UNIVERSIDADE FEDERAL DO PARANÁ

LÍGIA MORAES BARIZON DE SOUZA

PERFORMANCE EVALUATION OF SELECTED AND RECOMBINANT ANTIGENS FOR DETECTION OF LEISHMANIASES BY ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

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PERFORMANCE EVALUATION OF SELECTED AND RECOMBINANT ANTIGENS FOR DETECTION OF LEISHMANIASES BY ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Engenharia de Bioprocessos e Biotecnologia.

Orientadora: Professora Dra Vanete Thomaz Soccol

Coorientador: Professor Dr Paul A. Bates

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RESUMO

Métodos eficazes para o diagnóstico de leishmaniose são urgentemente necessários, seja para prevenção e controle da leishmaniose em áreas endêmicas ou direcionamento do tratamento de pacientes infectados. Este estudo descreve um imunoensaio ligado à enzima (ELISA) utilizado para medir os níveis séricos de anticorpos anti-α-Gal em indivíduos com leishmaniose cutânea (LC) e a avaliação de uma proteína recombinante relacionada com o gene da cinesina de *Leishmania braziliensis* (Lbk39) para o diagnóstico de leishmaniose. Para isso, duas neoglicoproteínas contendo a fração terminal Galα1-3Gal (Galα1-3Galβ1-4GlcNAc-HAS, identificado como NGP 2334, e Galα1-3Gal-HAS, identificado como NGP2203) e o análogo de neoglicoproteína contendo a fração terminal Galα1-3Gal (Galα1-3Galβ1-3GlcNAc-HAS, identificado como NGP2333) foram testados. Já o gene sintético Lbk39 de *L. braziliensis* foi concebido com base no gene relacionado com a cinesina de *L. infantum*. O gene sintético foi, então, inserido no vetor recombinante pLEXSY-SAT2, com adição de cauda de histidina (6×His-tag) e transfectados para células hospedeiras de *L. tarantole*. Após a produção e purificação da proteína recombinante, a mesma foi avaliada quanto à sua capacidade de reconhecer anticorpos anti-*L. braziliensis* e anti-*L. infantum.* Condições ótimas de ELISA foram estabelecidas para todos os antígenos testados. As médias de títulos de anticorpos anti-α-Gal de pacientes com LC foram significativamente maiores ($p<0.05$) do que as médias dos indivíduos saudáveis para todos os NGPs testados. Notou-se tambem que todos osantígenos de NGPs foram capazes de ser mais bem detectados por anticorpos anti-α-Gal de pacientes com LC que estavam sob observação pós-tratamento. Nenhum dos antígenos de NGPs foi reconhecido por anticorpos de indivíduos saudáveis, indicando que o dissacarídeo Galα1-3Galβ é o sacarídeo imunodominante na superfície de células de *Leishmania* e é o único com estrutura terminal de glicoesfingolípidos não redutor reconhecidos por anticopos anti-α-Gal. O antígeno Lbk39 foi fracamente reconhecido por pacientes com LC, apesar do alto valor de sensibilidade obtido (88.9%). Tal antígeno tambem demonstrou capacidade de detecção de níveis de anticorpos de pacientes com LV significativamente ($p<0.05$) mais elevada do que pacientes com LC, com alta sensibilidade (80%) e especificidade (95.5%). Apenas reações cruzadas com soro de pacientes com doença de Chagas foram observadas para todos os antíegnos testados. Pode concluir-se que as glicoproteínas provaram ser uma ferramenta muito mais confiável para o diagnóstico serológico de LC, apesar dos altos valores de sensibilidade e especificidade obtidos para o antígeno recombinante Lbk39 para pacientes com LC e LV.

Palavras-chave: Anticorpos. Diagnóstico sorológico. *L. braziliensis*. Neoglicoproteínas. Proteína recombinante. Sacarídeos.

ABSTRACT

Rapid methods for effective leishmaniases diagnosis and species identification are urgently needed, either by the prevention and control of leishmaniases in endemic areas or the treatment of infected patients. This study describes an enzyme-linked immunosorbent assay (ELISA) used to measure serum levels of anti-α-Gal antibodies in individuals with cutaneous leishmaniasis (CL) and the evaluation of the *Leishmania braziliensis* kinesin-related recombinant protein (Lbk39) for the diagnostic of leishmaniases. For that, two neoglycoproteins (NGP) containing the Galα1-3Gal terminal fraction (Galα1-3Galβ1-4GlcNAc-HAS, identified as NGP 2334; and Galα1-3Gal-HAS, identified as NGP2203) and one Galα1-3Gal NGP analogue (Galα1-3Galβ1-3GlcNAc-HAS, identified as NGP2333) were tested. In addition, the synthetic Lbk39 gene was designed based on the kinesin-related gene of *L. infantum* and the homology search was performed by BLAST similarity in tritrypDB database website. The Lbk39 gene was inserted into pLEXSY-sat2 recombinant vector, cloned with 6×His-tag and transfected into *L. tarantole* host cells. Optimal ELISA conditions were established for all antigen tested. Means of anti-α-Gal antibody titres of CL patients were significantly ($p<0.05$) higher compared to the healthy individuals for all of NGPs tested and could be detected at the same level when crude extract from *L. braziliensis* as antigen was used. It can also be noticed that all NGPs were able to be better detected by anti-α-Gal antibodies from CL patients that had just finished the anti-*Leishmania* treatment. None of the NGPs was significantly ($p<0.05$) recognized by the healthy individuals, indicating that the disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal. The Lbk39 recombinant antigen was weakly recognized by CL patient's antibodies, despite its high sensitivity value (88.9%). The antigen also demonstrated significantly ($p<0.05$) higher antibody detection levels for VL patients, with high sensitivity (80%) and specificity (95.5%). Cross-reaction only with the Chagas Disease patients was observed for all antigens tested. It can be concluded that glycoproteins have proven to be a much more reliable tool to the serological diagnosis of CL, despite of the high values of sensitivity of the Lbk39 recombinant antigen for both CL and VL.

Key words: Antibodies. *L. braziliensis*. Neoglycoproteins. Saccharide. Serological diagnosis,. Recombinant vector.

FIGURE LIST

GENERAL INTRODUCTION

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ACRONYMS AND ABBREVIATIONS LIST

AA/ aa	-	Amino acid
AUC	-	Area under the curve
Ala	-	Alanine
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine serum albumin
CBAG	-	Coffee bean α-galactosidase
CD	-	Chagas disease
CD4	-	Cluster of differentiation 4 - glycoprotein found on the surface of immune cells
CD8	-	Cluster of differentiation 8 - transmembrane glycoprotein that serves as a co-receptor for the T cell receptor
CL	-	Cutaneous leishmaniasis
DNA	-	Deoxyribonucleic acid
ELISA	-	Enzyme-linked immunosorbent assay
GIPL	-	Glycoinositol phospholipids
GPI	-	Glycosylphosphatidylinositol
H_2SO_4	-	Sulphuric acid
IFN	-	Interferon (cytokine)
IgG, IgA, IgM	-	Immunoglobulins types A, G, and M
IgG HRP	-	Immunoglobulin G bonded to horseradish peroxidase enzyme
IL	-	Interleukin (cytokine)
LEXSY	-	*Leishmania* Eukaryotic Expression System
LPG	-	Lipophosphoglycan
Met	-	Methionine
ML	-	Mucocutaneous leishmaniasis
NaCl	-	Sodium Chloride
NGP	-	Neoglycoprotein
NO	-	Nitric oxide
PBS	-	Phosphate buffer saline
PCR	-	Polymerase Chain Reaction
PKDL	-	Post-kala-azar dermal leishmaniasis
PNV	-	Predictive negative value
PPV	-	Predictive positive value
RNA	-	Ribonucleic acid
ROC	-	Receiver Operating Characteristics
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	-	Serine
TC (flask)	-	Tissue culture (flask)
Th	-	T helper lymphocyte
Thr	-	Threonine
TNF	-	Tumor necrosis factor
UTR	-	Intergenic untranslated regions

VL - Visceral leishmaniasis

SYMBOL LIST

bp/ kbp	-	Base of pairs/ a thousand base of pairs
g	-	Grams
kDa	-	Kilo Dalton
L	-	litre
Mb	-	Megabases (10^6 bases)
mg	-	Milligram
mL	-	Millilitre
ng	-	Nanogram
nm	-	Nanometre
U	-	Unit
ºC	-	Celsius degree
µg	-	Microgram
µL	-	Microlitre
%	-	Percentage
™	-	Trade mark

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1. PRESENTATION

This thesis is organized into a general introduction, objectives, followed by the chapters one and two, and, lastly, the final considerations and perspectives. The general introduction is where general information about *Leishmania* parasites and the group of diseases that it causes are going to be addressed, as well as their main characteristics, host-parasite relationships, disease immunology and, principally, about the leishmaniases diagnosis.

Following the objectives of this study, the subsequent two chapters will explore different serological diagnosis antigens, and their manner of obtention and production, in order to detect the presence of *Leishmania* parasites in patients stricken with the cutaneous form of the disease. The chapters' titles are arranged as follows:

Chapter 1: Analysis of *Leishmania* mimetic neoglycoproteins for the cutaneous leishmaniasis diagnosis;

Chapter 2: Evaluation of an Enzyme-linked immunosorbent assays based on the *Leishmania braziliensis* kinesin-related recombinant antigen for serodiagnosis of leishmaniases in Brazil.

Finally, the final considerations and perspectives will point out new possibilities of future works in this field based on the findings of this study.

1. GENERAL INTRODUCTION

Leishmaniases are a group of parasitic diseases caused by the protozoan of the genus *Leishmania*, transmitted by the bite of female sandflies. It is known that there are at least 21 species of *Leishmania* causing disease in humans worldwide and each one show different levels of virulence, pathogenicity, and clinical manifestations. The leishmaniases have been separated into two groups, according to the geographic region where the infection is acquired: Old World leishmaniases, found in the Mediterranean basin, Middle East, and Africa; and New World leishmaniases, found in Mexico, Central America, and South America. (DAVID & CRAFT, 2009; DE VRIES, REEDIJK & SCHALLIG, 2015).

Leishmaniases are divided into three distinct forms of clinical presentation. The major factor that determines the development of each form of the disease is the specie of parasite associated with the host's specific immune responses - not all those infected by the parasites will develop the disease. In the most common form of the disease, cutaneous leishmaniasis (CL), the parasites remain only at the site of the sandfly bite and cause localized, long-term ulceration with no systemic symptoms. In some cases, inadequate treatment of a CL lesion may lead to later development of mucocutaneous leishmaniasis (ML). Visceral leishmaniasis (VL) is the most serious form of the disease in which parasites migrate from the inoculation site to multiply in the host's liver and spleen macrophages and bone marrow, causing immunosuppression and death if not treated. Lastly, but no less important, post-kala-azar dermal leishmaniasis (PKDL) is a dermal manifestation of visceral and can be present any time after remission of the infection. (PEACOCK, 2007; CAMPOS *et al.*, 2008; DAVID & CRAFT, 2009; BIFELD & CLOS, 2015; VAN DER AUWERA & DUJARDIN, 2015).

The control of leishmaniases is presently a serious problem due to the high death rates involved and the economic losses resulting from morbidity. In addition, the disease is strongly linked to poverty in the tropical and subtropical areas and with ever increasing cases worldwide each year. Ninety-eight countries are officially considered endemic for leishmaniases and estimates show that more than 58,000 VL and 220,000 CL cases are notified per year. More than 90% of global VL cases occurring in six countries - India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia - and around 75% of global CL cases occurring in ten countries - Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru. (ALVAR *et al.*, 2012; DE VRIES, REEDIJK & SCHALLIG, 2015).

Leishmania parasite infections are originally zoonotic and currently three different epidemiological cycles of leishmaniases are known: 1) The sylvatic cycle, where infection in humans is accidental and transmission occurs in wild foci; 2) The peridomestic cycle, where the reservoir is a peridomestic or domestic animal and the parasite is transmitted to humans by anthropophilic sandflies; and 3) The anthroponotic cycle, where the animal reservoir is unknown and the sandflies are completely anthroponotic. (BHARGAVA & SINGH, 2012; GEORGIADOU, MAKARITSIS & DALEKOS, 2015). Different environmental conditions that influence the mammalian reservoir distribution and create appropriate conditions for development of the sandfly vectors are closely related to the leishmaniases distribution. It can be seen in the case of deforestation in tropical and subtropical regions where the re-emergence of leishmaniases has been happening due to the invasion of peridomestic animals and the sandfly in urban areas. (COSTA *et al.*, 2015). A summary of the main information about parasites of the genus *Leishmania* is given in Table 1 below:

Table 1. Species* of *Leishmania* that causes human disease.

	Main clinical pathology	Transmission cycle	Main geographical distribution
New World *Leishmania* spp			
L. (Viannia) braziliensis	LCL and mucosal	Zoonotic	South America ,parts of Central America, Mexico
L. (V.) panamensis	LCL and mucosal	Zoonotic	Northern South America and southern Central America
L. (V.) peruviana	LCL	Zoonotic	Peru
L. (V.) guyanensis	LCL	Zoonotic	South America
L. (V.) lainsoni	LCL	Zoonotic	South America
L. (V.) colombiensis	LCL	Zoonotic	Northern South America
L. (Leishmania) amazonensis	LCL and DCL	Zoonotic	South America
L. (L.) mexicana	LCL and DCL	Zoonotic	Central America, Mexico, USA
L. (L.) pifanoi	LCL	Zoonotic	South America
L. (L.) venezuelensis	LCL	Zoonotic	Northern South America
L. (L.) garnhami	LCL	Zoonotic	South America
Old World *Leishmania* spp			
L. (L.) aethiopica	LCL and DCL	Zoonotic	Ethiopia, Kenya
L. (L.) killicki	LCL	Zoonotic	North Africa
L. (L.) major	LCL	Zoonotic	Central Asia, north Africa, middle east, East Africa
L. (L.) tropica	LCL	Anthroponotic	Central Asia, middle east, parts of north Africa, southeast Asia
L. (L.) donovani	Visceral and LCL	Anthroponotic	Africa, central Asia, southeast Asia
Old and New World *Leishmania* spp			
L. (L.) infantum	Visceral, LCL	Zoonotic	Europe, north Africa, Central America, South America

LCL = localised cutaneous leishmaniasis. DCL = diffuse cutaneous leishmaniasis. *Subgenus is given in parentheses. Southeast Asia includes the Indian subcontinent and China. Source: Reithinger *et al*., 2007.

Once a *Leishmania* infected sandfly takes a blood meal on another mammal, the promastigotes forms of *Leishmania* are inoculated into the host's skin, where they are phagocytosed by macrophages and dendritic cells (Figure 1). Both cells are simultaneously host cells for the parasite and antigen-cell presenting, but only macrophages are also capable of killing cells infected with the parasite. Inside the macrophages and dendritic cells, the promastigote is subject to a morphological differentiation into the amastigote form induced by the temperature of the host and the acidification of the formed parasitophorous vacuole. (BIFELD & CLOS, 2015).

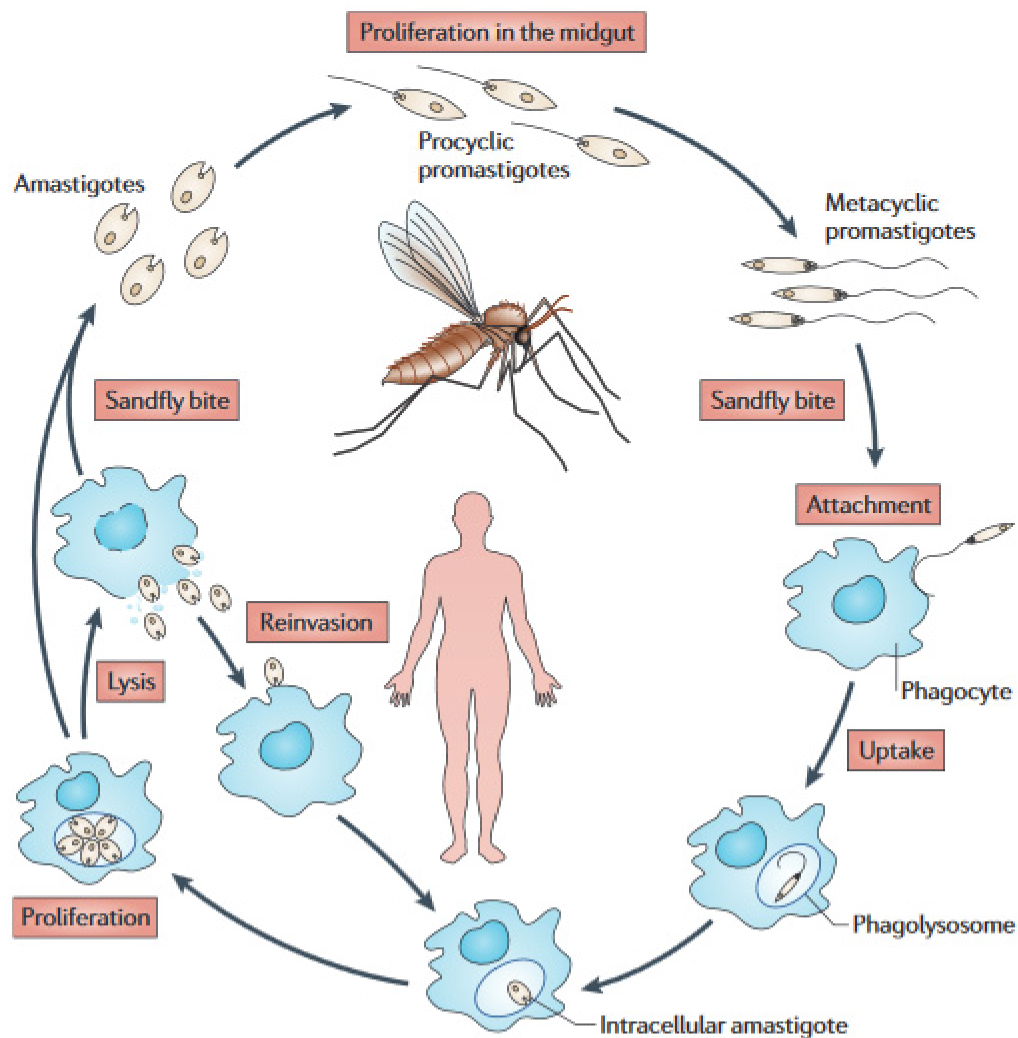

Figure 1. The life cycle of *Leishmania* parasites. During blood feeding, the sandfly regurgitates metacyclic promastigotes that are phagocytosed by one of several possible cell types found in the local environment. After establishing an intracellular residence, metacyclic promastigotes transform into aflagellate amastigotes that undergo replication within host cells. Adapted from Kaye & Scott (2011).

The ability of the host to control infection requires the generation of cell-mediated immune responses able to eliminate intracellular parasites by activation of macrophages

(Figure 2). This process initiates as a strong local inflammatory response with the recruitment of neutrophils and monocytes by the production of chemoattractant by macrophages. While neutrophils are recruited to the inflammatory site within minutes after infection, a massive quantity of monocytes, cells that will differentiate into macrophages or dendritic cells in the tissue, are seen from the second week (Figure 3). (MOUGNEAU, BIHL & GLAICHENHAUS, 2011; CARLSEN *et al.*, 2015; HURRELL, REGLI & TACCHINI-COTTIER, 2016).

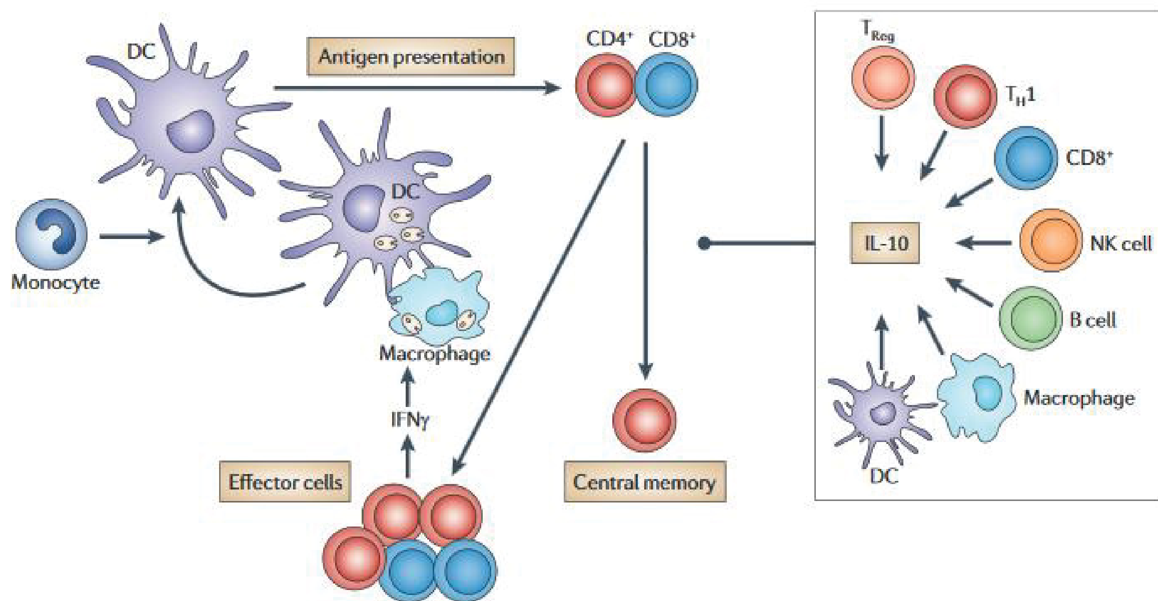

Figure 2. Cellular components of the anti-leishmanial immune response. Monocytes infiltrate the site of infection and differentiate into dendritic cells (DCs). DCs become infected but fail to become activated, whereas local uninfected DCs upregulate major histocompatibility complex class II. CD4+T cells are then activated and differentiate into T helper (Th1) cells, which produce interferon-γ (IFNγ), and this promotes parasite killing by infected cells and also further promotes the development of Th1 cells. CD8+T cells recognizing leishmanial antigens are also activated and also produce IFNγ. Adapted from Kaye & Scott (2011).

For CL, the clinical cure happens when macrophages are activated by the Th1 cell response, which is triggered by antigen-presenting cells and responds with the production of CD4 and CD8 cells and secretion of cytokines, especially IFN-γ, IL-12 and IL-4. Although leishmaniases are characterized by a mixture of Th1 and Th2 cell immune responses, the primary activation of the cell immune response is important for the subsequent course of infection. (ALMEIDA *et al.*, 2003; MURRAY *et al.*, 2005; REITHINGER *et al.*, 2007; KAYE & SCOTT, 2011; MOUGNEAU *et al.*, 2011; BRELAZ *et al.*, 2012; FERRAZ *et al.*, 2015). In the initial period of dendritic cell activation by the presence of the parasites, IL-4 directs the cells to produce IL-12, which promotes Th1 cell maturation, and leads the host to control the disease. But later, IL-4 can induce naive CD4 cells into Th2 differentiation and

this immune response is associated with a non-healing and progressive form of leishmaniases. At the same time, IL-4 stimulates the production of the cytokines IL-5, IL-10, IL-13 by Th2 cells, promotes anti-*Leishmania* antibody production by B cells, and inhibits the action of IFN-γ in producing CD4 cells, suppressing the protective Th1 immune responses. (AL-QADHI, MUSA & HUMMADI, 2015). Although the disease resolution is mediated by T cells, infected subjects by *Leishmania* exhibit high serum levels of parasite-specific IgG and B-cell immune response is dependent on CD4 cells. (MOUGNEAU, BIHL & GLAICHENHAUS, 2011).

