitate the usage of Lbk39 as a general immunodiagnostic antigen for both VL and CL, since the main issue is a lack of existing tools for the latter. A greater sampling of patients with cutaneous leishmaniases coming from different regions of Latin America will be needed to confirm if this is a useful approach. The cross-reactivity between the CL and VL patients likely arises from two factors, the first of which is the conservation between Lbk39 and the homologue from *L. infantum* (Suppl. Fig 1), which, while only 59% at the amino-acid level, is concentrated in several immunogenic repeat motifs. The second factor is the very high antibody response stimulated by VL infection compared to CL, which also explains the relatively high values seen in the ELISA results for VL sera with Lbk39, and the high sensitivity.

The sensitivity and specificity of a leishmaniasis immunodiagnostic test is influenced by various factors such as antigenic and structural properties of the antigen itself, the duration of the infection, number of lesions, and variation in the parasite and host population. Regarding properties of the antigen, although no previous studies have investigated the levels of antibodies against *L. braziliensis* kinesin-related proteins in CL and VL patients until this study, a few have investigated the reaction of antibodies from CL patients against *L. infantum* kinesin-related recombinant proteins. For example, Molinet et al. (2013) found that all of 272 serum samples from patients with CL in Brazil

were negative when using one commercially available rK39 rapid test. Hartzell et al. (2008) observed that both the rK39 rapid test and ELISA using the rK39 antigen demonstrated a positivity of only 10.2 and 28.8%, respectively, in United States soldiers stationed in Afghanistan and Iraq who had contracted CL (mostly due to *L. major*). Likewise, Oliveira et al. (2011) evaluated several recombinant antigens that demonstrated the ability to identify *Leishmania infantum*-infected patients and found that CL patients were generally less well identified. Interestingly, only 3 out of 26 CL patients showed a positive result using an antigen that encoded a C-terminal fragment of an *L. infantum* kinesin. One potentially important difference to the current study is that the recombinant antigens described by Oliveira et al. were expressed in *E. coli*, perhaps compromising their sensitivity for CL diagnosis. According to Moreno et al. (2009), the high titres of the anti-rK39 antibody in patients with acute VL are explained by expression in the high number of amastigotes present, compared to asymptomatic patients that have lower numbers of amastigotes. However, here we show that Lbk39 is capable of detecting antibodies in CL patients, which also have low numbers of amastigotes. Another factor to consider is the potential diversity of the diagnostic antigen. In that regard, Bhattacharyya et al. (2013) showed that there is diversity in rK39 sequences between *L. infantum* and *L. donovani*, which may explain the poorer performance of rK39 in diagnosis of East African VL due to *L. donovani*. Therefore, potential diversity in Lbk39 should also be investigated in further studies (Cupolillo et al., 2003). Finally, the reasons why kinesin-related cytoskeletal proteins have been found to be good antigens for serodiagnosis is not fully understood but presumably is related to their structure, as they contain repetitive amino acid sequences thus presumably providing multiple stimulation of antibody responses.

Regarding other factors that affect sensitivity and specificity in the current context, duration of the infection is significant. In patients with recent lesions (1 to 6 months of progression), serological negativity is higher, and parasitological tests are more sensitive and specific (Costa et al., 1996). Also, in the case of positive serology, the mean titres are significantly higher in patients with multiple lesions, reflecting the higher antigenicity induced by a larger number of parasites. Even within the same species, genetic variability of responses can be high and the antigen used for serological testing can give different results. For example, the rK39 antigen used to detect VL antibodies in several regions of the world, shows variable sensitivity and specificity according to geographical region and ethnic groups (Singh et al., 2009; Mohapatra et al., 2010). Different host immune responses may also be responsible for the variability of serological test results. Goto et al. (2009) analysed antibody responses to the rK39 antigen in humans and dogs with VL, and they noticed that humans showed much stronger immune responses to the rK39 antigen than dogs, concluding that the rK39 recombinant antigen is very specific towards detecting VL in humans only. However, Porrozzi et al. (2007) revealed that the IgG response to the rK39 antigen was variable in asymptomatic dogs (sensitivity of 66%) and significantly higher in symptomatic dogs (sensitivity of 100%). Likewise, 33% of *L. braziliensis*-infected dogs were positive for the rK39, and 11% of dogs with leptospirosis were also positive showing cross reactivity. For intervention programmes of leishmaniases in humans and dogs, an ideal serodiagnostic tests must be able to identify infected and non-infected reservoirs, specifically in dogs (Quinnell et al., 2013), thus providing the possibility of guided control and treatment.

In summary, this study showed that the recombinant Lbk39 protein produced was able to recognize *Leishmania* infection in the serum of humans with cutaneous or visceral leishmaniases in Brazil. This is a particularly important result for the *L. braziliensis* CL

patients, where there is a lack of serological tests. Further work is required to investigate the potential use of this antigen in different population sera, as well as in different geographical regions in order to determine the specific ability to detect anti-*Leishmania* antibody levels in patients with CL or VL. In addition, analysis of the response to Lbk39 by sera of dogs infected with *L. infantum* should be considered in order to determine how the antigen behaves in different hosts.

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598 **Figure captions**

599

600 **Figure 1. A.** Linearization and partial digestion of the Lbk39 plasmid with the *Swa*I
601 (*Smi*I) enzyme. MM: molecular mass markers (1kb DNA ladder); Lane 1 (a): 2.9 kbp
602 fragment related to the *E coli* part; Lane 1 (b): a larger fragment (approximately 5 kbp)
603 related to the Lbk39 plasmid; Lane 1 (c): the entire Lbk39 plasmid, linearised, with
604 8486bp. **B.** PCR for confirmation of the Lbk39 genomic integration into the chromosomal
605 18SrRNA (*ssu*) locus of *L tarentolae* resulted in two DNA fragments of different sizes:
606 2.3 kbp fragment size for F2999/F3002 pair of primers (lane 1) and 1.1 kbp fragment size
607 for F3001/A1715 pairs of primers (lane 2) MW: molecular weight markers.

608

609 **Figure 2.** The purified Lbk39 recombinant protein expression confirmed by SDS-PAGE.
610 Lane 1: molecular mass markers (10-225 kDa); Lane 2: crude extract of *L. tarentolae*
611 non-transfected; Lane 3: purified Lbk39 recombinant protein; Lane 4: crude extract of *L.*
612 *tarentolae* transfected.

613

614 **Figure 3.** Receiver operating characteristic (ROC) curve analysis for Lbk39 antigen
615 compared to the positive control *L. braziliensis* crude extract of *Leishmania (Viannia)*
616 *braziliensis* (strain MHOM/BR/84/LTB300). A to C = Lbk39 antigen ROC
617 curve comparing results of the group healthy non-endemic individuals vs. the group with
618 active skin lesions (A); visceral leishmaniasis (B) and Chagas disease (C). D to F = ROC
619 curve results from crude extract of the *L. (V.) braziliensis* in healthy non-endemic
620 individuals vs. group with active skin lesions (D); visceral leishmaniasis (E) and Chagas
621 disease (F). AUC = area under curve.

622

623 **Figure 4.** Levels of anti-Lbk39 antibodies detected by means of indirect ELISA in
624 individuals from CL endemic and non-endemic areas in Brazil compared to levels of anti-
625 *L braziliensis* antibodies detected by the same technique. Group 0: healthy individuals from
626 non-endemic areas; Group 1: CL patients with active lesion and culture positives; Group
627 2: VL: positive patients for VL; Group 3: serum from patients with Chagas disease.
628 ANOVA p values are shown for each antigen, together with pairwise Tukey's tests for
629 comparisons between groups for the same antigen.