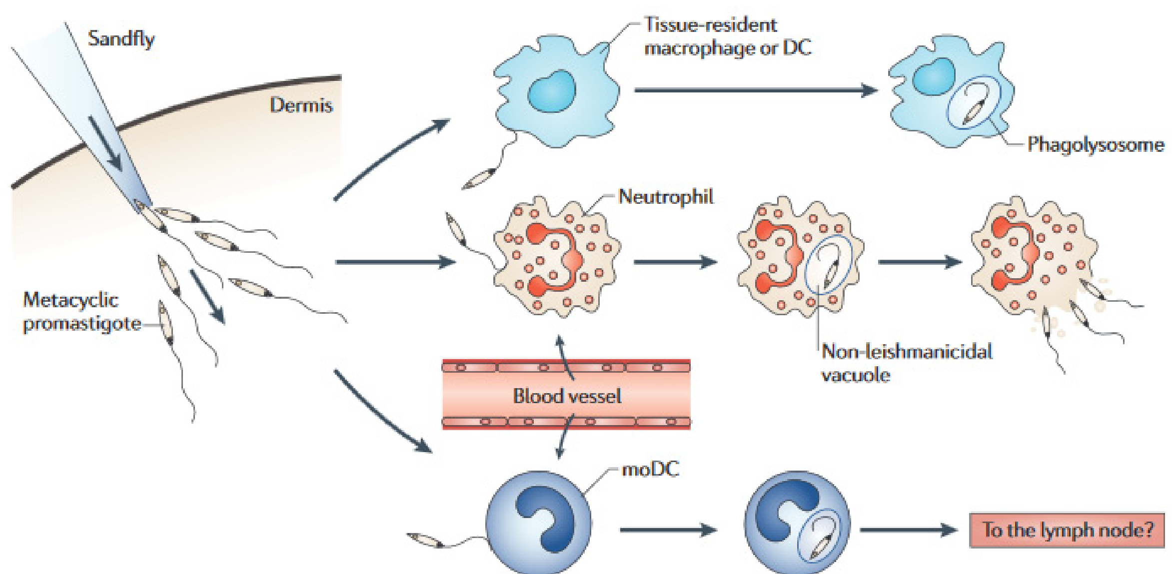

Figure 3. Multiple cell types are involved in the uptake of *Leishmania* parasites. Capillary and other tissue damage resulting from the mechanical trauma of the bite may result in the release of endothelial alarmins, such as interleukin-33 (IL-33), which facilitates the recruitment of neutrophils. The neutrophils swarm around the extracellular metacyclic promastigotes, engulfing many in non-leishmanicidal vacuoles. The death of neutrophils releases metacyclic promastigotes that may be pre-conditioned for survival in other myeloid cells. It is not known whether neutrophils are involved in amastigote uptake after the initial infection. Adapted from Kaye & Scott (2011).

While in CL IL-4 has been implicated in disease progression, in VL its importance has been eliminated. Once inside the host, the presence of promastigotes forms activates the complement system. Although procyclic promastigotes are extremely susceptible to the complement system action, the metacyclic forms can prevent its cell lysis mediated by the complement due to the presence of lipophosphoglycans (LPG) on the surface of *Leishmania* membrane cells. The LPGs are longer in the metacyclic promastigotes membrane cells and are able to prevent the attachment of C5b to C9 subunits of the complement complex, avoiding its lytic action. Also, opsonisation of the promastigotes forms with *Leishmania*

parasite-specific IgG facilitates its uptake by macrophages and dendritic cells, leading to the establishment of infection (Figure 4). (SANTAREM *et al*., 2007; DAS & ALI, 2012).

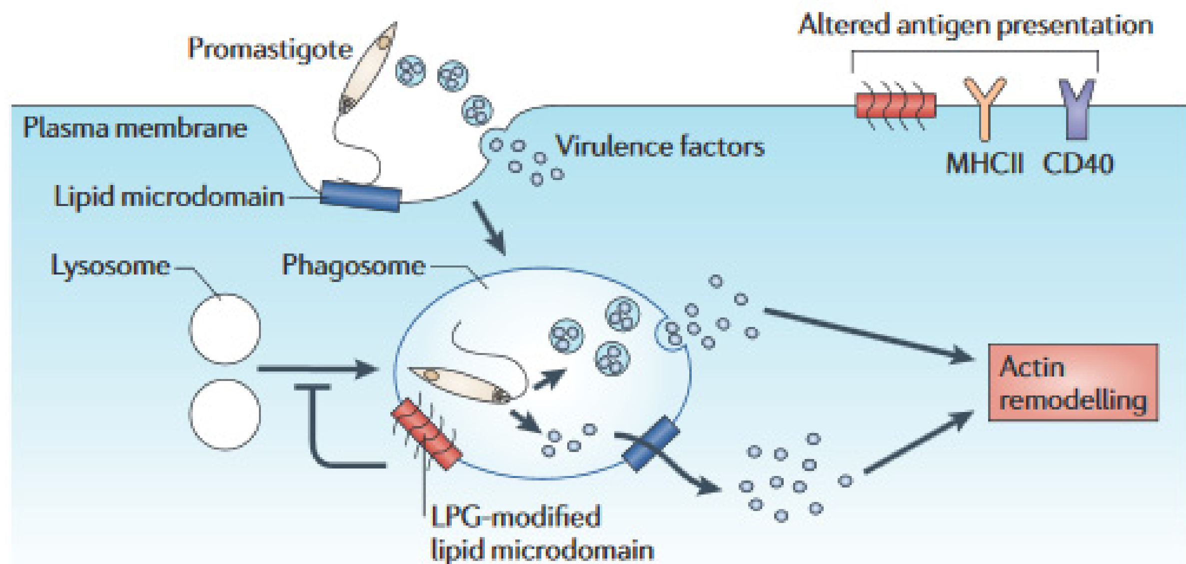

Figure 4. Lipid microdomains during *Leishmania* infection of macrophages. During the initial encounter, lipid microdomains on the plasma membrane of the macrophage have a role in directing parasite uptake and the entry of specific virulence factors such as major surface protein (MSP, also known as GP63).Once the promastigote has entered the phagosome, lipophosphoglycan (LPG) inserts into lipid rafts and inhibits phagosome–lysosome fusion. Adapted from Kaye & Scott (2011).

Rapid methods for the effective leishmaniases diagnosis and species identification are urgently needed, either by prevention and control of leishmaniases in endemic areas or treatment of infected patients. The definitive diagnosis of all suspected *Leishmania* infections should be performed in an association of clinical symptoms, and parasitological and immunologic findings. Several diagnostic methods have been described to detect the presence of the *Leishmania* parasites, with a huge variation in diagnostic accuracy, including direct parasitological examination by microscopy, histopathology and/or parasite culture, indirect testing by serology and molecular diagnostics. The demonstration of the presence of the parasite by parasitological diagnosis is still considered the gold standard in leishmaniases diagnosis because of its high specificity. However, the sensitivity of the direct examinations is low for the diagnosis of CL and ML, with a range from 15% to 70%, whilst in the case of VL the sensitivity varies depending on the tissue used, with a range from 93% to 99% for spleen, 53% to 86% for bone marrow and 53% to 65% for lymph node aspirates. Moreover, parasite

culture in the culture media is difficult, requires technical skills, is prone to contamination, and is time-consuming. As with all other parasitological methods for *Leishmania* detection, the parasite culture does not allow *Leishmania* species determination. The sensitivity of cultures depends on the parasite quantity, but is estimated to be between 60% and 85%. (BHARGAVA & SINGH, 2012; REZVAN, 2014; DE VRIES, REEDIJK & SCHALLIG, 2015; GEORGIADOU, MAKARITSIS & DALEKOS, 2015; PAIVA-CAVALCANTI *et al.*, 2015).

Alternative and complementary diagnosis techniques also include the evaluation of indirect parameters. The Montenegro skin test has been successfully used in the diagnosis of cutaneous and mucocutaneous forms of the disease. Its sensitivity range from 86.4% to 100%, but it is not very reliable in detecting the presence of parasites in recent lesions, in diffuse forms of disease and in immuno-suppressed patients, as well as not differentiating between past and present infection. (GOTO & LINDOSO, 2012; DE VRIES, REEDIJK & SCHALLIG, 2015; HANDLER *et al.*, 2015; PAIVA-CAVALCANTI *et al.*, 2015).

Immunological diagnoses, another important indirect method for detecting leishmaniases, are based on the detection of either *Leishmania* antigens or anti-*Leishmania* antibodies in the host serum samples. The optimal test for serologic diagnosis is one that is easy to use, cheap to make and has both a high sensitivity and specificity. Most of the immunological techniques for the detection of anti-*Leishmania* antibodies have been based on the Enzyme-Linked Immunosorbent Assay (ELISA) technique and the sensitivity and specificity of this method depends on the antigen employed. Considering the variations of the individual immune responses to the infection, several antigens have been identified for potential use in the diagnosis of leishmaniases and modern molecular biology techniques allowing these antigens to be manufactured as a recombinant protein in a standardized manner. (GOTO & LINDOSO, 2012; MAIA *et al.*, 2012; SÁNCHEZ-OVEJERO *et al.*, 2016). Although recombinant antigens have considerably improved the sensitivity and specificity of immunological diagnosis when compared to the crude extract antigens, some of them have significant disadvantages, such as the inability to differentiate between clinically active and asymptomatic infections and long periods of positivity after the cure. (BHARGAVA & SINGH, 2012).

Serological diagnosis is the technique more commonly used for VL and rarely used for CL and ML. High anti-*Leishmania* antibody levels can be found in both asymptomatic and active VL and remain in the host serum for many years, but it does not occur in CL and ML. The rK39, a recombinant protein with several repeated sequences of 39amino acid of kinesin-

related antigen from *L. infantum* (synonym *L. chagasi*), is the most reliable antigen for the serological VL diagnosis. A rapid strip test using this recombinant antigen has been quite successful in VL detection in Asia and South American, reaching between 86% and 100% sensitivity and between 82% and 100% specificity. However, the same antigen has not been able to detect *Leishmania* infections in African patients. Due to its low sensitivity in some regions, the new antigens rK26, rK28 and rK18, all kinesin-related antigens from *L. infantum*, were developed with improved sensitivity without any changes to its specificity. The rK28 antigen is also known to be useful in the diagnosis of VL in Brazil and the rK26 and rK18 antigens are suitable to be used as indicators of parasite clearance after treatment. (RICCIARDI & NDAO, 2015; SAVOIA, 2015; SINGH & SUNDAR, 2015). Other candidates for the serological diagnosis of several forms of leishmaniases are recombinant or purified membrane glycoproteins: gp63, gp70, gp72, and A2 protein. (BHARGAVA & SINGH, 2012; PAIVA-CAVALCANTI *et al.*, 2015). Heat shock proteins (HSPs), and in particular HSP83, have also appeared as potential candidates in serodiagnosis of all forms of leishmaniases. Furthermore, *Leishmania* α-Gal epitopes have been useful in detecting anti-α-Gal antibodies in CL patients by ELISA, showing a higher sensitivity than microscopy analysis. (DE VRIES, REEDIJK & SCHALLIG, 2015).

Most antigens used in serological tests are able to detect high levels of anti-*Leishmania* antibodies in active VL patients and also in truly cured VL subjects, once those individuals continue to produce antibodies against *Leishmania* epitopes even after the elimination of the parasite. For this reason, such results must be interpreted with caution, taking into account both the clinical and epidemiological information about each patient. Also cross-reaction with other diseases can occur. In addition, the serodiagnostic of CL patients is limited, because the sensitivity and specificity are variable due to the low anti-*Leishmania* antibody levels. (GEORGIADOU, MAKARITSIS & DALEKOS, 2015).

It is known that the ability of a host to control and resolve infection caused by *Leishmania* parasites is cell mediated and the outcome of infection will depend on which cell responses the host will first produce. Anti-*Leishmania* IgE antibodies were documented in more than 70% of patients infected with CL, specifically in those infected for less than 2 months, but were not documented in late infected patients, as well as in those atopic patients. The results also showed that patients with multiple lesions had higher levels of this isotype of antibody than patients with a single lesion. The IgE antibodies are produced from the Th2 immune response after the stimulus of a specific cell receptor on mast cells, basophils, and Langerhans cells. The presence of IgE antibodies can be seen as a protective mechanism to

stop the cutaneous dissemination of the infection and to modulate the skin inflammatory response mediated by Th1 cytokines, as well as a serum marker of the presence of the disease. (AL-QADHI, MUSA & HUMMADI, 2015).

Several other cells contribute to the resolution of infections with *Leishmania*. Natural Killer (NK) cells respond soon after *L. major* entrance into the host skin and promote increased resistance to the parasite, apparently due to their production of IFN-γ. However, neutrophils play a controversial role in the outcome of the infection, as in some studies they are essential for initiating an infection with *L. major*, while in other studies, they promote parasite killing. Several studies had examined the role of B cells and antibodies in leishmaniasis and most of them had suggested that antibodies play no role in protection against *Leishmania* spp., although in some situations, antibodies may be detrimental to the parasites. (REITHINGER *et al*., 2007; NOVAIS & SCOTT, 2015).

The nature of the humoral immune response against antigens of *L. amazonensis* differs to the response against antigens of *L. braziliensis* and these differences were most evident in the IgG1 immunoglobulin isotype, promoted by a Th2-dependent immune response, in the *L. amazonensis* infection. These antigens lead to the generation and maintenance of a predominant Th2 immune response that would stop an effective Th1 response, allowing the progression of the disease. The presence of IgG1 antibodies against one or more antigens of the *L. amazonensis* can be used as a marker of the presence of the disease. Another interesting fact observed was that the IgG2a isotype antibodies produced in the *L. braziliensis* infection also recognizes *L. amazonensis* antigens, meaning that they are preferentially produced by the Th1 immune response. (SILVA *et al*., 2015).

Diagnoses based on molecular methods are highly sensitive and specific for the detection of all *Leishmania* DNAs, although it does not distinguish asymptomatic infections from true active forms of the disease. Polymerase Chain Reaction (PCR) is a rapid diagnostic method with high specificity and sensitivity - from 89% to 100% - that uses only small amounts of parasite DNA, obtained from the lesions, to perform the test. Despite this being an invasive technique (much more invasive to VL patients than to CL or ML patients), as a biopsy is necessary to extract DNA from the parasites, it has proven to be better than conventional methods in the diagnosis of CL and ML, showing a better sensitivity than the direct visualisation of parasites in biopsies, immunohistochemical testing of biopsy sections, culture, or serological testing. (SCHWARTZ *et al*., 2006)

Given the need for a fast, inexpensive, reliable and effective serological diagnostic tool, able to detect anti-*Leishmania* antibodies in patients with CL and VL, this study aimed to

develop new serological approaches that will be addressed for the leishmaniasis diagnosis, taking into consideration all performance characteristics of all antigens tested.

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3. OBJECTIVES

3.1. GENERAL

Analysis of *Leishmania* mimetic neoglycoproteins for the CL diagnosis and production and analysis of the *L. braziliensis* kinesin-related recombinant protein for the detection of anti-*Leishmania* antibody levels in individuals with leishmaniases.

3.2. SPECIFICS

- Analysis and selection of *Leishmania* mimetic neoglycoproteins commercially available with high potential in detecting anti-*Leishmania* antibody levels in patients with CL;

- Standardisation of an enzyme-linked immunosorbent assay (ELISA) to measure serum levels of *Leishmania* anti-α-Galactose (anti-α-Gal) antibodies in CL individuals from different endemic areas in Brazil;

- Analysis, selection, production and cloning of synthetic *L. braziliensis* kinesin-related gene sequence based on the reference *L. infantum* kinesin-related;

- Use of *L. tarentolae* as host for the expression of *L. braziliensis* kinesin-related recombinant protein (Lbk39);

- Purification and analyse of the efficiency of the Lbk39 antigen in anti-*Leishmania* antibody detection in CL and VL patients by ELISA;

- Determination of the sensitivity and specificity of all selected *Leishmania* mimetic neoglycoproteins and of the Lbk39 recombinant protein.

CHAPTER 1

ANALYSIS OF *Leishmania* MIMETIC NEOGLYCOPROTEINS FOR THE CUTANEOUS LEISHMANIASIS DIAGNOSIS

ABSTRACT

Once anti-Gal antibodies are widely produced in healthy humans it has the potential for many clinical uses. This study describes an ELISA used to measure serum levels of anti-α-Gal antibodies in patients with cutaneous leishmaniasis (CL) in Brazil. A total of 154 serum samples were collected from CL endemic and non-endemic regions in Brazil. Optimal ELISA conditions were established and two neoglycoproteins (NGP) containing the Galα1-3Gal terminal fraction (Galα1-3Galβ1-4GlcNAc-HAS, identified as NGP 2334; and Galα1-3Gal-HAS, identified as NGP2203) and one Galα1-3Gal NGP analogue (Galα1-3Galβ1-3GlcNAc-HAS, identified as NGP2333) were used as antigens. β-Gal NGP β1-4-Galactosyl-Galactose-BSA (NGP 0204) was used as a negative control and crude extract from *L. braziliensis* (strain MHOM/BR/84/LTB300) was used as a positive control. To determine the α-Gal specific activity, α-galactosidase from green coffee beans was used. Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney analyses. ROC curve, sensitivity and specificity analyses were also performed. Means of anti-α-Gal antibody titres of CL patients were significantly higher than the healthy individuals for all NGPs tested and could be detected at the same level of the crude extract from *L. braziliensis*. However, it can be noticed that all NGPs were able to be better detected by anti-α-Gal antibodies from CL patients that were under observation post-treatment. Sensitivity and specificity of all NGPs ranged from 70% to 91.7% and 60.9% to 73.9%, respectively. The presence of *Leishmania* anti-α-Gal levels were detected by the immune reaction of anti-α-Gal antibodies against Galα1-3Galβ epitope from different neoglycoproteins in CL infected patients. Finally, none of the NGPs were significantly recognized by the healthy individuals, which gives solid support to the conclusion that the disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal.

Key words: ELISA, *L. braziliensis*, Neoglycoprotein, Saccharide, α-galactosidase enzyme.

1. INTRODUTION

Oligosaccharides are broadly present on cell surfaces arranged as glycan arrays, responsible for regulating the interaction between cells. It can be useful for disease diagnosis and also helpful in identifying new disease-causing microbial cell markers. The interactions among pathogens and their host cells are guided by different cell-surface glycans and glycan binding receptors at each stage of the infectious process. Nevertheless, oligosaccharides must be conjugated to carrier proteins to increase immune response since they are themselves poorly immunogenic. (FERNÁNDEZ-TEJADA *et al*., 2015).

Glycoinositol phospholipids (GIPLs) are the main family of low molecular weight glycolipids synthesized by *Leishmania* parasites. It is expressed in abundance on promastigote and amastigote cell surfaces and is not linked to proteins, forming a protective surface revetment that provides essential host-parasite interactions. However, each parasite stage is already known to have different glycoconjugates attached on its surface beyond GIPLs. Biochemical analyses reveal that, unlike amastigotes, the procyclic promastigotes surface coat expresses two other glycoconjugates that is less abundant than GIPLs and is responsible for protecting parasites from hydrolytic enzymes in the sandfly gut: 1) glycosylphosphatidylinositol (GPI) anchored to macromolecules such as metalloprotease and; 2) protein-free lipophosphoglycan complex (LPG), a GPI-anchored macromolecules underlying layer composed of densely, free-packed glycolipids. Altogether, these molecules create an effective barrier which protects promastigotes from cell death processes like lysis mediated by complement system, oxygen radicals and hydrolases in the mammalian and insect host environments. (ILGOUTZ; MCCONVILLE, 2001; MUKHOPADHYAY; MANDAL, 2006; BARRETO-BERGTER; VERMELHO, 2010; GALILI, 2013a).

A previous study has shown that sera of healthy and trypanosomatid-infected individuals have immunoreactivity against GIPLs from *Leishmania major* and have presented clear evidence of the existence of GIPLs in American *Leishmania* and *Trypanosoma* spp. as well as in *L. donovani*. The authors have also demonstrated that GIPLs are present in *T. cruzi*, *T. rangeli*, *L. mexicana*, *L. braziliensis*, and *L. donovani* culture forms that were partially characterized as glycan from predominant GIPLs. The major GIPLs in these parasites include tetraglycosylinositol, pentaglycosylinositol, and hexaglycosylphosphatidylinositol and sugar analysis of *L. mexicana* and *L. braziliensis* GIPLs revealed a monosaccharide composition of Manose (Man), Galactose (Gal), Glucosamine (GlcN), and inositol. (ÁVILA *et al*., 1991).

Remarkably, another piece of research has demonstrated that anti-α-Gal, the most abundant natural circulating human antibody, recognizes specifically the unique glycosphingolipids structures with non-reducing terminal Galα1–3Galβ1–4GlcNAc-R, which is also known as the GPI-anchored mucins in the trypomastigote stage of *Trypanosoma* spp. (GALILI, 1993; MARCHER; GALILI, 2008; SCHOCKER *et al.*, 2016). However, the parasites escape through anti-Gal action by penetrating tissues and continue to produce and release GIPLs and LPG with α-Gal epitopes, stimulating the immune system to continuously produce anti-α-Gal antibodies at high titres. (GALILI, 2013). High levels of anti-α-Gal antibodies have also been described in patients infected with trypanosomatid parasites such as *L. mexicana* spp., *L. braziliensis* and *Leishmania* spp. which cause visceral leishmaniasis. (ÁVILA *et al.*, 1988; 1989).

Anti-α-Gal antibodies were originally isolated from normal human AB sera type and it was found that they belong to the IgG class. Its concentration ranged from 50 to 100 µg/mL, although other authors have detected concentrations between 30 and 70 µg/mL using Mancini radial immunodiffusion assay to isolate the antibody. (GALILI *et al.*, 1984). This anti-α-Gal concentration represents approximately one percent of circulating IgG in human serum. (TOWBIN *et al.*, 1987; GALILI, 1988; GALILI, 1993). In more than 95% of the normal adult population, anti-α-Gal titres ranged between 1:800 and 1:1,600 titres and it is not related to blood group type. (GALILI *et al.*, 1984).

The presence of Galα1-3Gal epitope and anti-α-Gal binding sites on the cell membrane of many mammalian species has been evolutionarily conserved. It can explain the large amount of anti-α-Gal antibodies found in Old World monkeys, apes and human serum - capable of immediately agglutinating in the presence of erythrocytes of all non-primate mammals and New World monkey species - and also its absence from the serum of non-primate mammalian. Quantities of anti-α-Gal antibodies constantly produced can represent a continuous immune response to Galα1-3Gal structures found in various gastrointestinal bacteria (GALILI, 1984; GALILI *et al.*,1987; GALILI, 2013b), confirming the polyreactive nature of these antibodies in human serum. (SATAPATHY; RAVINDRAN, 1999).

Since anti-α-Gal binds specifically to Galα1–3Gal glycosphingolipids, it is assumed that the majority of the anti-α-Gal binding sites expressed in the cell membranes of many organisms have this non-reducing terminal structure. (GALILI *et al.*, 1988; GALILI, 1993; GALILI; ANDREW, 1995; GALILI, 2013a; GALILI, 2013b). It is also known that anti-α-Gal does not bind either to glycosphingolipids with a non-reducing terminal GalNAcα1–3GalNAc disaccharide, or to the specific α-Gal structure GalNAcαl–3(Fucαl–2)Gal-R (GALILI, 1993;

MARCHER; GALILI, 2008), which is expressed in glycolipids of erythrocyte membrane from blood group B. The GalNAcαl–3(Fucαl–2)Gal-R structure shows some similarity to Galα1-3Gal epitope, but has 10 to 600 fold less affinity for the anti-α-Gal antibodies. (TOBWIN *et al.*, 1987; GALILI, 1988; GALILI, 1993). Purified anti-α-Gal from sera of subjects belonging to A and O blood group binds to blood group B α-Gal epitopes expressed on erythrocytes membrane and it is called anti-B reactivity. Purified anti-α-Gal from blood group B and AB sera binds to the α-Gal epitope, but not to the specific blood group B α-Gal antigen. Most anti-B reactivity is due to the much larger natural antibody fraction with α-galactosyl specificity that reacts primarily with the Galα1-3(Fucα1-2)Gal epitope. (GALILI, 1988; MARCHER; GALILI, 2008). There is no evidence that the Rh factor induces immune reactions against anti-α-Gal antibodies. (GALILI *et al.*, 1984).

Once anti-Gal antibodies are widely produced in healthy humans and α-gal epitopes can be easily synthesized by several methods, anti-Gal has the potential for many clinical uses. This study describes an enzyme-linked immunosorbent assay (ELISA) used to measure serum levels of anti-α-Gal antibodies in individuals with cutaneous leishmaniasis (CL) from different endemic regions in Brazil and compares them to those from healthy individuals living in the same endemic areas (HIEA) and non-endemic areas (HINEA) using different neoglycoproteins (NGPs) as the antigen.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 154 serum samples from females and males of different age groups were collected from the following CL endemic and non-endemic regions in Brazil: endemic region from Pará and Espírito Santo state and endemic and non-endemic regions from Paraná state. The main inclusion criterion for this study was the proven diagnosis of CL by parasite presence on the direct examination of lesion smears obtained from the edge of the active lesion with a punch biopsy tool and/or positivity in the parasite culturing. The patients were divided into ten groups (Table 1):

Table 1. Identification of samples used in this study

Group	N	Classification
H1	23	Healthy individuals from non-endemic areas
H2	37	Healthy individuals from endemic areas
CL1	12	CL patients with active lesion and no treatment
CL2	05	CL patients with active lesion under the treatment
CL3	13	CL patients that had finished treatment and were under observation for the subsequent 3 months
OD	54	Serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis
CD	05	Serum reagent patients for Chagas disease
CLR	02	Patients with a reincident lesion and who had the same treatment several times lasting approximately one year
CLP	02	Patients that had CL for longer than five years prior to this study
VL	01	Positive patients for visceral leishmaniasis (VL) after treatment and were under observation

N: number of patients in each group

Individuals from H2 group were medically examined to discard any previous CL infection. CL and VL patients were treated according to the following Brazilian Health Ministry guidelines: Manual of surveillance and control of Visceral Leishmaniasis (2014) and Manual for Surveillance of American Integumentary Leishmaniasis (2010). Patients with other diseases such as Chagas disease and visceral leishmaniasis (VL) were also studied to evaluate the chance of cross-reactivity in these tests.

Ethical approval was obtained from the Universidade Federal do Paraná Ethical Committee under number 684.244 (Attachments 2 and 3).

2.2. Enzyme-linked immunosorbent assay (ELISA)

To determine whether levels of anti-α-Gal antibodies in human serum, both non-infected and infected with CL and other diseases can identify *Leishmania* α-Gal epitopes, optimal ELISA conditions were established, such as dilutions of sera (1:100, 1:200, 1:400 and 1:800), conjugate (1:5,000, 1:10,000 and 1:20,000) and antigens (0.1 µg, 0.5 µg and 1 µg/well). Polystyrene microtiter plates with 96 wells (NUNC C96 Immuno Plate Maxisorp Surface, Thermo Scientific, Denmark) were coated overnight at 4°C with 100 µL of 0,1 µg/well of two Galα1-3Gal neoglycoprotein series (Galα1-3Galβ1-4GlcNAc-HAS, identified as NGP 2334; and Galα1-3Gal-HAS, identified as NGP2203) and one Galα1-3Gal analogue neoglycoprotein (Galα1-3Galβ1-3GlcNAc-HAS, identified as NGP2333) (Dextra Laboratories), diluted in carbonate–bicarbonate buffer (pH 9.6). In addition, the β-Gal NGP

β1-4-Galactosyl-Galactose-BSA (NGP 0204) was also included in the study as a negative control and soluble proteins from the crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300) as a positive control. Human albumin serum was chosen for the generation of NGPs because of its absence of immunological reaction with human serum and its adequacy as a carrier protein.

The following day, the plates were washed twice with 200 μL/well of washing solution (NaCl 0.9% - Tween 20 0.05%), then the wells were blocked with 120 μL of blocking solution (Pierce Protein - Free T20 – PBS Blocking Buffer, Thermo Scientific) for one hour at 37°C, and after were washed twice again with 200 μL/well of washing solution. Immediately after, serum samples were diluted to 1:800 in a solution of PBS – 0.25% casein + 0.5% Tween 20 and were added in their respective wells and incubated at 37 °C for one hour. Then the plates were washed four times with 200 μL/well of washing solution and polyclonal rabbit anti-human IgG HRP conjugate (1.3g/L, Dako) was diluted to 1:5,000 and was added to each well for one hour at 37°C. Finally, the reaction was developed by adding 100 μL of SureBlue TMB™ Microwell Peroxidase Substrate to each well at room temperature for 15 minutes, avoiding light, and to interrupt the reaction 20 μL of a solution 1:20 of H_2SO_4 was added. Plates were read in the Infinite F200 PRO multimode reader (Tecan) at 450 nm and values were expressed in absorbance. Each sample was measured in triplicate and the whole assay described above was performed in duplicate.

2.3. Determination of α-Gal specific activity

Based on Al-Salem *et al*. (2014) CBAG treatment protocol to determine the α-Gal specific activity, 0.1 μg/well of each NGP previously tested, except for NGP 2204, were treated overnight at 28°C with 0.04 U/well of α-galactosidase from green coffee beans (Sigma). After incubation, the plates were washed five times with washing solution and the ELISA was performed as described above. A pool of seven serums from CL1 and CL2 and 10 from H1 individuals was also used as positive and negative controls.

2.4. Statistical analysis

The homogeneity of variance analysis and the Kolmogorov-Smirnov test for the normal condition of variables evaluation were performed using the Statistica 7 and MedCalc 16.1 software, respectively. Once data showed non-parametric distribution, a Kruskal-Wallis

one-way analysis with a Dunn post-test was performed to validate the significant difference among groups, except for CLR, CLP and VL due to an insufficient number of patients in each group to perform a reliable statistical analysis. The Mann-Whitney analysis was used to compare the means of each group between antigens and positive and negative controls. All non-parametric analyses were performed using GraphPad Prism 6 software, assuming the significant level of $p<0.05$. Finally, ROC (Receiver Operating Characteristics) curve together with sensitivity and specificity analyses were performed using MedCalc 16.1 software and a significant level of $p<0.05$ was adopted.

3. RESULTS

3.1. ELISA standardisation

The ELISA optimization procedures were performed to improve the test performance. The optimal condition found for all antigens tested was 0.1 μg of antigen, sera dilution of 1:800 and conjugate dilution of 1:5,000. For those conditions, the absorbance of CL patient samples was 16-fold higher than healthy individual's samples.

3.2. Determination of the sensitivity and specificity of all NGPs

Following the ELISA standardisation, all serum samples collected were analysed in two different moments. First, absorbance values of samples from H1 and CL1 groups were used to perform the ROC curve analysis, sensitivity, specificity, predictive positive value (PPV) and predictive negative value (PNV) of all antigens, except for the negative control NGP 0204. The results obtained were compared with the same parameters of the same sample groups for the positive control. All parameters were evaluated considering the cut-off values calculated by MedCalc software (Table 2 and Figure 1), with 2 standard deviation (2SD) (Table 3) and with 3 standard deviation (3SD) (Table 4).

Comparing all parameters evaluated above, the better conditions obtained were those calculated by the MedCalc software, where the cut-off values ranged from 0.1068 for *L. braziliensis* crude extract to 0.2090 for NGP 2334, whilst sensitivity and specificity ranged from 70% (for the NGP 2334 antigen) to 91.7% (for the NGP 2203 antigen) and 60.9% (for the NGP 2203 antigen) to 82.6% (for the positive control), respectively.

Table 2. ROC curve analysis for NGP 2334, NGP 2333, NGP 2203 antigens compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed using MedCalc software.

	MedCalc					
	Cut-off	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.1068	0.909±0.0625	90.9	82.6	0.41	0.98
NGP 2334	0.2090	0.735±0.111	70.0	73.9	0.27	0.95
NGP 2333	0.1069	0.791±0.0861	81.8	65.2	0.24	0.96
NGP 2203	0.1092	0.844±0.0669	91.7	60.9	0.24	0.98

NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

Table 3. Sensitivity, specificity, PPV and PNV analysis for NGP 2334, NGP 2333, NGP 2203 antigens compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed considering 2SD.

	2 SD				
	Cut-off	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.2057	75.0	95.8	0.71	0.97
NGP 2334	0.4550	66.7	95.8	0.68	0.96
NGP 2333	0.2102	66.7	95.8	0.68	0.96
NGP 2203	0.2294	66.7	95.8	0.68	0.96

NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

Table 4. Sensitivity, specificity, PPV and PNV analysis for NGP 2334, NGP 2333, NGP 2203 antigens compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed considering 3SD.

	3 SD				
	Cut-off	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.2651	75.0	95.8	0.71	0.97
NGP 2334	0.5951	63.2	100.0	1.00	0.95
NGP 2333	0.2665	66.7	100.0	1.00	0.96
NGP 2203	0.2917	63.2	100.0	1.00	0.95

NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

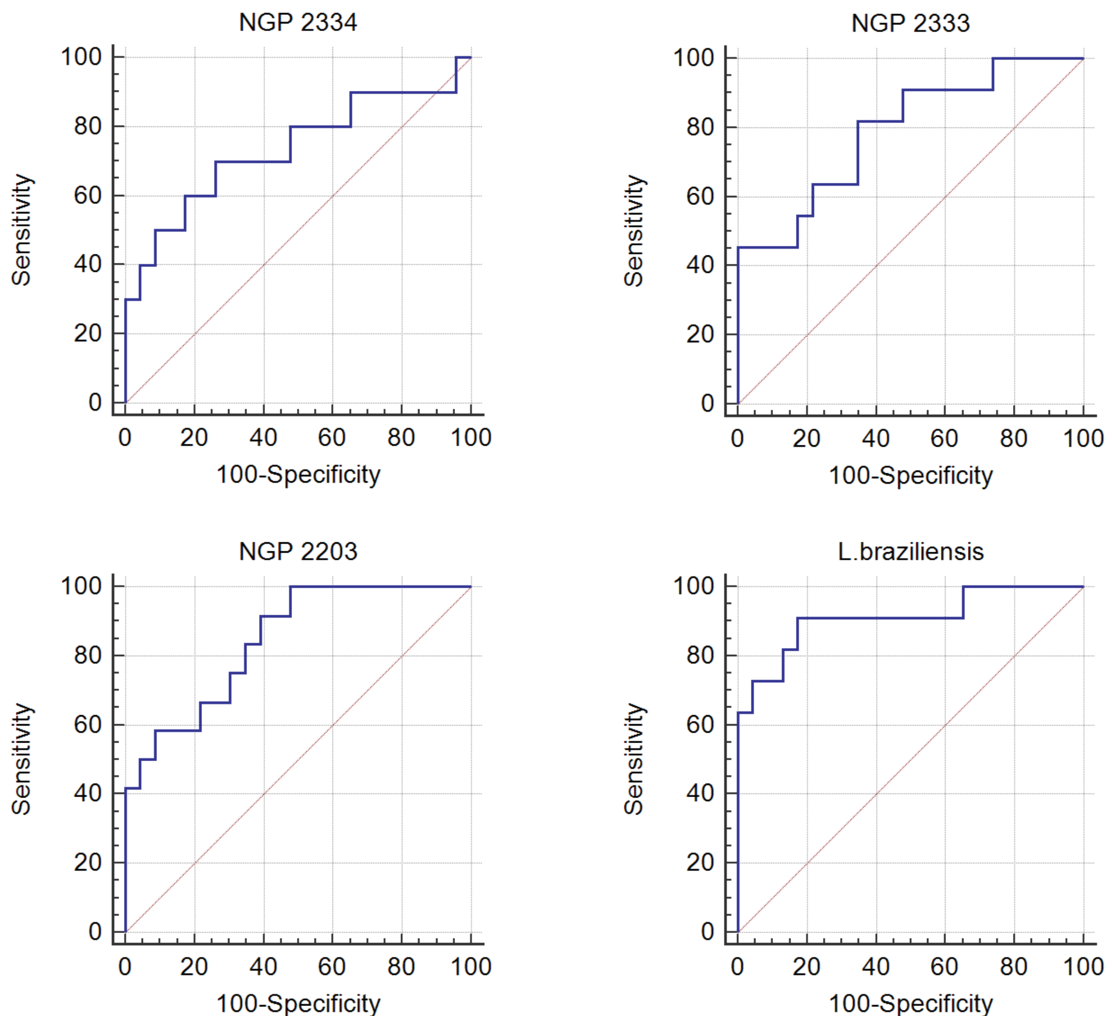

Figure 1. ROC curve analysis for NGP 2334, NGP 2333, NGP 2203, and *L. braziliensis* antigens. NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300).

Considering the high values of sensitivity and specificity obtained for all antigens and compared to the positive control group, the Kruskal-Wallis analysis was performed to validate the significant difference among all sera groups per antigen.

The presence of *Leishmania* anti-α-Gal antibodies was determined by comparing anti-α-Gal levels in CL patients and healthy individuals living in the same endemic area, non-endemic area and individuals with other diseases (Table 5 and Figure 2). It was found that means of anti-α-Gal antibody titres of only CL2 and CL3 groups were significantly higher ($p<0.05$) than the H1 group for NGP 2234 and NGP 2333. For NGP 2203, all CL groups presented significantly higher means of antibody levels ($p<0.05$) compared to H1 group.

There was no significant difference between groups for the negative control NGP 0204, whereas for the positive control - *L. braziliensis* crude extract - only CL1 and CD

groups showed significant difference (p<0.05) when compared to the H1 group. No cross-reaction between the NGPs antigens and antibodies from patients with other diseases was detected, except for the Chagas disease (CD group), since the etiological agent of this disease is another trypanosomatid parasite.

Table 5. Kruskal-Wallis statistical analyses of levels of anti-α-Gal antibodies found in individuals from CL endemic and non-endemic areas. The p values with an asterisk show significant difference between groups (p<0.05).

			p values					
	Groups	**Positivity (%)**	**H2**	**CL1**	**CL2**	**CL3**	**OD**	**CD**
NGP 2334	**H1**	21.7	>0.9999	0.2477	0.0032*	<0.0001*	>0.9999	0.0496*
	H2	51.4		>0.9999	0.0645	0.0073*	0.3576	0.6020
	CL1	75.0			>0.9999	>0.9999	0.0625	>0.9999
	CL2	100.0				>0.9999	0.0008*	>0.9999
	CL3	100.0					<0.0001*	>0.9999
	OD	37.0						0.0190*
	CD	100.0						
NGP 2333	**H1**	34.8	>0.9999	0.1806	0.0201*	0.0005*	>0.9999	0.0071*
	H2	27.0		0.0375*	0.0059*	<0.0001*	>0.9999	0.0018*
	CL1	83.3			>0.9999	>0.9999	0.1294	>0.9999
	CL2	100.0				>0.9999	0.0167*	>0.9999
	CL3	100.0					0.0001*	>0.9999
	OD	35.2						0.0055*
	CD	100.0						
NGP 2203	**H1**	34.8	>0.9999	0.0205*	0.0089*	0.0004*	>0.9999	0.0037*
	H2	54.1		0.1769	0.0525	0.0047*	>0.9999	0.0237*
	CL1	91.7			>0.9999	>0.9999	0.1571	>0.9999
	CL2	100.0				>0.9999	0.0504	>0.9999
	CL3	92.3					0.0033*	>0.9999
	OD	46.3						0.0223*
	CD	100.0						
N.C.	**H1**	26.1	>0.9999	0.1310	0.2780	>0.9999	>0.9999	>0.9999
	H2	37.8		0.0547	0.1843	>0.9999	>0.9999	0.9806
	CL1	75.0			>0.9999	>0.9999	0.4284	>0.9999
	CL2	100.0				>0.9999	0.7235	>0.9999
	CL3	53.8					>0.9999	>0.9999
	OD	44.4						>0.9999
	CD	60.0						
P.C.	**H1**	17.4	>0.9999	0.0004*	0.0545	0.1902	>0.9999	0.0113*
	H2	16.2		<0.0001*	0.0099*	0.0160*	0.2131	0.0016*
	CL1	91.7			>0.9999	>0.9999	0.0063*	>0.9999
	CL2	80.0				>0.9999	0.3525	>0.9999
	CL3	61.5					>0.9999	>0.9999
	OD	40.7						0.0871
	CD	100.0						

NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; N.C.: β1-4-Galactosyl-Galactose-BSA (NGP 02040); P.C.: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; OD: serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis; CD: serum reagent patients for Chagas disease.

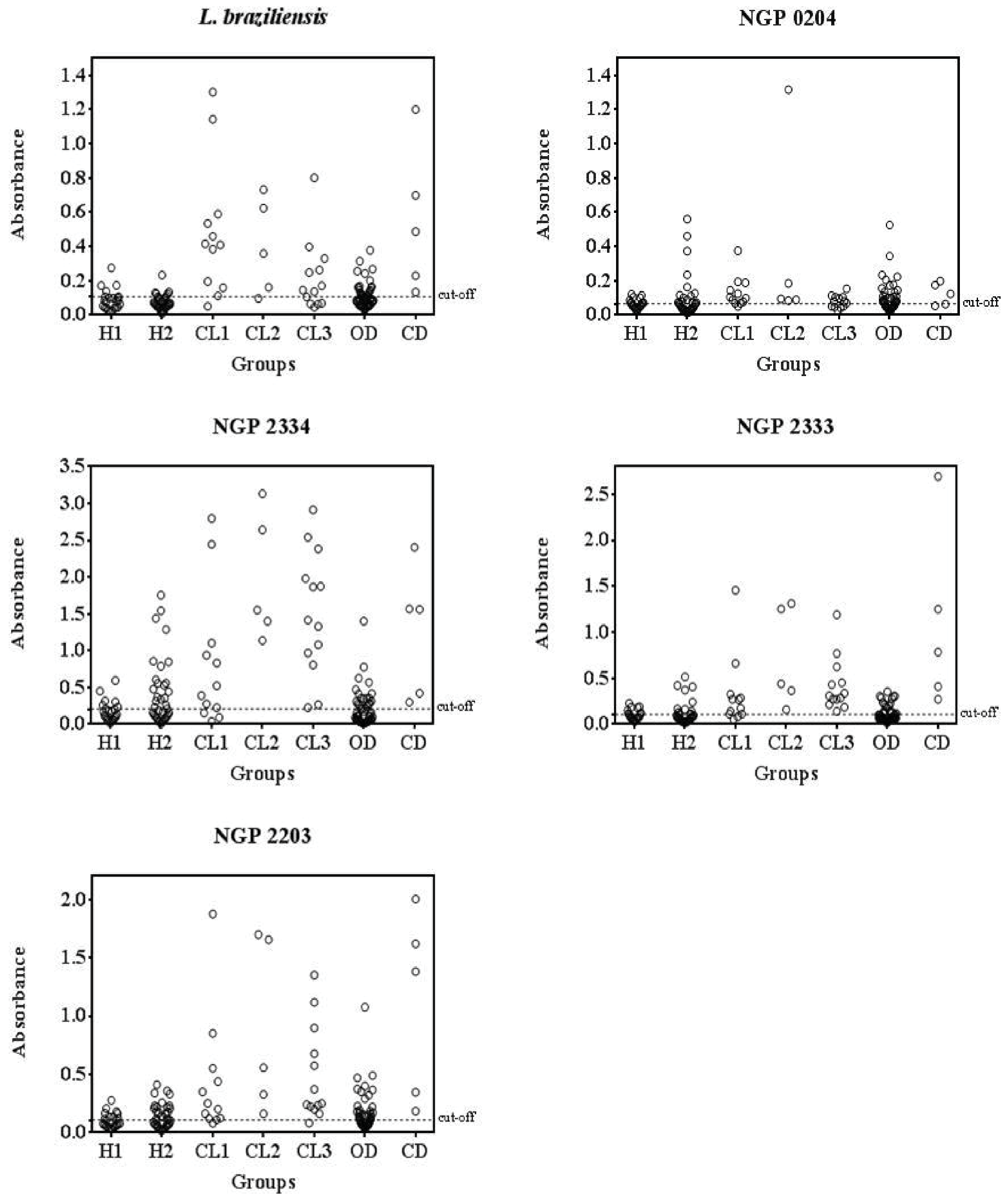

Figure 2. Levels of anti-α-Gal antibodies detected by ELISA in individuals from CL endemic and non-endemic areas in Brazil using NGP 2334, NGP 2333, NGP 2203, NGP 0204 (negative control) and proteins from crude extract from *L. braziliensis* culture as antigens. NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; NGP 0204: β1-4-Galactosyl-Galactose-BSA; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; OD: serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis; CD: serum reagent patients for Chagas disease.

Although no statistical analysis has been performed for CLP, CLR and VL groups due to an insufficient number of patients in each group, the CLP and CLR groups showed absorbance values above the cut-off line for all antigens tested, whilst VL patient showed absorbance values below the cut-off line for all antigens tested (data not shown).

Based on the positive and negative controls results, the Mann-Whitney analysis was performed to compare means of each sera group for each antigen (Table 6). Means of antibody titres of CL1 group from all NGPs showed no significant difference compared to the same group from positive control. However, means of antibody titres of CL2 from NGP 2334 and CL3 groups from all NGPs showed higher difference compared to the same groups from positive control. No statistical difference was observed between CD and OD groups from all antigens compared to the positive control. Nonetheless, only means of antibody titres of CL1 group for NGP 2233 and CL2 for NGP 2333 and NGP 2203 showed no significant difference compared to the same group from negative control.

Table 6. Mann-Whitney statistical analysis between groups of individuals from CL endemic and non-endemic areas and α-Gal antigens. The p values with an asterisk show significant difference between groups ($p<0.05$).

		p values	
	Antigen	***L. braziliensis***	**NGP 0204**
H1	**NGP2334**	0.0082*	0.0002*
	NGP 2333	0.4167	0.0299*
	NGP 2203	0.2921	0.0127*
H2	**NGP2334**	<0.0001*	<0.0001*
	NGP 2333	0.3025	0.1682
	NGP 2203	0.0010*	0.0031*
CL1	**NGP2334**	0.7430	0.0045*
	NGP 2333	0.1584	0.0679
	NGP 2203	0.4704	0.0100*
CL2	**NGP2334**	0.0079*	0.0159*
	NGP 2333	0.4127	0.2222
	NGP 2203	0.4127	0.0952
CL3	**NGP2334**	<0.0001*	<0.0001*
	NGP 2333	0.0101*	<0.0001*
	NGP 2203	0.0337*	<0.0001*
OD	**NGP2334**	0.2954	0.0121*
	NGP 2333	0.6829	0.0514
	NGP 2203	0.1497	0.0002*
CD	**NGP2334**	0.2222	0.0079*
	NGP 2333	0.3095	0.0079*
	NGP 2203	0.3095	0.0159*

NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; NGP 0204: β1-4-Galactosyl-Galactose-BSA; *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; OD: serum reagent patients for other diseases – such as Hepatitis B and C, Syphilis and truly positive patients for Tuberculosis; CD: serum reagent patients for Chagas disease.

To determine the specific immunogenic activity of the α-Gal residues, all NGPs, except for NGP 2204, were treated with α-galactosidase enzyme from green coffee beans, which specifically unlinks Galα1-3Gal into two parts - Galα1 and 3Gal – and, consequently, abrogates the recognition of α-Gal epitope by anti-α-Gal antibodies. CL1 and CL2 mixed group and H1 groups were used as positive and negative controls for non-enzymatic treatment (Figure 3). Although it is clear there is still an antigen-antibody reaction detected in the assay performed with the presence of the α-galactosidase enzyme, compared to the controls, patients from the CL1/CL2 and CL3 groups used in enzymatic treatment assay with NGP 2334 showed a 2- and 3-fold decrease in the antibody titres than positive control, respectively. The same was found for the other antigens: a 2.5- and almost 4-fold decrease in the antibody titres for CL1/CL2 and CL3 groups for NGP 2333 antigen, respectively; and almost a 2.5- and almost 4-fold for the CL1/CL2 and CL3 groups NGP 2203 antigen, respectively. CLR, CLP, H2 and even H1 groups used in enzymatic treatment also presented a decrease in the antibody titres when compared to the positive and negative controls from non-enzymatic treatment.

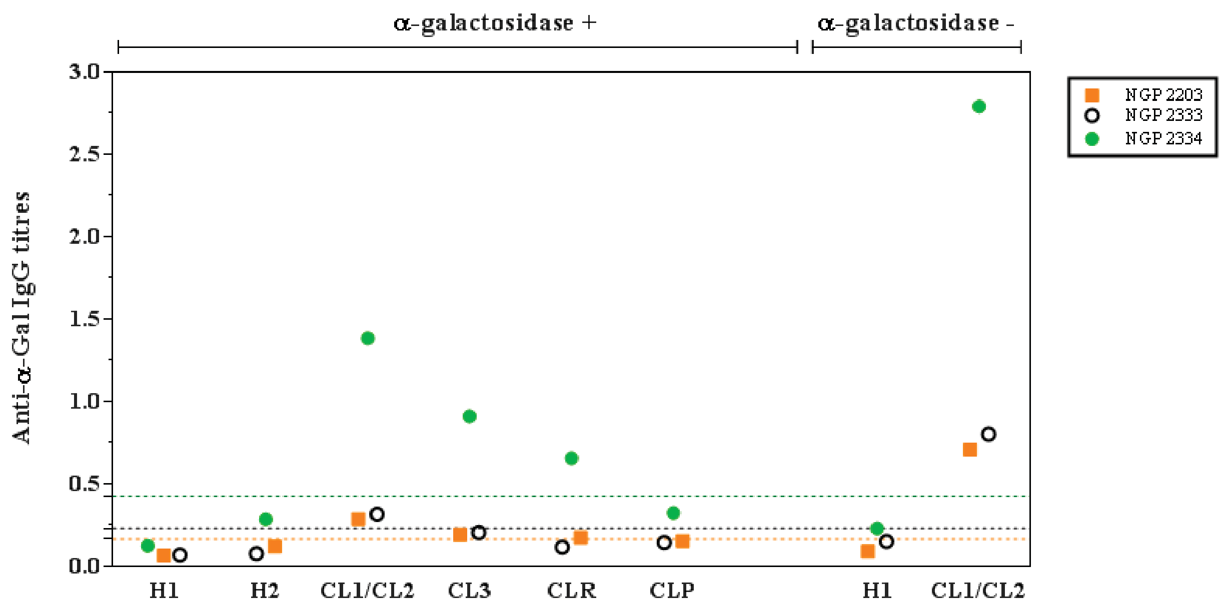

Figure 3. Levels of anti-α-Gal antibodies detected in individuals from CL endemic and non-endemic areas in Brazil after cleavage specific with α-galactosidase enzyme from Green Coffee beans on α-galactosylated NGPs antigen. NGP 2334: Galα1-3Galβ1-4GlcNAc-HAS; NGP 2333: Galα1-3Galβ1-3GlcNAc-HAS; NGP 2203: Galα1-3Gal-HAS; H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; CLR: patients with reincident lesion and had the same treatment several times lasting approximately one year; CLP: patients that had CL for more than five years prior to this study.

4. DISCUSSION

The presence of anti-*Leishmania* antibodies was determined by comparing anti-α-Gal levels in CL patients and healthy individuals using NGPs as antigens. All antigens were able to be better detected by anti-α-Gal antibodies from CL patients under treatment and those that were under observation post-treatment. However, means of antibody titres of CL patients with an active lesion for all NGPs showed no significant difference compared to the same group for positive control, meaning that all NGPs were able to detect anti-*Leishmania* antibody level in CL patients with an active lesion at the same level of the crude extract from *L. braziliensis*. The sensitivity and specificity values of all antigens ranged from 70% to 91.7% and 60.9% to 73.9%, respectively.

Some studies also suggest that sera from patients with an active lesion and cured individuals are able to recognize α-Gal epitopes. Al-Salem *et al*. (2014) observed that patients from Kingdom of Saudi Arabia infected with *L. major* and *L. tropica* showed an increase of 12-fold and 8-fold in the anti-α-Gal IgG titres, respectively, when compared to healthy individuals from the same area. Ávila *et al*. (1989) found that anti-α-Gal titres were 10-fold higher in the sera of patients with Chagas disease and *Leishmania* infection than that of healthy individuals. Ávila & Rojas (1990) demonstrated that high anti-GPL antibody activity was present in 84% of active CL patients, as well as 80% of patients with skin lesions with only 15 days of clinical evolution - this percentage increased to 91% after 3 months of clinical evolution. It can explain why few infected individuals had presented anti-α-Gal values below the cut-off line. Either total parasite clearance could take several years to complete or the treatment could allow a parasite cell lysis, releasing more α-Gal epitopes into the host's immune system.

Patients with reincident cutaneous lesions and healed patients (CLP and CLR groups) showed absorbance values above the cut-off line for all antigens tested, whilst VL patient showed absorbance values below the cut-off line for all antigens tested. It can lead to some conclusions: 1) No NGP antigens were able to detect anti-α-Gal levels from patients with VL, while the positive control antigen was, indicating that NGP antigens can be specific to diagnose only CL; 2) CLR patients present high levels of anti-α-Gal antibodies, suggesting that, even after being treated several times, some strains of *Leishmania* parasites have already developed resistance to antiparasitic drugs used in Brazil; 3) CLP patients also presented levels of anti-α-Gal antibodies above the cut-off line, suggesting that serum anti-α-Gal antibodies levels do not disappear by the death of the *Leishmania* parasite.

No cross-reaction between the NGPs antigens and antibodies from patients with other diseases was detected, except for the Chagas disease (CD group), since the etiological agent of this disease is another trypanosomatid parasite. Ashmus *et al.* (2013) also showed that chagasic anti-α-Gal antibodies strongly recognize saccharides containing the non-reducing terminal disaccharide Galα1-3Galβ moiety; whilst Bretaña *et al.* (1992) reported the distinct localization of α-Gal epitopes in the external surface of the plasma membranes of members of the Trypanosoma family: in the lips of the flagellar pocket in *Leishmania* amastigotes and promastigotes and dispersedly in *T. cruzi* trypomastigotes membranes.

The presence of different antibodies binding to similar epitopes, either in patients with CL and VL or patients with chronic *T. cruzi* and *T. rangeli* infections, can indicate the strong presence of highly immunogenic oligosaccharide antigens linked to phosphatidylinositol in trypanosomatid parasites. (ÁVILA *et al.* , 1988; ÁVILA *et al.*,1991). Anti-α-Gal antibodies specifically interact with glycoconjugates that have Galα1-3Galβ1-4GlcNAc residues but do not interact with glycoconjugates that have Galα1-4Galβ1-4GlcNAc residues, β-galactosyl residues, or glycoconjugates with other carbohydrate residues. The α-Gal binding site has a size corresponding to the free trisaccharide Galα1-3Galβ1-4GlcNAcα-BSA and it exhibits a 20-fold differential between chagasic patients and healthy individuals antibodies, 7-fold higher in affinity of human anti-α-Gal than that to the disaccharide Galα1-3Gal, and much more strongly than α-Gal alone. Others saccharides such as Galβ1-4GlcNAcα-BSA, GlcNAcα-BSA and GlcNAcβ-BSA show minimal binding between the same chagasic patients and healthy individuals antibodies. (GALILI, 1989; OBUKHOVA, RIEBEN & BOVIN, 2007; SCHOCKER *et al*., 2016).

Anti-α-Gal only recognizes the free non-reducing terminal α1-3-linked to galactose residue, but not the α1-4-linked to the penultimate galactose neither free Galβ1-4GlcNAc. (GALILI *et al.,* 1985; GALILI, 2013b). Other galactose disaccharides with different α-glycosidic linkage, i.e., αl-4 and αl-6, are from 100 to 1,000-fold less competent anti-α-Gal binding inhibitors than Galαl-3Gal. (TOBWIN *et al*., 1987). The presence of the saccharide α1-2-fucose linked to the second galactose, either in red cell membranes or in its purified form from the B blood group, blocks anti-α-Gal binding action. (GALILI *et al*., 1985).

A decrease of antibody titres from CL patients and for all NGPs was noticed when the α-Gal residues specific immunogenic activity protocol was applied. The same results were observed by Galili *et al.* (1984), Ávila *et al.* (1989), Ávila *et al.* (1988), Ávila & Rojas (1990) and Ávila *et al.* (1991) which suggest that GPIs oligosaccharide chains have terminal α-galactose residues but not β-galactoside linkages.

During the *Leishmania* spread through the mammalian host cells, the parasites are rarely exposed directly to the humoral immune response and the presence of antibodies is induced by the complement system. Some *Leishmania* membrane structures can stimulate high levels of antibody production, like the LPG, which is the most abundant GPI-anchored molecule on the promastigote surface. The promastigotes forms attach to the sandfly gut epithelium via LPGs and later, during the sandfly infection period, the LPG molecules of the then metacyclic promastigotes become longer and more complex compared with those of procyclic promastigotes, enabling the parasite to detach from the insect gut and migrate towards the midgut prior to transmission. In addition, the modified LPG molecules of metacyclic promastigotes confer resistance to the human complement system. Amastigotes express very little LPG on their surface but they still express other GIPLs that are host-derived glycosphingolipids. (BIFELD & CLOS, 2015).

The LPG exhibits a wide variation in sugar composition between and within *Leishmania* species, e.g., LPG from *L. braziliensis* is devoid of oligosaccharide side chains whereas LPG from *L. infantum* contains side chains, and they both trigger distinct immune responses in macrophages. The LPG of *L. braziliensis* also induce the production of higher levels of TNF, IL-1β, IL-6 and nitrite oxide (NO) than LPG of *L. infantum*. The changes in cytokine levels affect the activation of macrophage and more systemic inflammatory pathways. (PODINOVSKAIA & DESCOTEAUX, 2015).

5. CONCLUSION

The presence of *Leishmania* anti-α-Gal levels was detected by the immune reaction of anti-α-Gal antibodies against Galα1-3Galβ epitope from different neoglycoproteins in CL infected patients. Means of anti-α-Gal antibody titres of individuals infected with CL were significantly higher than healthy individuals from non-endemic areas for all of NGPs tested. The values of sensitivity and specificity for all NGPs were as good as the values of sensitivity and specificity for the positive control and have proven to be good antigens to detect anti-α-Gal antibody levels in patients infected with CL, especially those recently treated. None of the NGPs were significantly recognized by the H1 group, which gives solid support to the conclusion that the disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal.

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CHAPTER 2

EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAYS BASED ON THE *Leishmania braziliensis* KINESIN-RELATED RECOMBINANT ANTIGEN FOR SERODIAGNOSIS OF LEISHMANIASES IN BRAZIL.

ABSTRACT

Owing to the absence of an appropriate diagnostic test for Leishmaniases, the development of a reliable diagnostic tool still needs to be done. Some purified and recombinant antigens of many *Leishmania* species have been produced and used for serologic assays as the example of the rK39 antigen for the diagnosis of visceral leishmaniasis (VL). The aim of this study was to explore the use of *L. tarentolae* as a host for the expression and secretion of *L. braziliensis* kinesin-related recombinant protein (Lbk39) and evaluate the cutaneous leishmaniasis (CL) diagnostic efficiency of this new antigen by ELISA in patients from Brazil. A total of 108 serum samples were collected from CL and VL endemic and non-endemic regions in Brazil. The synthetic Lbk39 gene was designed based on the kinesin-related gene of *L. infantum* and the a homology search was performed by BLAST similarity on the ??? tritrypDB database website. The Lbk39 gene was inserted into a pLEXSY-sat2 recombinant vector, cloned with 6×His-tag and transfected into *L. tarantole* cells by electroporation. The culturing was carried out according to the manufacturer's instruction and the protein purification was performed using a nickel affinity column. Optimal ELISA conditions were established for Lbk39 antigen. Crude extract from *L. braziliensis* culture (strain MHOM/BR/84/LTB300) was included as positive control. ANOVA one-way analysis was performed to validate the significant difference among groups and student's t test was used to compare the means of each group between antigens. ROC curve, sensitivity and specificity analysis were also performed and for all analyses the significant level of 5% was adopted. The Lbk39 antigen was weakly recognized by CL patients compared to healthy individuals from non-endemic areas, but demonstrated significantly higher antibody levels for VL patients compared to the same control group. Cross-reaction with the Chagas Disease patients was noticed. The ROC curve showed a strong accuracy for VL patients (AUC = 0.918±0.0753), but a weak accuracy for CL patients (AUC = 0.631±0.117). Values of sensitivity and specificity for VL patients were 80% and 95.5%, respectively, whilst sensitivity and specificity for CL patients were 88.9% and 40.9%, respectively. These findings show that, despite the good outcome of production and expression of the target protein and despite it being produced from a kinesin-related gene of *L. braziliensis*, it is still greatly recognisable only by antibodies from patients stricken with the visceral form of the disease.

Key words: rK39. Homology. *L. infantum*. *L. tarantole*. Recombinant vector. Antibody.

1. INTRODUTION

Leishmaniases are a group of vector-borne diseases caused by the heteroxenous parasites belonging to the genus *Leishmania* (Trypanosomatidae), which are capable of inducing the host immune system by the constant alteration of the antigenic epitopes and of the mechanisms of immunosuppression. The ability of the host to control the infection requires the production of the cell-mediated immune responses that are able to activate macrophages responsible for eliminating those intracellular parasites. Although the resolution of the infection is mediated by Th1 cells by secreting IFN-γ in response to the increase of IL-12, the development of a Th2 cells response, in effect to the increase of IL-4, can result in the progression of the lesions and in the course of the systemic disease. Some *Leishmania* antigens lead the host immune system differentiation into active T-cells, which secrete several cytokines, including IL-4, that also activate B-lymphocytes to produce immunoglobulins. It is also known that antibodies are produced by the neutrophil stimulation in the very beginning of the infection; however some studies suggest that antibodies play no role in host protection, but can be useful to determine the presence of the parasite. (AL-QADHI, MUSA & HUMMADI, 2015; MARTINS *et al*., 2016).

Heretofore, the correct diagnosis of the leishmaniases is performed by a combination of clinical, epidemiological and laboratory findings. Owing to the absence of an appropriate and specific diagnostic test for this group of diseases, the development of a reliable diagnostic tool still needs to be made and should be easy to handle, cheap to produce and has high sensitivity and specificity. (MAIA *et al.*, 2012). Some purified and recombinant antigens of many *Leishmania* species have been produced and used for serologic assays and have demonstrated an increase in the operational characteristics of these tests, as in the example of the rK39 antigen for the serodiagnosis of Visceral Leishmaniasis. (DE VRIES, REEDIJK & SCHALLIG, 2015). The rK39 is a recombinant protein isolated from *Leishmania infatum* that contains 6.5 tandem copies of the B-cell antigenic epitope arranged in 39 amino acids. This antigen is related to the kinesin protein - a motor protein - which is conserved between *L. infantum* and *L. donovani*, and reveals a single open reading frame that encodes a total of 298 amino acids with a predicted molecular mass of 32.7 kDa. The *Leishmania* motor protein is involved in various intracellular processes and is present in the amastigote forms of many species. (BURNS *et al.*, 1993).

Isolation of antigenic proteins of *Leishmania* species and heterologous expression of its specific epitopes in a prokaryotic system as *Escherichia coli* is a technique that is easy to

handle, cheap to culture and fast to produce the target recombinant protein, but has limited eukaryotic post-translational activity, especially glycosylation, which is considered the main disadvantage for the production of the eukaryotic proteins. Likewise, a high concentration of the unfolded protein can lead to a decline in refolding yields, as well as culturing at the *E. coli* optimal temperature inhibits high yields of recombinant proteins and allows more expressed protein degradation. (KHOW & SUNTRARACHUN, 2012). The protozoan *L. tarentolae,* that is no pathogenic to mammalians, has been explored as a eukaryotic host of a protein expression platform that allows complex eukaryotic proteins expression at high levels and has the ability to correctly produce proteins post-translationally. Another advantage of this system is that the genes encoding the target recombinant protein can be expressed when transcribed by RNA polymerase I or an external polymerase once RNA processing is detached from DNA transcription. Moreover, the host is easy to manipulate, can be cultivated on a cheap medium between 6 and 8 hours doubling time and is as effective as the prokaryotic system, making the parasite a reliable expression system for expression of eukaryotic proteins. Finally, the maintenance of the transfected culture of *L. tarantolae* is performed under specific antibiotic selection and keeps the same level of the proteins expression after several months of culturing. (BREITLING *et al*., 2002; KUSHNIR *et al.*, 2011; KLATT & KONTHUR, 2012). Presently, recombinant proteins from parasites have been used for many applications as the development of new diagnostic devices and immunogenic antigens for vaccination, analysis of the structure and function of proteins from parasites and screening and profiling of candidate drugs, but also presents challenges and limitations of the technique as the scale of production, immunogenicity of the recombinant product and post-translational modifications. (FERNÁNDEZ-ROBLEDO & VASTA, 2010).

Due to the lack of knowledge of the way in which different *Leishmania* species can cause diverse clinical manifestations in mammals, the identification of gene distribution in each parasite species can help to elucidate their mechanisms of action in the disease development. Few genes of species-specific parasites are considered important in the manner that the disease develops because, in addition to the levels of parasite, gene expression differs between species. The parasite genome can also play only a small participation in the clinical presentation. When comparing the genome of the two most common species of *Leishmania* causing disease in Brazil, *L. infantum* which causes Visceral Leishmaniasis (VL) and *L. braziliensis* which causes Cutaneous Leihsmaniasis (CL), similarities have been found between them, as the quantity of genes in each species (8,195 genes are presented in the *L. infantum* genome and 8,314 genes in the genome of *L. braziliensis*) and the average amino

acid and nucleotide identities (77% and 81%, respectively). However, it is known that *L. infantum* has 36 chromosomes, whereas *L. braziliensis* has 35 chromosomes, owing to an apparent fusion of chromosomes 20 and 34. Despite the broad differences between both disease phenotypes, few are the specific genes of each individual *Leishmania* species: there are only 26 *L. infantum*–specific genes and approximately 47 *L. braziliensis*–specific genes, distributed throughout the genome. (PEACOCK, 2007).

Based on what is exposed above, the aim of this study was to explore the use of *L. tarentolae* as a host for the expression and secretion of *L. braziliensis* kinesin-related recombinant protein, designed based on the reference kinesin-related gene of *L. infantum*. The diagnostic efficiency of this new antigen was evaluated by ELISA for specific detection of CL patients from Brazil. Until now, no studies have been reported on the levels of antibodies against *L. braziliensis* kinesin-related protein in CL patients.

2. MATERIAL AND METHODS

The flowchart presented in Figure 1 describes briefly each step of this study.

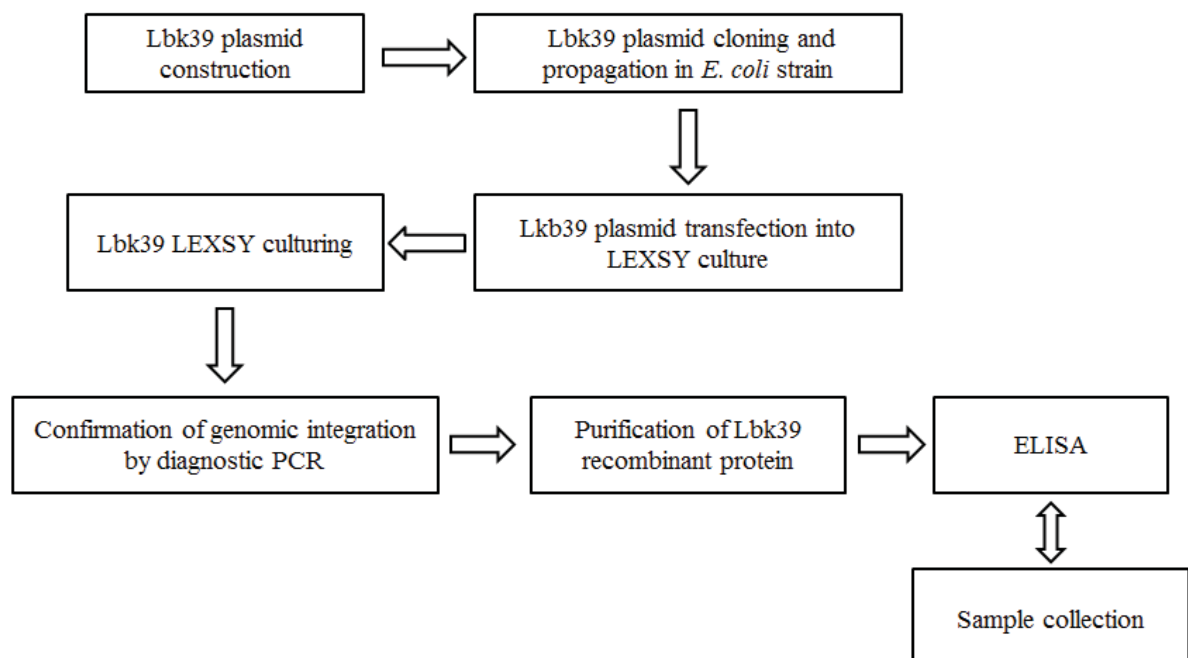

Figure 1. Methodological approach flowchart

2.1. Sample collection

A total of 108 serum samples from females and males of different age groups were collected from the following CL and VL endemic and non-endemic regions in Brazil: endemic region from Pará and Espírito Santo state and endemic and non-endemic regions from Paraná state. The main inclusion criterion for this study was the proven diagnosis of CL by parasite presence on the direct examination of lesion smears obtained from the edge of the active lesion with a punch biopsy tool and/or positivity in the parasite culturing. The patients were divided into groups according to the following classification (Table 1):

Table 1. Identification of samples used in this study

Group	N	Classification
H1	22	Healthy individuals from non-endemic areas
H2	37	Healthy individuals from endemic areas
CL1	09	CL patients with active lesion and no treatment
CL2	05	CL patients with active lesion under the treatment
CL3	13	CL patients that had finished treatment and were under observation for the subsequent 3 months
CD	13	Serum reagent patients for Chagas disease
CLR	02	Patients with a reincident lesion and who have had the same treatment several times lasting approximately one year
CLP	02	Patients that had CL for longer than five years prior to this study
VL	05	Positive patient for visceral leishmaniasis (VL) after treatment and were under observation

N: number of patients in each group

All serum samples were stored frozen (−20°C). Individuals from H2 group were medically examined to discard any previous CL infection. CL and VL patients were treated according to the following Brazilian Healthy Ministry guidelines: Manual of surveillance and control of Visceral Leishmaniasis (2014) and Manual for Surveillance of American Integumentary Leishmaniasis (2010). Patients with other diseases such as Chagas disease and visceral leishmaniasis were also studied to evaluate the chance of cross-reactivity in these tests.

Ethical approval was obtained from the Universidade Federal do Paraná Ethical Committee under number 684.244 (Attachments 2 and 3).

2.2. Lbk39 plasmid construction

The gene fragment of kinesin-related gene of *L. braziliensis*, henceforth called Lbk39, contains 843 base pairs (bp) and is responsible for encoding immunologic dominant repeated amino acids. It is designed based on the kinesin-related gene of *L. infantum* (synonymous *L. chagasi*) - Genebank: L07879, described by Burns *et al.* (1993) and comprising 46 amino acid regions followed by 6.5 times of 39 amino acid repeats. The homology search was performed by BLAST similarity (ALTSCHUL *et al.*, 1997) on the tritrypDB database website.

For expression of the target recombinant protein, the synthetic gene Lbk39 was assembled from synthetic oligonucleotides by Invitrogen (by Thermo Fischer Scientific, Germany) and the fragment was inserted into pLEXSY-sat2 recombinant vector, developed by Jena Bioscience (Germany), and cloned with 6×His-tag into the corresponding site of the above mentioned recombinant vector.

According to the manufacturer, Jena Bioscience, the pLEXSY-sat2 recombinant vector belongs to the LEXSY protein expression platform and was developed to be inserted into the eukaryotic host *L. tarentolae*, a parasite isolated from *Tarentola mauritanica* (Moorish gecko). The referred expression vector was designed for integration into the chromosomal 18SrRNA (*ssu*) locus of the parasite, allowing the true eukaryotic protein expression and, for this specific study, the target protein was selected to be secreted into the culturing medium. (BREITLING *et al*., 2002).

2.3. Lbk39 plasmid cloning and propagation in E. coli strain

Following the construction of the Lbk39 plasmid, One Shot™TOP10 Chemically Competent *Escherichia coli* strain (Invitrogen) was chosen for the plasmid cloning and propagation and the procedure for culturing was followed according to the manufacturer's instructions, except for the incubation temperature, which was 30°C for plasmid stability reasons.

After that, the plasmid was purified from the *E. coli* strain using the Geneflow Q-Spin Plasmid DNA Purification Kit and was sent for sequencing. To confirm the plasmid identity the forward P1442 (5'-CCGACTGCAACAAGGTGTAG-3') and reverse A264 (5'-CATCTATAGAGAAGTACACGTAAAAG-3') sequencing primers, included in the LEXSY kit were used.

2.4. *Lkb39 plasmid transfection into LEXSY culture and Lbk39 LEXSY culturing*

The propagated and purified amount of Lbk39 plasmid from *E. coli* strain was linearized by digestion with *SwaI* (*SmiI*) enzyme, from *Streptococcus milleri* S - 10U/µL (Thermo Fischer Scientific), to prepare for plasmid transfection into LEXSY host *L. tarantole*, 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. When ready for transfection by electroporation, the culturing cells were handled according to the same manual mentioned above. Another aliquot of LEXSY cells was electroporated without DNA under the same conditions as a negative control. Then the electroporated cells were transfected with a capillary to the tissue culture (TC) flasks with 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 started to get slightly turbid (24 hours after electroporation), the specific Streptothricin-class of aminoglycoside antibiotic Nourseothricin (LEXSY NTC, Jena Bioscience) for the pLEXSY-sat2 vector was added and the culturing were carried out in the same conditions every 4 days.

2.5. *Confirmation of genomic integration by diagnostic PCR*

The genomic integration of the Lbk39 plasmid into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* strain was confirmed by PCR. The genomic DNA was extracted from 2 mL of a dense Lbk39 LEXSY culture by DNeasy Blood & Tissue Quick-start kit (Qiagen) according to the manufacturer's recommendation. After that, 200 ng of genomic DNA was added into two 0.2 mL microtubes (100 ng in each), containing both 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 the specific primers (provided by Jena Bioscience) for the genomic integration diagnostic: into the first tube the F3001 forward primer (5'-GATCTGGTTGATTCTGCCAGTAG-3'), responsible for integration of all *ssu* expression vectors from Jena Bioscience, and A1715

reverse primer (5’-TATTCGTTGTCAGATGGCGCAC-3’), responsible for integration of all "AP" expression vectors from Jena Bioscience with 5'utr *aprt*; and into the second tube the F2999 forward primer (5’-CCTAGTATGAAGATTTCGGTGATC-3’), responsible for integration diagnostics of all *sat* expression vectors from Jena Bioscience, and the F3002 reverse primer (5’-CTGCAGGTTCACCTACAGCTAC-3’), responsible for integration diagnostics of all *ssu* integration vectors from Jena Bioscience.

The PCR conditions for the F3001/A1715 pair of primers were: 1 cycle of 95°C for five minutes for the initial denaturation, 35 cycles of 95°C for 30 seconds + 60°C for 30 seconds + 72°C for one minute for the annealing stage, and 1x 72°C for 10 minutes for final extension; whilst the PCR conditions for the F2999/F3002 pair of primers were: 1 cycle of 95°C for five minutes for the initial denaturation, 35 cycles of 95°C for 30 seconds + 53°C for 30 seconds + 68°C for four minutes for the annealing stage, and 1x 72°C for 10 minutes for final extension.

2.6. Purification of Lbk39 recombinant protein

The Lbk 39 recombinant protein purification was carried out using the HisTrap HP 1 ml column (GE HealthCare) by loading the culture media into the column, according to the manufacturer’s instructions. Afterwards, salts and imidazole were removed by dialysis in PBS buffer at 4°C, twice for two hours and after once, overnight. Then, lyophilisation was performed to concentrate the purified Lbk39 recombinant protein.

To analyse whether the purification process had worked, the purified and dialysed Lbk39 recombinant protein was concentrated with trichloroacetic acid (TCA), as indicated in the LEXSYcon2 Expression kit manual, loaded on a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with nitrate silver.

Finally, the protein determination was performed using the Micro BCA™ Protein Assay Kit (Thermo Fischer Scientific) following the manufacturer’s procedure.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To determine whether levels of anti-*Leishmania* antibodies in human serum non-infected and infected with CL, VL, and other diseases can identify Lbk39 epitopes, optimal ELISA conditions were established, such as dilutions of sera (1:100, 1:200, 1:400 and 1:800), conjugate (1:5,000, 1:10,000 and 1:20,000) and antigens (0.1 µg, 0.5 µg and 0.85 µg/well).

Different high binding polystyrene microtiter plates (96 well EIA/RIA 1x8 Stripwell Plate, Costar, USA) were coated overnight at 4°C with 100 μL/well solution of 0,1 μg/well of Lbk39 antigen-diluted in carbonate–bicarbonate buffer (pH 9.6). In addition, soluble proteins from crude extract of *L. braziliensis* culture (strain MHOM/BR/84/LTB300) were also included in the study as a positive control. On the following day, the plates were washed twice with 200 μL/well of washing solution (NaCl – 0.9% + Tween 20 – 0.05%), then the wells were blocked with 120 μL of blocking solution (PBS + 0.1% casein) for one hour at 37°C, and after were washed twice again with 200 μL/well of washing solution. Right after, serum samples were diluted to 1:200 in an incubation solution (PBS + 0.25% casein) and were added in their respective wells and incubated at 37 °C for one hour. Then the plates were washed four times with 200 μL/well of washing solution and polyclonal goat anti-human IgG HRP conjugate (2mg/mL, SanBio Científica) was diluted to 1:10,000 and was added to each well for 1 h at 37°C. Finally, the reaction was developed by adding 100 μL of solution of 10.5 mL 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) + 2μL H_2O_2 at 30% to each well at room temperature for 15 minutes, avoiding light, and to interrupt the reaction 20 μL of a solution 1:20 of H_2SO_4 was added. Plates were read in the Powerwave HT reader (BioTek) at 492 nm and values were expressed in absorbance. As control, the pooled positive and negative serum were included in each plate, when testing individual sera, and each sample was measured in triplicate and the whole assay described above was performed in duplicate.

2.8. Statistical analysis

The homogeneity of variance analysis and Kolmogorov-Smirnov test for normal condition of variables evaluation were performed using the MedCalc 16.1 software. Once data showed a parametric distribution, ANOVA one-way analysis with Tukey post-test was performed to validate the significant difference among groups, except for CLR and CLP due to insufficient number of patients in each group to perform a reliable statistical analysis. The student's t test was used to compare the means of each group between antigens. All analyses were performed using GraphPad Prism 6 software and assuming the significant level of $p<0.05$. For the last, ROC (Receiver Operating Characteristics) curve together with sensitivity and specificity analysis and Comparison of independent ROC curves were performed using MedCalc 16.1. software and a significant level of $p<0.05$ was adopted.

3. RESULTS

3.1. *Lbk39 plasmid construction*

BLAST similarity sequence analysis of the cloned fragment revealed an 843 bp product from the locus LBRM_14_1110 of *L. braziliensis* strain MHOM/BR/75/M2904, which exhibited 84% bp of identity with kinesin-related gene of *L. infantum* (synonymous *L. chagasi*) and encodes a protein of 281 amino acids (AAs) with a predicted molecular mass of 30.2 kDa, with 4 identical copies encoding 39 AAs (Figure 2 and Attachment 1). Then the synthetic oligonucleotides produced were inserted into pLEXSY-sat2 recombinant vector and cloned with 6×His-tag (Figure 3).

```
    GTTGCAGCGGCCGTGTCGGTCGACGCTGGCGCCTCTCTAGACGCAGCAGCAGGCAAGGCA
  1 ---------+---------+---------+---------+---------+---------+
    CAACGTCGCCGGCACAGCCAGCTGCGACCGCGGAGAGATCTGCGTCGTCGTCCGTTCCGT
                                    L__D__A__A__A__G__K__A__

    GAGAATACAGCTTCAGAGACAATATCAAGAATAACAGAACAGCTGCGGGGTGCCGAGGAG
 61 ---------+---------+---------+---------+---------+---------+
    CTCTTATGTCGAAGTCTCTGTTATAGTTCTTATTGTCTTGTCGACGCCCCACGGCTCCTC
    E__N__T__A__S__E__T__I__S__R__I__T__E__Q__L__R__G__A__E__E__

    CGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAG
121 ---------+---------+---------+---------+---------+---------+
    GCGCGGCGCCTCGACCGCTCGGTCGACCGCAGGTGACGACGGCGCTTCTGCGACGACCTC
    R__A__A__E__L__A__S__Q__L__A__S__T__A__A__A__K__T__L__L__E__

    CAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGC
181 ---------+---------+---------+---------+---------+---------+
    GTCCTCGCACTGTCGTGCTGCCGCCTGGACCTCCTCGCCGACGCCCCACGGCTCCTCGCG
    Q__E__R__D__S__T__T__A__D__L__E__E__R__L__R__G__A__E__E__R__

    GCCGCGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGAAGACGCTGCTGGAGCAG
241 ---------+---------+---------+---------+---------+---------+
    CGGCGCCTCGACCGCTCGGTCGACCCCCGGTGACGACGACGCTTCTGCGACGACCTCGTC
    A__A__E__L__A__S__Q__L__G__A__T__A__A__A__K__T__L__L__E__Q__

    GAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCC
301 ---------+---------+---------+---------+---------+---------+
    CTCGCACTGTCGTGCTGCCGCTTGGACCTCCTCGCCGACGCCCCACGGCTCCTCGCGCGG
    E__R__D__S__T__T__A__N__L__E__E__R__L__R__G__A__E__E__R__A__

    GCGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGAAGACGCTGCTGGAGCAGGAG
361 ---------+---------+---------+---------+---------+---------+
    CGCCTCGACCGCTCGGTCGACCCCCGGTGACGACGACGCTTCTGCGACGACCTCGTCCTC
    A__E__L__A__S__Q__L__G__A__T__A__A__A__K__T__L__L__E__Q__E__

    CGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCG
421 ---------+---------+---------+---------+---------+---------+
    GCACTGTCGTGCTGCCGCTTGGACCTCCTCGCCGACGCCCCACGGCTCCTCGCGCGGCGC
    R__D__S__T__T__A__N__L__E__E__R__L__R__G__A__E__E__R__A__A__
```

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    GAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGAAGCAGGTGGAGCAGGAGCGT
481 ---------+---------+---------+---------+---------+---------+
    CTCGACCGCTCGGTCGACCGCAGGTGACGACGACGCTTCTTCGTCCACCTCGTCCTCGCA
    E__L__A__S__Q__L__A__S__T__A__A__A__K__K__Q__V__E__Q__E__R__

    GACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAG
541 ---------+---------+---------+---------+---------+---------+
    CTGTCGTGCTGCCGCTTGGACCTCCTCGCCGACGCCCCACGGCTCCTCGCGCGGCGCCTC
    D__S__T__T__A__N__L__E__E__R__L__R__G__A__E__E__R__A__A__E__

    CTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGAAGCTGGTGGAGCAGGAGCGTGAC
601 ---------+---------+---------+---------+---------+---------+
    GACCGCTCGGTCGACCGCAGGTGACGACGGCGCTTCTTCGACCACCTCGTCCTCGCACTG
    L__A__S__Q__L__A__S__T__A__A__A__K__K__L__V__E__Q__E__R__D__

    AGCACGACGGCGAACCTGGAGGAGCGGATGAGGGATGCCAAGAATCGCGCCGCGGAGCTG
661 ---------+---------+---------+---------+---------+---------+
    TCGTGCTGCCGCTTGGACCTCCTCGCCTACTCCCTACGGTTCTTAGCGCGGCGCCTCGAC
    S__T__T__A__N__L__E__E__R__M__R__D__A__K__N__R__A__A__E__L__

    GCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGC
721 ---------+---------+---------+---------+---------+---------+
    CGCTCGGTCGACCGCAGGTGACGACGACGCTTCTGCGACCACCTCGTCCTCGCACTGTCG
    A__S__Q__L__A__S__T__A__A__A__K__T__L__V__E__Q__E__R__D__S__

    ACGACGGCGAAGCTGGAGGAGCGGCTGCGGGAGTCCGAGAGAAAGGTTTCTGTGATAGAA
781 ---------+---------+---------+---------+---------+---------+
    TGCTGCCGCTTCGACCTCCTCGCCGACGCCCTCAGGCTCTCTTTCCAAAGACACTATCTT
    T__T__A__K__L__E__E__R__L__R__E__S__E__R__K__V__S__V__I__E__

    GCGGTGTTGAGGGAGACAAAGTGTAAGTGTGGTACCCACCACCATCACCACCACTAGGCG
841 ---------+---------+---------+---------+---------+---------+
    CGCCACAACTCCCTCTGTTTCACATTCACACCATGGGTGGTGGTAGTGGTGGTGATCCGC
    A__V__L__R__E__T__K__C__K__C__G__T__

    GCCGCCCTCCT
901 ---------+-
    CGGCGGGAGGA
```

Figure 2. The synthetic Lbk39 gene fragment of kinesin-related gene of *L. braziliensis* produced by Invitrogen and its encoded immunologic dominant repeated amino acids.

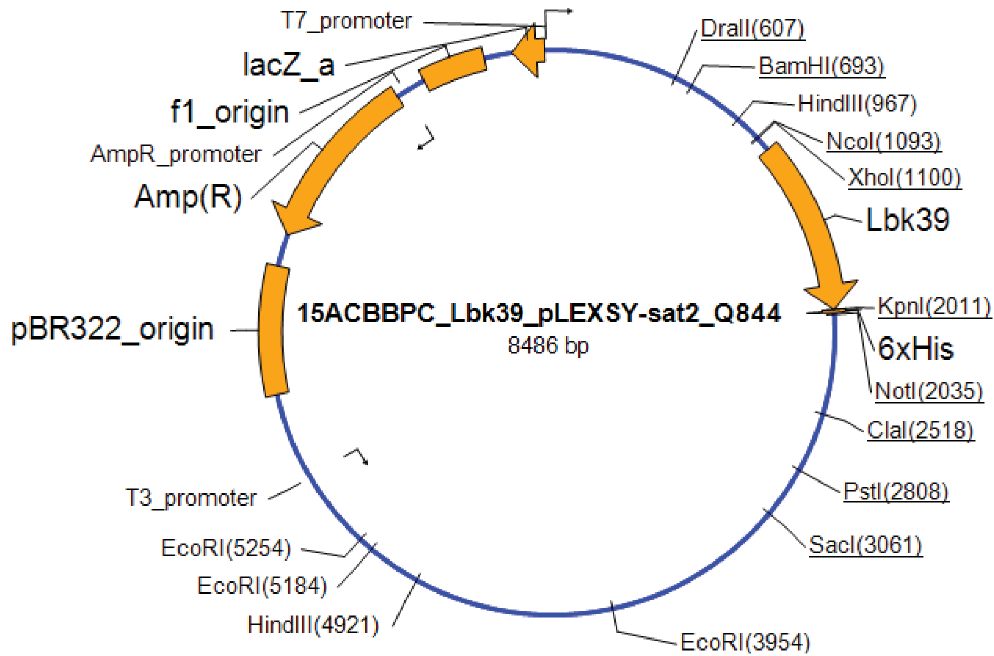

Figure 3. The synthetic gene Lbk39 inserted into the pLEXSY-sat2_Q844 recombinant vector and cloned with 6×His-tag.

3.2. Lbk39 plasmid cloning and propagation in E. coli strain and transfection into LEXSY culture

The purification of the Lbk39 plasmid yielded 1.5 µg/µL of DNA and the confirmation of the plasmid identity by sequencing the purified product showed a 100% of identity for both forward P1442 and reverse A264 sequencing primers.

Finally, the linearization of the Lbk39 plasmid by digestion with *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 (Figure 4).

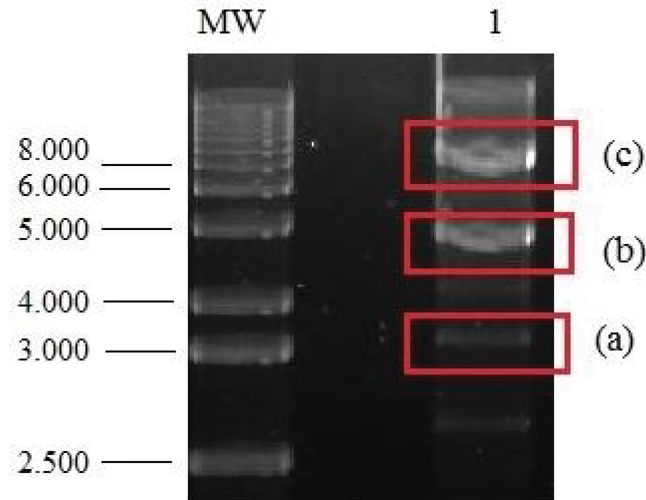

Figure 4. Linearization of the Lbk39 plasmid by digestion with *SwaI* (*SmiI*) enzyme. MW: molecular weight (1kb DNA ladder); Lane 1 (a): 2.9 kbp fragment related to the *E. coli* part; Lane 1 (b): a larger fragment (approximately 5 kbp) related to the Lbk39 plasmid; Lane 1 (c): the entire Lbk39 plasmid, not digested, with 8486bp.

3.3. Confirmation of genomic integration by diagnostic PCR

After approximately 10 days of Lbk39 LEXSY culturing, the cultures started to get turbid (10^7 cells/mL), as well as no parasite growth noticed in the negative control TC flasks. On the 12^{th} day of culturing another passage was made and 2 mL of that dense culture was withdrawn to perform the confirmation of Lbk39 genomic integration by PCR. For this objective two pairs of primers were used, one of them being hybridizing within the expression cassette and the other hybridizing to a chromosomal *ssu*-flanking sequence not present on the plasmid.

The PCR reactions resulted in two DNA fragments of different sizes, one for each pairs of primers, as expected and indicated by the manufacturer: 1.1 kbp fragment size for F3001/A1715 primers and 2.3 kbp fragment size for F2999/F3002 (Figure 5).

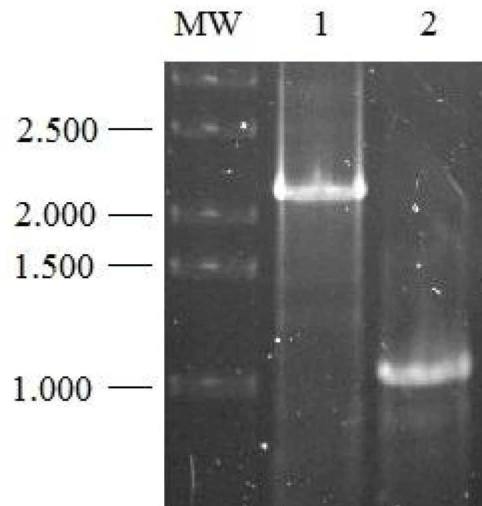

Figure 5. PCR reactions for confirmation of the Lbk39 genomic integration into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* resulted in two DNA fragments of different sizes: 2.3 kbp fragment size for F2999/F3002 pair of primers (lane 1) and 1.1 kbp fragment size for F3001/A1715 pairs of primers (lane 2). MW: molecular weight.

3.4. Purification of Lbk39 protein

The Lbk39 was expressed as 6xHis-tagged recombinant protein in the pLEXSY-sat2 vector, inserted into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* and selected to be secreted into the culturing media. The purification procedure and the protein expression were confirmed by SDS-PAGE and the most probable molecular weight of the target protein was 30 kDa, as expected (Figure 6). The total quantity of purified Lbk39 recombinant protein obtained was 40 µg/mL.

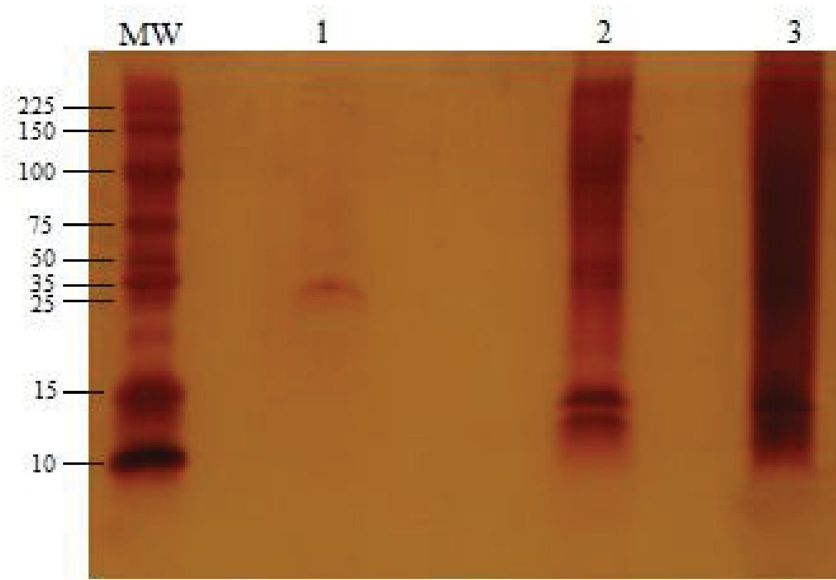

Figure 6. The purified Lbk39 recombinant protein expression confirmed by SDS-PAGE. MW: molecular weight (225 kDa length); Lane 1: purified Lbk39 recombinant protein - the most probable molecular weight is 30 kDa as shown; Lane 2: no-purified Lbk39-LEXSY protein secreted in culture media; Lane 3: *LEXSY-L. tarantole* proteins secreted in culture media.

3.5. Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA using the Lbk39 recombinant protein as antigen for detection of anti-*Leishmania* specific antibodies in CL and VL patient's serum was performed. All serum samples collected were analysed at two different times. First, absorbance values of samples from H1 and CL1 groups were used to perform the ROC curve analysis, sensitivity, specificity, predictive positive value (PPV) and predictive negative value (PNV) of Lbk39. The results obtained were compared with the same parameters of the same sample groups for the positive control. All parameters were evaluated considering the cut-off values calculated by MedCalc software (Table 2 and Figure 7), with 2 standard deviation (2SD) (Table 3) and with 3 standard deviation (3SD) (Table 4).

Table 2. ROC curve analysis for Lbk39 antigen compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed using MedCalc software.

	MedCalc					
	Cut-off	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.1731	0.914±0.0723	77.8	100	1.0	0.97
Lbk39	0.2293	0.631±0.117	88.9	40.9	0.17	0.96

L. braziliensis: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

Table 3. Sensitivity, specificity, PPV and PNV analysis for Lbk39 antigen compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed considering 2SD.

	2 SD				
	Cut-off	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.1668	81.8	91.7	0.57	0.97
Lbk39	0.4857	50.0	95.7	0.61	0.93

L. braziliensis: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

Table 4. Sensitivity, specificity, PPV and PNV analysis for Lbk39 antigen compared to the ROC curve analysis for the positive control (*L. braziliensis*) performed considering 3SD.

	3 SD				
	Cut-off	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
L. braziliensis	0.2109	75.0	100.0	1.0	0.97
Lbk39	0.5877	50.0	95.7	0.61	0.93

L. braziliensis: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); PPV: predictive positive value; PNV: predictive negative value.

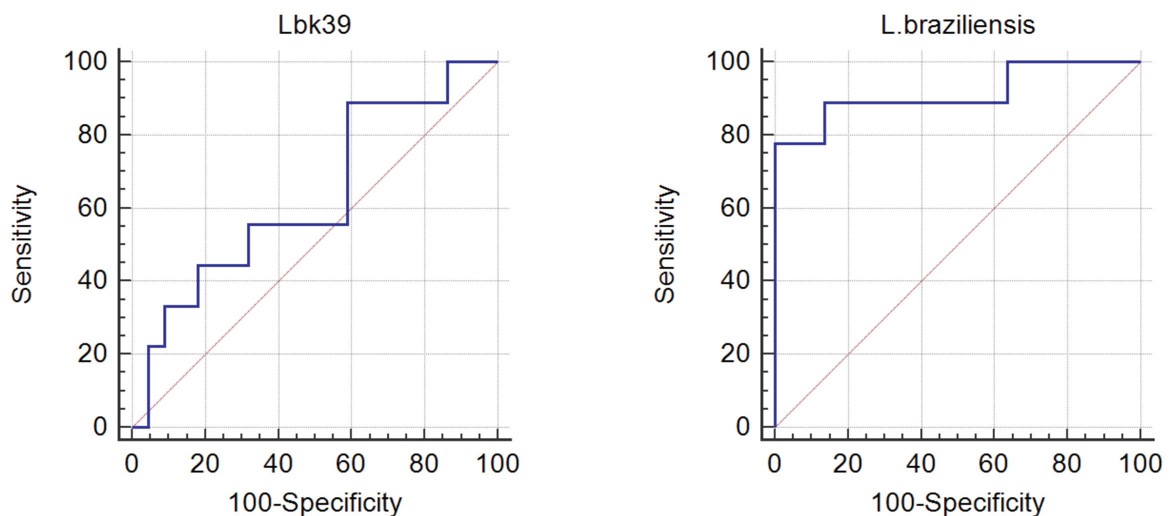

Figure 7. ROC curves analysis for Lbk39 antigen compared to the ROC curve analysis for the positive control. *L. braziliensis*: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300).

Considering the values of sensitivity (from 50% to 88.9%) and specificity (from 40.9% to 95.7%) obtained for Lbk39 using CL patients' samples, in all conditions tested and compared to the positive control group, the ANOVA one-way analysis was performed to validate the significant difference among all sera groups per antigen.

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, non-endemic area and individuals with other diseases, using the Lbk39 recombinant protein as antigen. It was found that only means of anti-Lbk39 antibody titres of

VL group were significantly higher than the H1 group (Table 5 and Figure 8). Also, based on the positivity percentage for all serum samples (Table 6) and sensitivity and specificity values of VL group (Table 7), it can be noticed that the Lbk39 antigen was able to be better detected by antibodies from VL patients. The positivity percentage of CL patients under treatment (CL2 group) was higher than the CL patients with active lesion (CL1 group) and CL patients under observation post-treatment (CL3 group), although it did not present a significant difference among the groups. There was a cross-reaction between the Lbk39 antigen and antibodies from patients with Chagas disease (CD group), since the etiological agent of this disease is another trypanosomatid parasite.

Table 5. ANOVA one-way analysis of levels of anti-Lbk39 antibodies found in individuals from CL endemic and non-endemic areas compared to positive control. The p values with asterisk show significant difference between groups (p<0.05).

		p values					
	Groups	**H2**	**CL1**	**CL2**	**CL3**	**VL**	**CD**
Lbk39	**H1**	0.9773	0.9939	0.3457	0.784	<0.0001*	0.7128
	H2		0.8329	0.1099	0.2686	<0.0001*	0.2154
	CL1			0.7931	0.9981	<0.0001*	0.9957
	CL2				0.9404	<0.0001*	0.9571
	CL3					<0.0001*	>0.9999
	VL						<0.0001*
	CD						
P.C.	**H1**	>0.9999	<0.0001*	0.0193*	0.361	<0.0001*	<0.0001*
	H2		<0.0001*	0.0101*	0.2134	<0.0001*	<0.0001*
	CL1			0.9262	0.0104*	0.5503	0.0002*
	CL2				0.5814	0.15	<0.0001*
	CL3					0.0001*	<0.0001*
	VL						0.4441
	CD						

P.C.: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; VL: positive patients for VL; CD: serum reagent patients for Chagas disease.

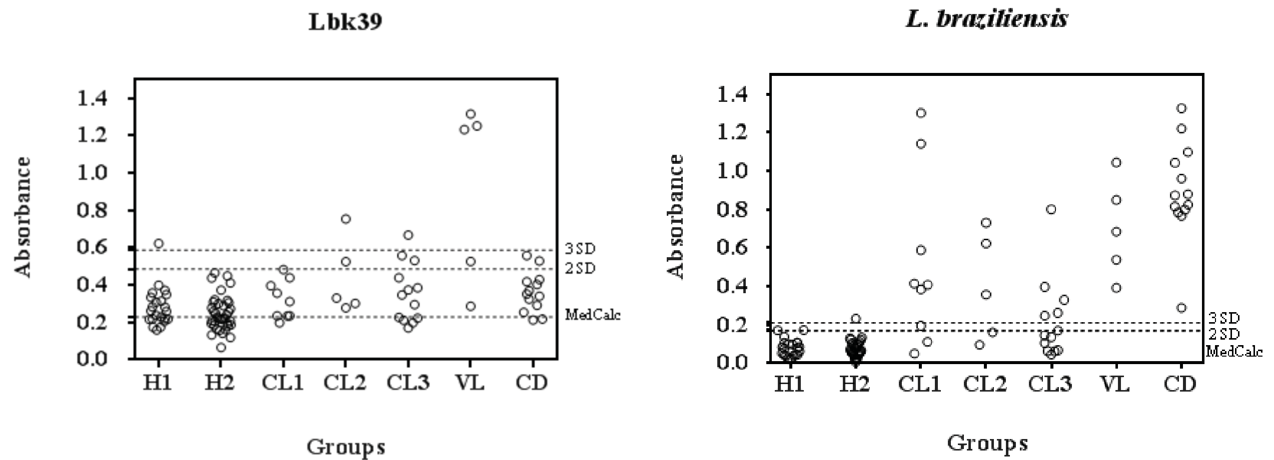

Figure 8. Levels of anti-Lbk39 antibodies detected by ELISA in individuals from CL endemic and non-endemic areas in Brazil compared to levels of anti-*L. braziliensis* antibodies also detected by the same technique and in the same region. *L. braziliensis*: *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; VL: positive patients for VL; CD: serum reagent patients for Chagas disease. MedCalc: cut-off value calculated by MedCalc software; 2SD: cut-off value calculated by 2x standard deviation; 3SD: cut-off value calculated by 3x standard deviation.

Table 6. Positivity percentage of anti-Lbk39 antibodies found in individuals from CL endemic and non-endemic areas performed considering cut-off calculated using the MedCalc software, 2SD and 3SD.

		Positivity (%)		
	Group	**MedCalc**	**2 SD**	**3 SD**
Lbk39	**H1**	59.1	4.5	4.5
	H2	45.9	0.0	0.0
	CL1	77.8	0.0	0.0
	CL2	100.0	40.0	20.0
	CL3	61.5	23.1	7.7
	VL	100.0	80.0	60.0
	CD	84.6	15.4	0.0
L. braziliensis	**H1**	0.0	9.1	0.0
	H2	2.7	2.7	2.7
	CL1	77.8	77.8	66.7
	CL2	60.0	60.0	60.0
	CL3	38.5	46.2	38.5
	VL	100.0	100.0	100.0
	CD	100.0	100.0	100.0

L. braziliesnsis: soluble proteins from crude extract from *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; VL: positive patients for VL; CD: serum reagent patients for Chagas disease. 2SD: 2x standard deviation; 3SD: 3x standard deviation.

Table 7. ROC curve analysis for Lbk39 antigen using absorbance values of samples from H1 and VL groups performed using MedCalc software.

	Lbk39 - VL					
	Cut-off	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**PPV**	**PNV**
2 SD	0.4857	N/A	83.3	95.7	0.24	1.00
3 SD	0.5877	N/A	71.4	95.7	0.21	1.00
MedCalc	0.3985	0.918±0.0753	80.0	95.5	0.70	0.97

2SD: 2x standard deviation; 3SD: 3x standard deviation; PPV: predictive positive value; PNV: predictive negative value.

Although no statistical analysis has been performed for CLP and CLR groups due to insufficient number of patients in each group, results obtained showed that both groups did not present a high level (above the cut-off line) of anti-Lbk39 antibodies (data not shown).

Finally, the student's t test was performed to compare means of each group for Lbk39 antigen compared to the positive control (Figure 9). The mean values for VL and all CL groups analysed for the Lbk39 antigen did not present a statistical difference with respect of means values of the same groups for the positive control. These findings show that, whilst the target protein is produced from a kinesin-related gene of *L. braziliensis*, the antibodies from patients stricken with visceral leishmaniasis are able to be as recognisable by Lbk39 as by *L. braziliensis* crude extract.

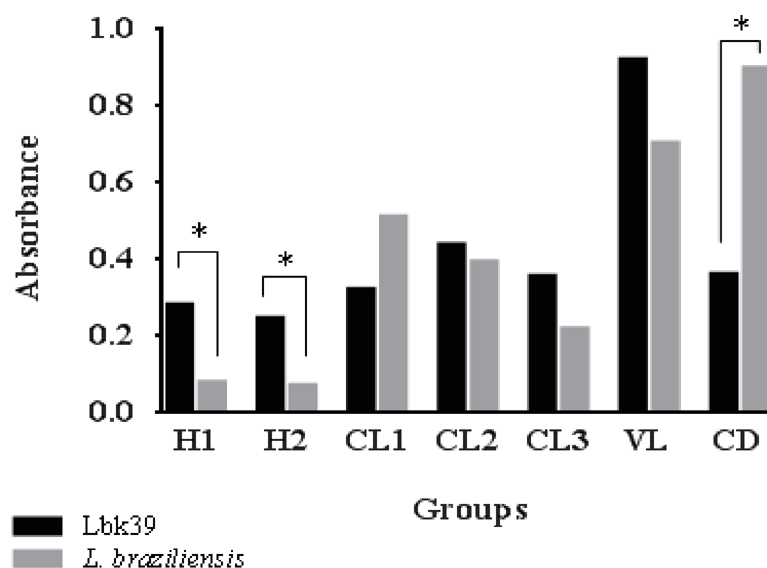

Figure 9. Student's t test between groups of individuals from CL endemic and non-endemic areas for both Lbk39 and positive control. The p values with asterisk show significant difference between groups ($p<0.05$). *L. braziliensis*: *Leishmania (Viannia) braziliensis* culture (strain MHOM/BR/84/LTB300); H1: healthy individuals from non-endemic areas; H2: healthy individuals from endemic areas; CL1: CL patients with active lesion and no treatment; CL2: CL patients with active lesion under the treatment; CL3: CL patients that had finished treatment and were under observation for the subsequent 3 months; VL: positive patients for VL; CD: serum reagent patients for Chagas disease.

4. DISCUSSION

Answering the aim of this study in exploring the use of *L. tarentolae* as a host for the expression and secretion of *L. braziliensis* kinesin-related recombinant protein, the total quantity of the 30 kDa purified Lbk39 obtained was 40 µg/mL. Despite the success obtained during all the procedure for the expression of the target protein, some improvement still needs to be made to achieve a high yield of the secretory protein fraction. The method used to produce the Lbk39 protein by the LEXSY platform can be supported by Kushnir *et al.* (2011). The authors demonstrated that proteins with molecular weights above 100 kDa are well expressed in the LEXSY system. However, they also compared the production of these proteins from linearized and circular plasmids and have found that nearly 100% of all *L. tarantole* colonies that were transfected with linearized plasmid displayed strong inducible overexpression of the target proteins, and only 10% of clones that were transfected with the circular plasmids could be induced to produce the same proteins. Mureev *et al*. (2009) and Kovtun *et al*. (2011) also worked with the trypanosomatid platform system and showed that protein production in a large-scale fermentation of *L. tarentolae* is not expensive and allows the yield of approximately 50 mL of cell-free protein extracts in a standard laboratory model; and Rooney *et al*. (2015) concluded that three potential *Trypanosoma brucei gambiense* antigens were able to be highly expressed and post-translationally processed and secreted by *L. tarentolae* in similar way of the *Trypanosoma* species.

Organisms from Trypanosomatidae family have been considered a very good model for the production of eukaryotic recombinant proteins owing to their mechanisms of gene expression, where messenger RNA (mRNA) is transcribed as a polycistronic precursor, which is a type of mRNA that can encode more than one polypeptide separately within the same RNA molecule, and is post-transcriptionally processed into individual mRNA. Although in all others eukaryotic cells the genes that encode proteins are transcribed by RNA polymerase type II, the high rate transcription process of recombinant proteins in trypanosomatid platforms is made by the RNA polymerase type I, ensuring high levels of recombinant gene expression due to trans-splicing of polycistronic precursors. Better expression can also be obtained if the target gene is followed by the 3'-UTR (intergenic untranslated regions) from a highly expressed gene, because the regulation of protein expression occurs mainly after the transcriptional process through the structure of the UTRs. (KUSHNIR *et al*., 2005; BASILE & PETICCA, 2009; NIIMI, 2012; SUGINO & NIIMI, 2012; FERNÁNDEZ & VEJA, 2013).

During the infection, secreted proteins of *Leishmania* primarily play a role in the establishment of the infection, and in a second moment it contributes to the survival of the

parasite into the host's body either by repression of some gene expression, post-translation modification or degradation of specific proteins important to battle the infection, or even by activation of pathways or substances of suppression. (SANTAREM *et al*., 2007).

When levels of anti-*Leishmania* antibodies in human serum infected with CL and VL were detected by the Lbk39 epitopes, it can be noticed that the antigen was able to be better detected by antibodies from VL than CL patients. These findings show that, whilst the target protein is produced from a kinesin-related gene of *L. braziliensis*, the antibodies from patients stricken with VL are just as able to be recognisable by Lbk39 as by *L. braziliensis* crude extract. However, among CL patients, the positivity of CL patients under treatment was higher than the CL patients with active lesion and under observation post-treatment.

Although until now no studies have been reported on the levels of antibodies against *L. braziliensis* kinesin-related protein in CL and VL patients, few studies have shown the reaction of CL individual antibodies against some *L. infantum* kinesin-related recombinant proteins: 1) Molinet *et al.* (2013) found that all of the 272 serum samples from patients with CL were negative when using one commercially available rK39 rapid test; 2) Hartzell *et al.* (2008) observed that both rK39 rapid test and ELISA using the rK39 antigen demonstrated a positivity of only 10.2% and 28.8%, respectively, to diagnosis the United States soldiers stationed in Afghanistan and Iraq that had contracted CL; and 3) Reiter-Owona *et al*. (2016) showed that one commercially available ELISA using rK39 as antigen was less sensitive in patients with CL, where 77% of patients' serums with imported CL were tested negative by ELISA and only 23% were found ELISA positive or borderline. Serological assays in general are more sensitive for VL diagnosis, whereas the serological CL diagnosis has a low predictive value due to low antibody concentration. Likewise, Oliveira *et al*. (2011), when tried to reunite all recombinant antigens that have shown to be able to identifying *Leishmania infantum*-infected patients, found that serum samples from 3 of 26 patients with CL showed a positive result only in the Lci2 antigen based ELISA; the mentioned antigen curiously encodes C-terminal fragments of the approximately 358kD protein that belongs to the *L. infantum* kinesin superfamily of motor proteins.

In addition to having identified the kinesin-related gene of *L. infantum* (synonymous *L. chagasi*), Burns *et al.* (1993) had already indicated that rK39 may be specific to identify the *L. chagasi* and *L. donovani* infection only, as well as Badaró *et al*. (1996). The recombinant antigen used in the immunoblot test and ELISA were strongly recognized by VL patients' antibodies, but not by neither CL nor CD patients' antibodies. Additionally, Moreno *et al*. (2009) observed that the presence of the kinesin gene expression predominantly on

amastigotes can explain the high titres of anti-rK39 antibody only in patients with acute VL, because patients without clinical signs have low numbers of amastigotes replicating in their lymphoid tissue, as well as in patients with CL.

Other studies performed using rK39 antigen to detect VL antibodies in several regions of the world have been also reported (table 8), showing that the variability in sensitivity and specificity has been associated with the geographic region and ethnic groups. (SINGH, SUNDAR & MOHAPATRA, 2009).

Table 8. Studies performed using *L. infantum* kinesin-related recombinant proteins as antigens to detect visceral leishamaniasis.

Author	Antigen	Method	Sensitivity (%)	Specificity (%)	Region
Maia et al. (2012)*	rK39	strip test	50 - 95	77 - 99	All regions
		ELISA	50 - 91	77 - 88	
Cunningham *et al.* (2012) **	rK39	RDT	73.2 - 87.2	96.4	East Africa
			79.2 - 92	95.6 - 98.8	Brazil
			98.8 - 100	96.8 - 100	Indian Subcontinent
	rKE16		36.8 - 67.6	98 - 90.8	East Africa
			61.5 - 84.7	96.8 - 98.4	Brazil
			92.8 - 99.6	96 - 99.2	Indian Subcontinent
Delgado *et al.* (2001)	rK39	strip test	87.8	100	Venezuela
de Assis *et al.* (2008)	rK39	RDT	93	97	North Eastern Brazil
		ELISA	97		
Ritmeijer *et al.* (2006)	rK39	RDT	89.6	99.2	Sudan
Vaish (2012)	rK39	strip test	100	100	India
		ELISA	100	97.7	
Salam (2008)	rk39	strip test	100	86.95	Bangladesh

RDT: rapid diagnostic tests; *Meta-analysis performed to evaluate the VL individual's recognition by the rK39 antigen; **Study performed to evaluate the VL individual's recognition by the rK39 and rKE16 antigens commercially available.

In a more specific study relating *Leishamania* variability from different geographic regions, Bhattacharyya, Boelaert & Miles (2013) investigated whether molecular divergence in the *L. donovani* kinesin-related gene, the main specie causing VL in East Africa, contributes to lower rates of the antibody detection in this region using the rK39 as antigen. Taking into consideration the reference genome K39 derived from *L. infantum* (*L. chagasi*), by analysing the genome of the isolates of *L. donovani* from those African endemic regions and compared to the South Asian *L. donovani* sequences, the authors discovered that there is, in fact, the presence of diversity of nucleotides and predicted amino acids among East African *Leishmania* strains. For example, in the region of the kinesin-related protein that encodes 46

non-repeated amino acids, there is only one divergence found in the 41st amino acid position, which is Cysteine in South Asian strains and changes to Serine in East African strains. However, in the region that encodes the 6.5 copies of the 39 amino acids sequence, the 2nd position in the repeats 4, 5 and 7 changes from Glutamine in East African strains to Glycine in South Asian strains; and the 11th position in the repeats 1, 2 and 4 changes from Arginine in East African strains to Histidine in South Asian strains. Conservatives changes, mostly in the latter half of the repeats, were also found, e.g. in repeat 1 for Ala→Ser^{21}, Ala→Thr^{23}, Ser→Met^{27}, Thr→Ala^{39} in East African and South Asian strains, respectively. Some other conservative changes were only a non-charged residue replaced by another non-charged residue. Finally, all rK39 homologous repeats from East African strains were compared with the reference amino acid sequence rK39 derived from *L. infantum* (*L. chagasi*) and residues 2, 6, 10, 16 and 18 were found affected three or four times by substitutions with charge changes, as well as residues 11 to 15 and 28 to 34 were totally conserved between reference protein rK39 across all the East African strains. All results found by the authors give support to the current study, considering that the Lbk39 recombinant protein exhibited 84% of identity with kinesin-related gene of *L. infantum* (*L. chagasi*) and encodes a protein of 281 amino acids with 4 identical copies encoding 39 amino acids.

Several *Leishmania* genes that encode potential antigens have been cloned and characterized aimed to improve the serological methods of anti-*Leishmania* antibodies detection, as well as to understand the alterations in the host immune response infected with the parasite and also to identify potential antigens that can be used in vaccines. Manifold *Leishmania* antigens belonging to evolutionarily conserved proteins have been described to be potential antigens for the diagnosis of leishmaniases because of their high frequency of recognition by antibodies in the sera from patients with the disease, such as the heat-shock proteins, ribosomal proteins and histones. However, their strong conserved protein sequence can be a source of cross-reactivity with the antibodies induced by the other infectious diseases or autoimmune processes. (REQUENA, ALONSO & SOTO, 2000).

Kubar & Fragaki (2005) stated in their review that sera from VL and CL patients contains substantial titres of antibodies capable of detecting several other *Leishmania* antigens, as *Leishmania major* stress inducible 1 (LmSTI1) protein, *Leishmania* thiol-specific antioxidant (TSA) protein, specific amastigote *L. donovani* A2 proteins, the repetitive sequence of *L. major* gene B protein (GBP) and the kinetoplastid membrane protein (KMP11). Also, according to the authors, only VL patients' sera carry antibodies against *L.*

donovani hydrophilic and acylated surface protein (HASPB1), nuclear proteins (papLe22 and Lepp12) and the promastigote central region surface antigen (PSA).

Abundance of protein markers varies considerably between cutanotropic and viscerotropic parasites, reflecting the different environments to which parasites have to adapt. However, in the beginning of the VL infection, the K39 antigen is recognised by the antibodies in the sera of infected humans and animals and may divert the immune response towards a less-protective Th2-driven reaction. (BIFELD & CLOS, 2015). Several other recombinant kinesin-related proteins, such as the rK9, rK26 and rK39, have also been investigated for the anti-*Leishmania* antibody response in order to determine the most suitable antigen for diagnostic purposes. Nonetheless, the sensitivity and specificity of tests using these different antigens are significantly different between them, and it could be explained by the fact that there are different *Leishmania* species causing leishmaniasis, leading to the difference in the antibody response. (DUJARDIN, 2009; MOHAPATRA *et al*., 2010).

Notwithstanding, the genomes of *Leishmania* species vary in size from 29 Mb, for *L. amazonensis*, to 33 Mb for *L. major*, *L. infantum* and *L. braziliensis*, and are organised into a variable number of chromosomes, i.e., 34 in *L. amazonensis* and *L. mexicana*, 35 in *L. brasiliensis*, and 36 in *L. major*, *L. donovani*, and *L. infantum*. However, in spite of the variability in pathogenicity and tissue tropism of different *Leishmania* species, their genomes are highly similar, with a high degree of gene conservation. (CANTACESSI *et al*., 2015).

The analysis of genes that encode proteins in *Leishmania* spp. performed by ROGERS *et al*. (2011) showed only a small number of species-specific genes, where 19 were *L. infantum*–specific genes of which 15 encode proteins of unknown function, 14 were *L. major*–specific genes of which 13 encode proteins of unknown function and 67 were *L. braziliensis*–specific genes of which 54 encode proteins of unknown function. According to the authors, species-specific genes in *Leishmania* are conserved among isolates within the same species complex and among strains of the same species isolated from different or similar geographical locations.

Nevertheless, Cupolillo *et al.* (2003) revealed that *L. braziliensis* strains are an extremely diverse population and can be classified into three distinct genotypic groups in Brazil, which could explain the ability of these parasites to adapt to changing ecological conditions. Molecular diversity found in parasites from the Amazon Basin is apparently related to the great number of sandfly and/or animal reservoir existing in the same region and that are involved in the transmission cycles. In opposition, the *L. braziliensis* populations found in the Brazilian Atlantic coast showed a lower level of heterogeneity and have

peridomestic sandfly species as the main vectors. Finally, parasites found in urban areas, where transmission of the disease is associated with the sandfly specie *Lutzomyia intermedia*, cluster together.

Although recombinant proteins have been extensively used for specific antibody detection, their use in diagnostic tests have revealed some problems: 1) They can be less immunoreactive than the corresponding purified antigen due to the absence of post-translational modifications depending on the expressed protein system; and 2) their production in high quality and quantity is almost always laborious and expensive to produce. The use of synthetic peptides as antigen is a valid alternative to decrease the non-specific antigen-antibody reactions and can be used to improve the reproducibility and remove the high variability observed in some serodiagnosis assays. However, the use of short linear peptides is not exempt from problems, as the low peptide absorption on the polystyrene titration plates, the Cysteinyl-glycyl-glycine (CGG) moiety derivatisation, the use of derivatised peptides with fatty acids hydrophobic tails, and the covalent bonding of the peptide to the solid medium. In addition, although biotin-avidin methodology has been widely applied to increase the sensitivity of the tests that use peptides as antigens, it leads to the masking of the peptide recognition by antibodies. (GÓMARA & HARO, 2007)

Finally, recognizing and understanding multiple host immune response may be the solution to the identification and control of zoonotic diseases like leishmaniasis. Goto *et al.* (2009) analysed antibody responses to rK39 antigen both in humans and dogs with VL, and noticed that humans showed much stronger immune responses to the rK39 antigen than dogs, concluding that the rK39 recombinant antigen is very specific to detect VL only in humans. However, Porrozzi *et al.* (2007) revealed that IgG responses to the rK39 antigen were very 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, as well as 11% of dogs with leptospirosis were also positive for the same antigen, indicating that rK39 is not a good antigen to detect leishmaniasis in dogs. Quinnell *et al.* (2013) also showed an overall sensitivity of 46% and specificity of 100% for detection of VL infection by rK39 RDT in dogs, although sensitivity of 77% was detected in the group of symptomatic infected dogs and had a strong association between rK39 RDT positivity and disease severity. For the intervention programmes of leishmaniases both in human and dogs, ideal serodiagnostic tests must be able to identify infected and non-infected reservoirs – such as dogs - giving the possibility of guided, controlled

treatment.

5. CONCLUSION

In trying to identify new ELISA antigens for specific detection of CL patients from Brazil, we explored the use of *L. tarentolae* as a host for the expression and secretion of *L. braziliensis* kinesin-related recombinant protein, the design based on the kinesin-related gene of *L. infantum* (Genebank: L07879). Similarity sequence analysis has revealed a product, named Lbk39, which exhibited 84% of identity with the reference kinesin-related gene and encodes a protein of 281 amino acids with a predicted molecular mass of 30.2 kDa and 4 identical copies encoding 39 AAs. Then, Lbk39 was synthesized, inserted into pLEXSY-sat2 recombinant vector and cloned with 6×His-tag, and then, inserted into the chromosomal 18SrRNA (*ssu*) locus of *L. tarentolae* and selected to be secreted into the culturing media, originating, in the end of the process, 40µg/mL of a 30 kDa protein. Despite the positive outcome of production and expression of this study's target protein, Lbk39 antigen has proven to be a better antigen in detecting anti-Lbk39 antibodies levels in patients infected with VL than in patients infected with CL, and has also shown cross-reaction with the CD patients. Identification of species-specific genes that encode potential antigens, capable of identifying anti-*Leishmania* antibodies, provides knowledge in identifying parasite features that hold the infection.

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5. FINAL CONSIDERATIONS AND PERSPECTIVES

Obeying proposed objectives of this study and after analysing some potential *Leishmania* antigens, it can be concluded that glycoproteins have proven to be a much more reliable tool to the serological diagnosis of CL than only protein antigens. Means of anti-α-Gal antibody titres of individuals infected with CL were significantly higher than healthy individuals from non-endemic areas for all of the NGPs tested. None of the NGPs were significantly recognized by the H1 group, which gives solid support to the conclusion that the disaccharide Galα1-3Galβ is the immunodominant saccharide in *Leishmania* cell surface and is the unique non-reducing terminal glycosphingolipids structure recognized by anti-α-Gal.

Despite the use of *L. tarentolae* as a host for the expression and secretion of *L. braziliensis* kinesin-related recombinant protein and the positive outcome of production of the target protein, Lbk39 antigen has proven to be a suitable antigen to better detect anti-Lbk39 antibodies levels in patients infected with VL than patients infected with CL.

The results obtained in this study were conclusive, indicating there are many differences among *Leishmania* species, from genome to pathogenicity and the ability to survive inside each host's organism, which can explain the different results found in comparison of antigens tested. Therefore, it is important to continue investigating the action of all antigens in other different population's sera, in different geographical regions, infected with *Leishmania* spp., in order to determine the specific ability of antigens to detect anti-*Leishmania* antibody levels only in patients with CL or VL.

The analysis of the action of these antigens against sera of dogs infected with *Leishmania* spp. must be considered in order to evaluate the usefulness of these antigens in detecting anti-*Leishmania* antibody levels also in dogs and how *Leishmania* species behave in different hosts.

Also, the antigens here tested could be used as a source of the intradermal reaction with the view to evaluate the ability of these antigens to detect other immunological reactions against *Leishmania* spp.

Finally, future research may be required to determine the production optimization of the Lbk39 antigen using the LEXSY platform, as well as subsequent steps, such as the purification methods and the antigen's feasibility.

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ATTACHMENTS

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ATTACHMENT 1- Lbk39 design

1. K39 *L. infantum* gene (*L. chagasi* kinesin-like protein partial gene, 3319 bp, DNA linear), GenBank code: L07879.1.

GCTCCCACGGCGCTACCCCCTTTCCCGCATGTGCGACAGTTTCACGCGTACAAACGTCTTTCTCTCTCCTTCGCG
CGTGTCGCTATGGGCGGCGGCGCGTCGGTGTCTTTGATTGCACAGCTCACCGCCTCGCCATATTTTCGTCGTGGC
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GTCGTCGGCGGAGCAGGACCGCGAGAACACGAGGGCCGCGTTGGAGCAGCAGCTTCGTGACTCCGAGGAGCGCGC
CGCGGAGCTGGCGAGCCAG

2. BLAST similarity sequence analysis of the kinesin-like fragment of *L. braziliensis* (strain MHOM/BR/75/M2904) based on the *L. infantum* kinesin-like protein partial gene (84% of identities - highlighted in step 1).

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Query  635   GAGTCGATGGGGACTGCAAGGCGGGTAGCGCAGGACTTTCAGTTCGACCACGTGTTCTGG  694
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             |    || |||   ||||| | ||| |||||| || | ||| |    |||  ||||||| 
Sbjct  1660  CAGGAGCGGCTCGTACAGGCTCTGAACGACCGTGAACAGCTCAGCAACGATCTGCAGCGC  1719

Query  2315  ATTCAGTACGAGTACGGGGAAACCGAGCTCGCGCGAGACGTGGCGCTGTGCGCCGCGCAG  2374
             |||||    |  |  | ||| |||||||| |  ||  | |  ||  |||||||   | ||
Sbjct  1720  ATTCAAGTGGTCTGTGAGGAGACCGAGCTGGGCCGCAAGGCTGCCTTGTGCGCTTTGGAG  1779

Query  2375  GAGATGGAGGCGCGCTACCACGCTGCTGTGTTTCACCTGCAAACGCTCCTGGAGCTCGCA  2434
             || |||||||  ||||| || || || |||||||||||||| || ||| ||||  | |||
Sbjct  1780  GAAATGGAGGGCCGCTATCATGCCGCAGTGTTTCACCTGCATACACTCATGGAAATAGCA  1839

Query  2435  ACCGAGTGGGAGGACGCACTCCGCGAGCGTGCGCTTGCAGAGCGTGACGAAGCCGCTGCA  2494
             || |||||||| ||||  || |||||||| |||||||  ||||  |||| ||  ||||| 
Sbjct  1840  ACGGAGTGGGAAGACGAGCTGCGCGAGCGCGCGCTTGACGAGCTCGACGCAGAAGCTGCT  1899

Query  2495  GCCGAACTTGATG--CCGCA-GCCTCTACTTCCCAAAACGCACGTGAA-AGCGCCTGCGA  2550
             || || |||||||  | | | ||  |  |   |   || ||| | ||| |  || |  ||
Sbjct  1900  GCTGAGCTTGATGAACTGAATGCAGCAGCAGGC---AAGGCA-GAGAATACAGCTTCAGA  1955
```

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Query  2551  GCGGCTAACCAGCCTTGAGCAG--CAGCTTCGCGAATCCGAGGAGCGCGCTGCGGAGCTG  2608
             |    || | ||  |  | |||  ||||| || |   ||||||||||||| |||||||||
Sbjct  1956  GACAATATCAAGAAT--AACAGAACAGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTG  2013

Query  2609  GCGAGCCAGCTGGAGGCCACTGCTGCTGCGAAGTCGTCGGCGGAGCAGGACCGCGAGAAC  2668
             ||||||||||||| | |||||||||| |||||| ||  |  ||||||||| || || | |
Sbjct  2014  GCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGC  2073

Query  2669  ACGAGGGCCACGCTAGAGCAGCAGCTTCGCGAATCCGAGGCGCGCGCTGCGGAGCTGGCG  2728
             |||| |||    || ||| ||| ||| || |   |||||| |||||| ||||||||||||
Sbjct  2074  ACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCG  2133

Query  2729  AGCCAGCTGGAGGCCACTGCTGCTGCGAAGATGTCAGC-GGAGCAGGACCGCGAGAACAC  2787
             |||||||||| |||||||||||||||||||| | | || ||||||||| || || | |||
Sbjct  2134  AGCCAGCTGGGGGCCACTGCTGCTGCGAAGACG-CTGCTGGAGCAGGAGCGTGACAGCAC  2192

Query  2788  GAGGGCCACGCTAGAGCAGCAGCTTCGTGACTCCGAGGAGCGCGCTGCGGAGCTGGCGAG  2847
             || ||| |  || ||| ||| ||| || |   ||||||||||||| ||||||||||||||
Sbjct  2193  GACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAG  2252

Query  2848  CCAGCTGGAGTCCACTACTGCTGCGAAGATGTCAGC-GGAGCAGGACCGCGAGAGCACGA  2906
             |||||||| | ||||| |||||||||||| | | || ||||||||| || || |||||||
Sbjct  2253  CCAGCTGGGGGCCACTGCTGCTGCGAAGACG-CTGCTGGAGCAGGAGCGTGACAGCACGA  2311

Query  2907  GGGCCACGCTAGAGCAGCAGCTTCGTGACTCCGAGGAGCGCGCTGCGGAGCTGGCGAGCC  2966
              ||| |  || ||| ||| ||| || |   ||||||||||||| ||||||||||||||||
Sbjct  2312  CGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCC  2371

Query  2967  AGCTGGAGTCCACTACTGCTGCGAAGATGTCAGCGGAGCAGGACCGCGAGAGCACGAGGG  3026
             |||||| ||||||| |||| ||||||| |   | ||||||||| || || ||||||| ||
Sbjct  2372  AGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGG  2431

Query  3027  CCACGCTAGAGCAGCAGCTTCGCGAATCCGAGGAGCGCGCTGCGGAGCTGGCGAGCCAGC  3086
             |    || ||| ||| ||| || |   ||||||||||||| ||||||||||||||||||||
Sbjct  2432  CGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGC  2491

Query  3087  TGGAGTCCACTACTGCTGCGAAGATGTCAGC-GGAGCAGGACCGCGAGAGCACGAGGGCC  3145
             ||| | ||||| |||||||||||| | | || ||||||||| || || ||||||| |||
Sbjct  2492  TGGGGGCCACTGCTGCTGCGAAGACG-CTGCTGGAGCAGGAGCGTGACAGCACGACGGCG  2550

Query  3146  ACGCTAGAGCAGCAGCTTCGTGACTCCGAGGAGCGCGCTGCGGAGCTGGCGAGCCAGCTG  3205
             |  || ||| ||| ||| || |   ||||||||||||| ||||||||||||||||||||
Sbjct  2551  AACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTG  2610

Query  3206  GAGGCCACTGCTGCTGCGAAGTCGTCGGCGGAGCAGGACCGCGAGAACACGAGGGCCGCG  3265
             | | |||||||||| |||||| ||  |  ||||||||| || || | ||||| |||
Sbjct  2611  GCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGAAC  2670

Query  3266  TTGGAGCAGCAGCTTCGTGACTCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAG  3319
              ||||| ||| ||| || |   ||||||||||||||||||||||||||||||||
Sbjct  2671  CTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAG  2724
```

3. *L. braziliensis* kinesin-like gene (length = 6647bp). Similar sequence to *L. infantum* kinesin-like gene is highlighted.

ATGAAGCCGATGGCAGGCAACAGGCAAAGCCCCGCTGGAGGAGAGTCATTGGCAAATACAAAGCGGCAAGCGACT
TACTTCCAGTTCGACCACGTGTTCTGGTCTGTAGAGACGCCGGACGCGTGCGGCGCGAGGCCTGCGACGCAGGCA
GACGTCTTCCGGACGATCGGGCACCCGCTGGTGCAGCACGCGTTCGACGGGTTCAACTCGTGCCTGTTCGCGTAC
GGGCAGACGGGGAGCGGGAAGACGTACACGATGATGGGTGCGGACGTGAAGACGCTTGGCGGTGAGGGCAGCGGT
GTGACGCCGCGGATCTGCCTGGAGATCTTTGAGCGGAAGGCGAGCGTGGAGGCGGAGGGGCACTCGCGGTGGAGC
GTGGAGCTCGGGTACGTGGAGGTGTACAACGAGCGCGTGTCGGACCTGCTTGGGAAGCGGAAGAAGGGCGCGAAG
GACGGTGGCGAGGAGGTGCACGTGGACGTGCGGGAGCACCCGAACCGCGGGGTGTTCCTGGAGGGGCAGCGGGTG
GTGGAGGTTGGGTGTCTGGACGACGTTGTGCGGCTGATCGAGCTGGGGAACGGCGTGCGGCACACTGCTGCGACG
AAGATGAACGAGCGGAGCAGCCGGAGCCACGCGATCATCATGCTGCTGCTGCGCGAGGAGCGGACGATGACGACG
ACGAGCGGGGAGACGATCCGGACTGCGGGCAAGAACAGCCGGATGAACCTTGTAGACCTTGCGGGGTCTGAGCGC
GTGGCGCAGTCGCAGGTGGAGGGGCAGCAGTTCAAGGAGGCGACGCACATCAACCTGTCGCTGACGACGCTGGGC
GCGTGATCGACGTGCTCGCGGACATGGCGAAGAAGGGCGCGAGGGCGCAGTACACCGTTGCGCCGTTCCGCGACT
CGAAGCTGACGTTCATCCTGCGGGACTCGCTTGGCGGGAACTCGAAGACGTTCATGATTGCGACTGTGAGCCCGA
GCGCGCTGAACTACGAGGAGACGCTGAGCACGCTGCGGTACGCGTCGCGCGCGCGCGACATTGTGAACGTCGCGC
AGGTGAACGAGGACCCGCGCGCGCGGCGGATCCGCGAGCTGGAGGAGCAGATGGCGGACATGCGGAAGGCGATGG
CCGGCGGCGACCCTGCGTACGTGTCTGAGCTGAAGGAGAAGCTTACGCTGCTGGAGTCGGAGGCGCAGAAGCGTG
CGGCGGATCTGCAGGCGCTGGAGCGGGAGCGCGAGCACAACCAAGTGCAGGAGCGGCTACTGCGCGCGACGGAGG
CGGAGAAGAGCGAGCTGGAGGCGCGCGCGGCTGCGCTACAGAAGGAGATGACAGCAACACGGCGGCAGGCGGACG
AGATGCAGACACTCAACATGCGGCTGAAGGAAGAGCAGGAGAGCAAGGAGCGCGAGCTGCTGGATGAGATGGCGA
AGAAGGACGCTGCGTTCCATGCTGTACAGCTACGCAAGGACGCCGAGATTGCGCGTGGGCGCGCAAAACTGCAGA
CGACTGTGGCGCAACTCGAGCGTGAACAGCACGACCGCGAAGCCGCACTAGACGCTCTGCAGACGCACCAAGCGA
AGCTGCAGGCTGCGCTTACGAGCTCAGCGCAAACAGCCGCAGAGCGCGATAAGCTTCAGCTACAACTTGCTGAAT
TGCTCTCTGAACAGGAGCGGCTCGTACAGGCTCTGAACGACCGTGAACAGCTCAGCAACGATCTGCAGCGCATTC
AAGTGGTCTGTGAGGAGACCGAGCTGGGCCGCAAGGCTGCCTTGTGCGCTTTGGAGGAAATGGAGGGCCGCTATC
ATGCCGCAGTGTTTCACCTGCATACACTCATGGAAATAGCAACGGAGTGGGAAGACGAGCTGCGCGAGCGCGCGC
TTGACGAGCTCGACGCAGAAGCTGCTGCTGAGCTTGATGAACTGAATGCAGCAGCAGGCAAGGCAGAGAATACAG
CTTCAGAGACAATATCAAGAATAACAGAACAGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGC
TGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGC
TGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGAAGACGCTGCTGG
AGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGG
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AGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGA
CGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCG
CGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGG
CGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTG
CCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGG
AGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACA
GCACGACGGCGAACCTGGAGGAGCGGCTGCAGGACGCTAAAAAGCGCGCCGCGGAGCTGGGGAGGCAGCTGGCGT
CCACTGCTACTGCGAAGAAGCTGTTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGG
GTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGG
AGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCC
AGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGC
GGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGG
TGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGC
TGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGGACC
TGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGA
AGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCG
CCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGA
CGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTG
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AGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGACGCTGGTGGAGCAGGAGCGTG
ACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGG
CGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGC
GGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGC
AGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGA
GCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGG
AGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGACGC

TGCTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGG
AGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGA
ACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTG
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CTGCTGCTGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTG
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TGGCGTCCACTGCTGCTGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGC
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AGCAGGAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGG
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AGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGA
CGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGATGAGGGACGCTAAAAAGCGCGCCG
CGGAGCTGGCGAGGCAGCTGGCGTCCACTGCTGCTGCGAAGAAGCAGGTGGAGCAGGAGCGTGACAGCACGACGG
CGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTG
CCGCGAAGACGCTGTTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGTGACGCCAAGA
AACGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGACGCTGCTGGAGCAGGAACTCATCA
TGATGAAGGCGAACCTGGAGGAGCGGCTGCGTGACGCCAAGAAACGTGCCGCGGAGCTGGCGAGCCAGCTGGCGT
CCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGCATCATGACGAGGGCGGACCTGGAGGAGCGGCTGCGTG
ACGCCAAGAAACGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGAAGCAGGTGGAGCAGG
AGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCC
AGCTGGCGTCCACTGCTGCCGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGC
GGCTGCGTGACGCCAAGAAACGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGAAGCAGG
TGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGC
TGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGAAGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACC
TGGAGGAGCGGATGAGGGATGCCAAGAATCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGA
AGACGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAAGCTGGAGGAGCGGCTGCGGGAGTCCGAGAGAAAGG
TTTCTGTGATAGAAGCGGTGTTGAGGGAGACAAAGTGTAAGTGTGAAGCTCGTGTTGCAGGGCTCAAAGAGGAGT
GCGAAAAGTTGAAGGTTGATACAGTGAAGTATGCGAAGAAGGTGCGAGTTCTGGAGTCTGAAAAGCGTATCGATG
TTGCCCGATTGCAAGCGCATCGTGATGCCCTTCACCGAAAGGAGTGA

4. *L. braziliensis* kinesin-like protein (length = 2214 amino acids). Amino acid sequence that encodes *L. braziliensis* kinesin-like protein fragment is highlighted in red.

MKPMAGNRQSPAGGESLANTKRQATYFQFDHVFWSVETPDACGARPATQADVFRTIGHPLVQHAFDGFNSCLFAY
GQTGSGKTYTMMGADVKTLGGEGSGVTPRICLEIFERKASVEAEGHSRWSVELGYVEVYNERVSDLLGKRKKGAK
DGGEEVHVDVREHPNRGVFLEGQRVVEVGCLDDVVRLIELGNGVRHTAATKMNERSSRSHAIIMLLLREERTMTT
TSGETIRTAGKNSRMNLVDLAGSERVAQSQVEGQQFKEATHINLSLTTLGA*STCSRMAKKGARAQYTVAPFRDS
KLTFILRDSLGGNSKTFMIATVSPSALNYEETLSTLRYASRARDIVNVAQVNEDPRARRIRELEEQMADMRKAMA
GGDPAYVSELKEKLTLLESEAQKRAADLQALEREREHNQVQERLLRATEAEKSELEARAAALQKEMTATRRQADE
MQTLNMRLKEEQESKERELLDEMAKKDAAFHAVQLRKDAEIARGRAKLQTTVAQLEREQHDREAALDALQTHQAK
LQAALTSSAQTAAERDKLQLQLAELLSEQERLVQALNDREQLSNDLQRIQVVCEETELGRKAALCALEEMEGRYH
AAVFHLHTLMEIATEWEDELRERALDELDAEAAAELDELNAAAGKAENTASETISRITEQLRGAEERAAELASQL
ASTAAAKTLLEQERDSTTADLEERLRGAEERAAELASQLGATAAAKTLLEQERDSTTANLEERLRGAEERAAELA
SQLGATAAAKTLLEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTADLEERLRGAEERAA
ELASQLGATAAAKTLLEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTTANLEERLRGAEE
RAAELASQLASTAAAKTLVEQERDSTTANLEERLQDAKKRAAELGRQLASTATAKKLLEQERDSTTADLEERLRG
AEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTTANLEER
LRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTADL
EERLRGAEERAAELASQLGATAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTT
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STTANLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLVEQ
ERDSTTADLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTL
LEQERDSTTADLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTTANLEERLRGAEERAAELASQLASTAAA
KTLLEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLAST
AAAKTLVEQERDSTTADLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQL
ASTAAAKTLVEQERDSTTADLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTADLEERLRGAEERAAELA
SQLASTAAAKTLVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTTANLEERMRDAKKRAA
ELARQLASTAAAKKQVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLLEQERDSTTANLEERLRDAKK
RAAELASQLASTAAAKTLLEQELIMMKANLEERLRDAKKRAAELASQLASTAAAKTLVEQERIMTRADLEERLRD
AKKRAAELASQLASTAAAKKQVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKTLVEQERDSTTANLEER
LRDAKKRAAELASQLASTAAAKKQVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKKLVEQERDSTTANL
EERMRDAKNRAAELASQLASTAAAKTLVEQERDSTTAKLEERLRESERKVSVIEAVLRETKCKCEARVAGLKEEC
EKLKVDTVKYAKKVRVLESEKRIDVARLQAHRDALHRKE

5. Lbk39 gene and protein encoding sequences.

TCTCTAGAC**GCAGCAGCAGGCAAGGCAGAGAATACAGCTTCAGAGACAATATCAAGAATAACAGAACAGCTGCGG**
GGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCGAAGACGCTGCTGGAGCAG
GAGCGTGACAGCACGACGGCGGACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGC
CAGCTGGGGGCCACTGCTGCTGCGAAGACGCTGCTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAG
CGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGGGGCCACTGCTGCTGCGAAGACGCTG
CTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAG
CTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGAAGCAGGTGGAGCAGGAGCGTGACAGCACGACGGCGAAC
CTGGAGGAGCGGCTGCGGGGTGCCGAGGAGCGCGCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCCGCG
AAGAAGCTGGTGGAGCAGGAGCGTGACAGCACGACGGCGAACCTGGAGGAGCGGATGAGGGATGCCAAGAATCGC
GCCGCGGAGCTGGCGAGCCAGCTGGCGTCCACTGCTGCTGCGAAGACGCTGGTGGAGCAGGAGCGTGACAGCACG
ACGGCGAAGCTGGAGGAGCGGCTGCGGGAGTCCGAGAGAAAGGTTTCTGTGATAGAAGCGGTGTTGAGGGAGACA
AAGTGTAAGTGTGGTACC

SLDAAAGKAENTASETISRITEQLRGAEERAAELASQLASTAAAKTLLEQERDSTTADLEERLRGAEERAAELAS
QLGATAAAKTLLEQERDSTTANLEERLRGAEERAAELASQLGATAAAKTLLEQERDSTTANLEERLRGAEERAAE
LASQLASTAAAKKQVEQERDSTTANLEERLRGAEERAAELASQLASTAAAKKLVEQERDSTTANLEERMRDAKNR
AAELASQLASTAAAKTLVEQERDSTTAKLEERLRESERKVSVIEAVLRETKCKCGT

ATTACHMENT 2 - Ethical approval

UNIVERSIDADE FEDERAL DO PARANÁ - SETOR DE CIÊNCIAS DA SAÚDE/ SCS -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico de leishmaniose

Pesquisador: LIGIA MORAES BARIZON DE SOUZA

Área Temática:

Versão: 2

CAAE: 30303714.7.0000.0102

Instituição Proponente:

Patrocinador Principal: Fundação Araucária

DADOS DO PARECER

Número do Parecer: 684.244

Data da Relatoria: 18/06/2014

Apresentação do Projeto:

Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico de leishmaniose LIGIA MORAES BARIZON DE SOUZA Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico de leishmaniose

AUTORIA:LIGIA MORAES BARIZON DE SOUZA (DOUTORANDA) E Profa. Dra. VANETE THOMAZ SOCCOL (orientadora). Trata-se de estudo visando ao desenvolvimento de método diagnóstico para a leishmaniose , hoje apenas diagnosticada pela presença da clínica da doença e demonstração direta da presença do parasita em amostras clínicas e cultura do protozoário, além de métodos moleculares e sorológicos. De modo geral,

muito poucos testes estão comercialmente disponíveis e a preços acessíveis, exigindo mão de obra qualificada, utilização de equipamentos de alto custo além de serem tecnicamente exigentes.

A hipótese apresentada pelas pesquisadoras é de que existem frações antigênicas específicas e de grande sensibilidade dentre as diferentes espécies de Leishmania capazes de serem isoladas e identificadas a fim de obter uma produção de antígenos específica, para inserção em kits diagnósticos rápidos para detecção de leishmaniose.

A pesquisa está em vigência desde março de 2012 e finalizará em fevereiro de 2016. Ao final do projeto (estima-se entre junho de 2014 e junho de 2015), será necessária a colheita de amostras

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UF: PR **Município:** CURITIBA
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UNIVERSIDADE FEDERAL DO PARANÁ - SETOR DE CIÊNCIAS DA SAÚDE/ SCS -

de soro de indivíduos portadores de leishmaniose para validação do processo biotecnológico de produção de antígeno para detecção de Leishmania sp. Ao mesmo tempo, deverá ser coletado, se possível, biópsia de lesões de pele causadas por Leishmania sp., a fim de proceder a identificação da espécie de Leishmania que está acometendo o indivíduo selecionado para participar deste projeto.

Objetivo da Pesquisa:

Objetivo geral: Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico imunológico de indivíduos portadores de leishmaniose, com técnica padronizada de detecção de anticorpos a partir de antígenos específicos, com alta especificidade e sensibilidade;

ESPECÍFICOS

1. Isolar frações protéicas antigênicas das formas promastigota e amastigota de Leishmania (Viannia) braziliensis, Leishmania (Leishmania) infantum e Leishmania (Leishmania) amazonenses;
2. Produção de antígenos específicos das formas promastigota e amastigota de Leishmania (Viannia) braziliensis, Leishmania (Leishmania) infantum e Leishmania (Leishmania) amazonenses através de soro hiperimunizado de coelhos;
3. Determinar a reprodutibilidade do processo biotecnológico para detecção imunológica de indivíduos portadores de leishmaniose, através do teste de diversos lotes do produto obtido para soro de pacientes com suspeita e confirmação da doença.

Avaliação dos Riscos e Benefícios:

Para a continuidade deste projeto, serão coletados cerca de 10 a 15 mL de sangue de pacientes clinicamente positivos para leishmaniose, ou por identificação laboratorial, e, se possível, um pequeno pedaço da lesão de pele ocasionada pela doença. Na ocasião da coleta o paciente poderá sentir a dor da picada da agulha, que é passageira e alguns pacientes poderão apresentar hematoma na região puncionada. Isto é perfeitamente normal e poderá ser solucionado com compressas de gelo no local. A pesquisa proporcionará o benefício futuro do desenvolvimento de método diagnóstico rápido e preciso de leishmaniose, contribuindo para a identificação eficaz da doença e consequente controle e erradicação da mesma.

Comentários e Considerações sobre a Pesquisa:

A pesquisa é relevante; a leishmaniose é considerada doença negligenciada. A casuística apontada pelas pesquisadoras indica que a cada ano, quase 2 milhões de novos casos de leishmaniose são registrados no mundo, segundo estimativas da Organização Mundial da Saúde (OMS). Entre 20 mil

e 40 mil pessoas morrem, por ano, no mundo, vítimas da leishmaniose. No Brasil, o Ministério da Saúde estima que quase 3 mil pessoas são contaminadas pela doença anualmente. A doença, que era restrita às áreas de floresta e
zonas rurais, tem avançado nas cidades, em função dos desmatamentos e da migração das famílias para os centros urbanos. Hoje, há casos da leishmaniose nas 27 unidades da Federação. Mais de 2,7 mil mortes foram relatadas entre 2000 e 2011, sendo os maiores índices de mortalidade registrados no Pará, Tocantins, Maranhão, Piauí, Ceará, São Paulo, Bahia e Minas Gerais.A pesquisa está em vigência desde março de 2012 e finalizará em fevereiro de 2016.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos exigidos por este CEP foram anexados exceto a análise de mérito por ser projeto aprovado pelo PRONEX/2009, FA.

Recomendações:

Solicitamos que sejam apresentados a este CEP, relatórios semestrais sobre o andamento da pesquisa, bem como informações relativas às modificações do protocolo, cancelamento, encerramento e destino dos conhecimentos obtidos, através da Plataforma Brasil - no modo: NOTIFICAÇÃO. Demais alterações e prorrogação de prazo devem ser enviadas no modo EMENDA. Lembrando que o cronograma de execução da pesquisa deve ser atualizado no sistema Plataforma Brasil antes de enviar solicitação de prorrogação de prazo.

Conclusões ou Pendências e Lista de Inadequações:

As pendências foram atendidas. É obrigatório retirar na secretaria do CEP/SD uma cópia do Termo de Consentimento Livre e Esclarecido com carimbo onde constará data de aprovação por este CEP/SD, sendo este modelo reproduzido para aplicar junto ao participante da pesquisa.

O TCLE deverá conter duas vias, uma ficará com o pesquisador e uma cópia ficará com o participante da pesquisa (Carta Circular nº. 003/2011CONEP/CNS)

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

UNIVERSIDADE FEDERAL DO PARANÁ - SETOR DE CIÊNCIAS DA SAÚDE/ SCS -

CURITIBA, 11 de Junho de 2014

Assinado por:
IDA CRISTINA GUBERT
(Coordenador)

Endereço: Rua Padre Camargo, 280
Bairro: 2ª andar **CEP:** 80.060-240
UF: PR **Município:** CURITIBA
Telefone: (41)3360-7259 **E-mail:** cometica.saude@ufpr.br

ATTACHMENT 3 - Informed consent

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA
SETOR DE TECNOLOGIA
DEPARTAMENTO DE ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nós, Lígia Moraes Barizon de Souza e Vanete Thomaz Soccol, pesquisadores da Universidade Federal do Paraná, estamos convidando o(a) Sr(a). a participar de um estudo intitulado "Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico de leishmaniose".

As leishmanioses são um complexo de doenças crônicas causadas por protozoários da família *Trypanosomatidae* e gênero *Leishmania.* Atualmente o método diagnóstico de detecção de leishmaniose baseia-se na apresentação clínica da doença, somado a demonstração direta da presença do parasita em amostras clínicas e cultura do protozoário, além de métodos moleculares e sorológicos. De modo geral, muito poucos testes estão comercialmente disponíveis e a preços acessíveis, requerem mão de obra qualificada, utilização de equipamentos de alto custo e são tecnicamente exigentes. A grande desvantagem da utilização de diagnósticos sorológicos para detecção da leishmaniose é a persistência de anticorpos contra o protozoário por longos períodos após a cura clínica da doença, dificultando a identificação de pacientes doentes e curados. Isto implica na busca de identificação de marcadores que possuem tanto potencial de diagnóstico quanto de monitoração do tratamento.

a) Visando a necessidade de um diagnóstico nacional mais específico e sensível, este trabalho teve como objetivo desenvolver um teste de diagnóstico para confirmação da leishmaniose, através de intradermorreação (que consiste na aplicação de uma proteína na pele do braço e verificação da presença de inchaço no local após 48 horas da aplicação) e exames para avaliar a presença de células de defesa contra leishmaniose no organismo. Os antígenos (proteínas para detecção da doença) utilizados neste trabalho foram (e continuam sendo) desenvolvidos pelo grupo de pesquisa orientado pela professora doutora Vanete Thomaz Soccol.

Rubricas:

Participante da Pesquisa e /ou responsável legal_________

b) Para ser incluído (a) na pesquisa, você precisa ter diagnostico positivo para leishmaniose, identificado através da sintomatologia clínica (espessamento das unhas, aumento do tamanho do fígado e baço, lesões de pele, perda acentuada de peso, febre, dentre outros), cultivo do parasito e testes para detecção de células de defesa do organismo contra o parasito. A coleta de sangue será coordenada pela pesquisadora Lígia Moraes Barizon de Souza e realizada por profissionais competentes. Na ocasião da coleta serão feitas algumas perguntas sobre o tempo que você tem a doença, os medicamentos que está utilizando, onde você contraiu a doença, seu nome e endereço e outras perguntas importantes relacionadas a esta enfermidade.

c) Serão coletados cerca de 10 a 15 mL de sangue de seu braço e, se possível, um pequeno pedaço da lesão de pele ocasionada pela doença. Este material será encaminhado ao laboratório de Biologia Molecular do Departamento de Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná (UFPR), onde será processado para os devidos ensaios.

d) Na ocasião da coleta o paciente poderá sentir a dor da picada da agulha, que é passageira e alguns pacientes poderão apresentar hematoma (mancha roxa) na região puncionada. Isto é perfeitamente normal e poderá ser solucionado com compressas de gelo no local.

e) Os benefícios esperados com essa pesquisa são: a identificação das espécies de *Leishmania sp*. que acometem os pacientes de acordo com as diferentes regiões geográficas onde se encontram, identificação de antígenos (proteínas) para o desenvolvimento de diagnóstico específico de leishmaniose e rapidez na detecção desta doença. No entanto, nem sempre você será diretamente beneficiado com o resultado da pesquisa, mas poderá contribuir para o avanço científico.

f) As pesquisadoras Lígia Moraes Barizon de Souza e Vanete Thomaz Soccol, responsáveis por este estudo poderão ser contatados pelo endereço do Laboratório de processos biotecnológicos e de biologia molecular da UFPR: Av. Francisco H dos Santos, 210 – Usina Piloto B - Jardim das Américas – Curitiba, Paraná ou pelos telefones (41) 3361-3272 ou (41) 9634-5773, de segunda a sexta-feiras, das 08:00 as 18:00h, para esclarecer eventuais dúvidas que o(a) Sr(a) possa ter e fornecer-lhe as informações que queira, antes, durante ou depois de encerrado o estudo.

g) A sua participação neste estudo é voluntária e se você não quiser mais fazer parte da pesquisa poderá desistir a qualquer momento e solicitar que lhe devolvam o termo de consentimento livre e esclarecido assinado. A sua recusa não implicará na interrupção de seu atendimento e/ou tratamento, que está assegurado.

Rubricas:

Participante da Pesquisa e /ou responsável legal_________

Comitê de ética em Pesquisa do Setor de Ciências da Saúde da FUFPR

Rua Pe. Camargo, 280 – 2º andar – Alto da Glória – Curitiba-PR –CEP:80060-240

Tel (41)3360-7259 - e-mail: cometica.saude@ufpr.br

h) As informações relacionadas ao estudo poderão conhecidas por autoridades sanitárias. No entanto, se qualquer informação for divulgada em relatório ou publicação, isto será feito sob forma codificada, para que a **sua identidade seja preservada e seja mantida a confidencialidade**.

i) As despesas necessárias para a realização da pesquisa não são de sua responsabilidade e pela sua participação no estudo você não receberá qualquer valor em dinheiro.

j) Quando os resultados forem publicados, não aparecerá seu nome, e sim um código.

Rubricas:

Participante da Pesquisa e /ou responsável legal__________

Comitê de ética em Pesquisa do Setor de Ciências da Saúde da FUFPR

Rua Pe. Camargo, 280 – 2º andar – Alto da Glória – Curitiba-PR –CEP:80060-240

Tel (41)3360-7259 - e-mail: cometica.saude@ufpr.br

Eu,____, li esse termo de consentimento e compreendi a natureza e objetivo do estudo do qual concordei em participar. A explicação que recebi menciona os riscos e benefícios desta pesquisa. Eu entendi que sou livre para interromper minha participação a qualquer momento sem justificar minha decisão e sem que esta decisão afete meu tratamento.

Eu concordo voluntariamente em participar deste estudo.

____

(Assinatura do participante de pesquisa ou responsável legal)

Local e data: ___________, ____/_______________/_________

____

Assinatura do Pesquisador

Comitê de ética em Pesquisa do Setor de Ciências da Saúde da FUFPR

Rua Pe. Camargo, 280 – 2º andar – Alto da Glória – Curitiba-PR –CEP:80060-240

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PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA
SETOR DE TECNOLOGIA
DEPARTAMENTO DE ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO

Título do Projeto: "Desenvolvimento de processo biotecnológico para produção de antígenos para diagnóstico de leishmaniose"
Investigador: Lígia Moraes Barizon de Souza e Vanete Thomaz Soccol

Local da Pesquisa: Laboratório de Biologia Molecular do Departamento de Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná

Endereço: Av. Francisco H dos Santos, 210 – Usina Piloto B - Jardim das Américas – Curitiba, Paraná

O que significa assentimento?
O assentimento significa que você concorda em fazer parte de um grupo de adolescentes/crianças, da sua faixa de idade, para participar de uma pesquisa. Serão respeitados seus direitos e você receberá todas as informações por mais simples que possam parecer.

Pode ser que este documento denominado TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO contenha palavras que você não entenda. Por favor, peça ao responsável pela pesquisa ou à equipe do estudo para explicar qualquer palavra ou informação que você não entenda claramente.

Informação ao Paciente:

O que é uma pesquisa?
Você está sendo convidado (a) a participar de uma pesquisa, com o objetivo de auxiliar no desenvolvimento de um método para diagnosticar leishmaniose.

Para que fazer a pesquisa? Como será feita? Quais os benefícios esperados com a pesquisa?

As leishmanioses são um complexo de doenças crônicas causadas por protozoários da família *Trypanosomatidae* e gênero *Leishmania.* Atualmente o método diagnóstico de detecção de leishmaniose baseia-se na apresentação clínica da doença, somado a demonstração direta da presença do parasita em amostras clínicas e cultura do protozoário, além de métodos moleculares e sorológicos. De modo geral, muito poucos testes estão comercialmente disponíveis e a preços acessíveis, requerem mão de obra qualificada, utilização de equipamentos de alto custo e são tecnicamente exigentes. A grande desvantagem da utilização de diagnósticos sorológicos para detecção da leishmaniose é a persistência de anticorpos contra o protozoário por longos períodos após a cura clínica da doença, dificultando a identificação de pacientes doentes e curados. Isto implica na busca de identificação de marcadores que possuem tanto potencial de diagnóstico quanto de monitoração do tratamento.
Visando a necessidade de um diagnóstico nacional mais específico e sensível, este trabalho teve como objetivo desenvolver um teste de diagnóstico para confirmação da leishmaniose,

através de intradermorreação e ensaios humorais, com a identificação de anticorpos presentes no soro de pacientes afetados pela doença. Os antígenos utilizados neste trabalho foram (e continuam sendo) desenvolvidos pelo grupo de pesquisa orientado pela professora doutora Vanete Thomaz Soccol.

Para ser incluído (a) na pesquisa, você precisa ter diagnostico positivo para leishmaniose, identificado através da sintomatologia clínica (onicogrifose – espessamento das unhas -, hepatoesplenomegalia, lesões de pele, perda acentuada de peso, febre, dentre outros), cultivo do parasito, teste de aglutinação direta, ELISA, IFI e/ou PCR. A coleta de sangue será coordenada pela pesquisadora Lígia Moraes Barizon de Souza e realizada por profissionais competentes. Na ocasião da coleta serão feitas algumas perguntas sobre o tempo que você tem a doença, os medicamentos que está utilizando, onde você contraiu a doença, seu nome e endereço e outras perguntas importantes relacionadas a esta enfermidade.

Que devo fazer se eu concordar voluntariamente em participar da pesquisa?

Caso você aceite participar, será coletado cerca de 10 a 15 mL de sangue de seu braço e, se possível, um pequeno pedaço da lesão de pele ocasionada pela doença. Este material será encaminhado ao laboratório de Biologia Molecular do Departamento de Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná (UFPR), onde será processado para os devidos ensaios.

Na ocasião da coleta o paciente poderá sentir a dor da picada da agulha, que é passageira e alguns pacientes poderão apresentar hematoma (mancha roxa) na região puncionada. Isto é perfeitamente normal e poderá ser solucionado com compressas de gelo no local.

A sua participação é voluntária. Caso você opte por não participar não terá nenhum prejuízo no seu atendimento e/ou tratamento.

Contato para dúvidas

Se você ou os responsáveis por você tiver (em) dúvidas com relação ao estudo, direitos do participante, ou no caso de riscos relacionados ao estudo, você deve contatar a Investigadora **Lígia Moraes Barizon de Souza, pelo telefone fixo (41) 3361-3272 e (41) 96345773, ou pelo endereço do Laboratório de processos biotecnológicos e de biologia molecular da UFPR: Av. Francisco H dos Santos, 210 – Usina Piloto B - Jardim das Américas – Curitiba, Paraná, de segunda a sexta-feiras, das 08:00h as 18:00h.** Se você tiver dúvidas sobre seus direitos como um paciente de pesquisa, você pode contatar o Comitê de Ética em Pesquisa em Seres Humanos (CEP) do Setor de Ciências da Saúde da Universidade Federal do Paraná, pelo telefone 3360-7259. O CEP é constituído por um grupo de profissionais de diversas áreas, com conhecimentos científicos e não científicos que realizam a revisão ética inicial e continuada da pesquisa para mantê-lo seguro e proteger seus direitos.

Rubricas:

Participante da Pesquisa e /ou responsável legal_________

DECLARAÇÃO DE ASSENTIMENTO DO PACIENTE:

Eu li e discuti com o investigador responsável pelo presente estudo os detalhes descritos neste documento. Entendo que eu sou livre para aceitar ou recusar, e que posso interromper a minha participação a qualquer momento sem dar uma razão. Eu concordo que os dados coletados para o estudo sejam usados para o propósito acima descrito.

Eu entendi a informação apresentada neste TERMO DE ASSENTIMENTO. Eu tive a oportunidade para fazer perguntas e todas as minhas perguntas foram respondidas.

Eu receberei uma cópia assinada e datada deste Documento DE ASSENTIMENTO INFORMADO.

NOME DO ADOLESCENTE ASSINATURA DATA

NOME DO INVESTIGADOR ASSINATURA DATA

Comitê de ética em Pesquisa do Setor de Ciências da Saúde da FUFPR
Rua Pe. Camargo, 280 – 2º andar – Alto da Glória – Curitiba-PR –CEP:80060-240
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