Universidade Federal de Pernambuco

Centro de Biociências

Programa de Pós-Graduação em Genética

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Avaliação e caracterização de proteínas antigênicas e de superfície de *Leishmania sp.* e seu papel no diagnóstico da leishmaniose e na patogenia do parasito.

Recife
2018

Catalogação na fonte
Elaine Barroso
CRB 1728

Castro Neto, Artur Leonel

Avaliação e caracterização de proteínas antigênicas e de superfície de *Leishmania* sp. e seu papel no diagnóstico da Leishmaniose e na patogenia do parasito/ Artur Leonel Castro Neto- 2018.

170 folhas: il., fig., tab.
Orientador: Osvaldo Pompílio de Melo Neto
Coorientador: Franklin Barbalho Magalhães
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Genética. Recife, 2018.
Inclui referências e anexos

1. Leishmaniose 2. Proteínas 3. Epidemiologia I. Melo Neto, Osvaldo Pompílio de (orient.) II. Magalhães, Franklin Barbalho (coorient.) III. Título

616.9364 CDD (22.ed.) UFPE/CB-2018-111

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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

Orientador: Dr. Osvaldo Pompiolio de Melo Neto

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À minha família.

AGRADECIMENTOS

Ao meu orientador, Dr. Osvaldo Pompílio de Melo Neto, por todo o conhecimento transmitido ao longo de todos os anos de orientação, por ter me proporcionado vivenciar várias etapas da vida de um pesquisador, desde a escrita de projetos para financiamento, a artigos científicos e co-orientação de alunos.

À minha família, sem os quais eu não estaria aqui. Especialmente a minha mãe Vanda, minhas irmãs Aída e Giselle, minhas tias Nevinha e Karla, minha avó Socorro e a Beta, por sempre me darem o apoio necessário e acreditarem em mim.

À minha esposa Nathaly Nascimento e ao meu cachorro Algodão pelo apoio e companheirismo e por sempre estarem ao meu lado no dia-a-dia, proporcionando felicidade, equilíbrio e tranquilidade para a vida agitada.

A todos que, gentilmente, contribuíram com minha formação profissional, através do ensinamento de técnicas que vieram a entrar nesta tese ou não. À Antonio Rezende por todos os ensinamentos de bioinformática que me permitiram realizar este trabalho. A Maria Ribeiro pela contribuição na execução do RNAseq. A Tatiany Romão e Gustavo Lima por todo suporte na realização dos ensaios de Southern Blotting. À equipe do biotério do Aggeu Magalhães pela solicitude e treinamento para manipulação e experimentação animal. A Éden Freire pelas produtivas discussões sobre evolução de tripanossomatideos.

A todos do grupo BiomolTrip pelas produtivas e enriquecedoras discussões científicas e suporte técnico durante todos dos anos.

Aos amigos mais próximos do laboratório Ludmila Arruda, Wagner Tenório, Igor Rocha, Isis Caroline e Maria Ribeiro pela troca de experiências e vivência diária.

A Wagner Tenório por todos os anos de parceria altamente produtiva na busca por um melhor diagnóstico e vacina para a leishmaniose visceral canina e humana.

Às minhas ótimas alunas Adriana Neuman e Allana Dutra, pelo desafio e auto superação que uma orientação exige. Por serem alunas dedicadas e compromissadas com seus trabalhos e por terem representado muito bem o grupo, sendo premiadas em eventos regionais e nacionais, ao longo de todos os anos de convivência.

A Pós-Graduação em Genética pelo apoio e solicitude do secretário Manassés que sempre está apto e disposto a ajudar os alunos.

A CAPES pela bolsa concedida e ao CNPq pelo financiamento que tornaram possível a execução deste trabalho.

Nada pode ser obtido sem uma espécie de sacrifício. É preciso oferecer algo em troca de valor equivalente.

(Hiromu Arakawa).

RESUMO

As leishmanioses são doenças infecto-parasitárias causadas por protozoários flagelados da família *Trypanosomatidae* e gênero *Leishmania*. Essas doenças apresentam aproximadamente 2 milhões de novos casos anuais mundialmente, com o Brasil representando grande parte deste número. Métodos de diagnóstico eficazes e o entendimento da patogênese são essenciais para um combate eficiente da Leishmaniose. Ambos dependem da identificação e caracterização de proteínas antigenicamente relevantes de *Leishmania*. Este estudo objetivou avaliar proteínas antigênicas previamente selecionadas quanto ao seu potencial para o diagnóstico da leishmaniose visceral provocada por *Leishmania infantum* e caracterizar a proteína de virulência GP63 de *Leishmania sp.* de um ponto de vista genético e proteico, observando similaridades e diferenças e suas implicações para o reconhecimento pelo sistema imune. Primeiramente, as proteínas recombinantes foram obtidas através de sistema de expressão em bactérias *Escherichia coli*, purificadas por colunas de afinidade e quantificadas pelo método de Bradford. As proteínas purificadas foram sensibilizadas em placas de ELISA e avaliadas quanto a seu reconhecimento por soros positivos de humanos e cães infectados por *L. infantum*, soros de outras enfermidades e soros de indivíduos sadios. A análise dos dados e testes estatísticos foi realizada pelos programas Medcalc e Prism3. A busca por novos parálogos de GP63 foi realizada através do modelo oculto de Markov e ensaios de PCR. A construção de árvores filogenéticas foi realizada a partir de alinhamentos produzidos no MAFFT, editados com o Trimal, avaliados quanto ao modelo evolutivo pelo ProTest e construídas pelo Phyml. As modelagens proteicas foram realizadas a partir de métodos de *threading* e a predição de epítopos foram obtidas através dos programas AAP12, Bcpred e Bepipred. Como resultados, observamos que proteínas foram identificadas com alto potencial para o diagnóstico da doença canina ou humana, e misturas das melhores proteínas tiveram melhor desempenho na detecção de ambas as formas da doença, sugerindo um teste único para o diagnóstico. Em um segundo momento passou se a investigar o principal antígeno de superfície de espécies de *Leishmania*, a protease gp63. Identificamos um grande número de parálogos em *Leishmania* e espécies relacionadas, categorizados em grupos distintos localizados nos

cromossomos 10, 28 e 31. Os genes dos dois últimos grupos parecem ser mais próximos de genes encontrados em parasitas de insetos ou plantas, enquanto que os do cromossomo 10 sofreram uma expansão em número de parálogos em *Leishmania* e em especial em *L. braziliensis*. Estes foram melhor estudados e apresentaram momentos de expressão distintos, expansão independente na evolução e eventos de recombinação intragênica. Variações de sequência e regiões de predição de anticorpos foram mapeados na superfície externa das proteínas. Esses dados sugerem um papel relevante para a variação antigênica da gp63 na biologia do parasito e na patogenia da doença.

Palavras-chave: Leishmaniose. Diagnóstico. Gp63.

ABSTRACT

Leishmaniasis are infectious-parasitic diseases caused by flagellate protozoa of the family *Trypanosomatidae* and genus *Leishmania*. These diseases cause approximately 2 million new cases annually worldwide, with Brazil accounting for a large part of this number. Effective diagnostic methods and the understanding of the pathogenesis are essential for an effective leishmaniasis control. Both depend on the identification and characterization of antigenically relevant *Leishmania* proteins. This study aimed to evaluate antigenic proteins previously selected as to their potential for the diagnosis of visceral leishmaniasis caused by *Leishmania infantum* and to contribute in the Leishmania sp. GP63 virulence protein characterization from a genomic and proteomic approach, observing similarities and differences and their implications for recognition by the immune system. First, the recombinant proteins were obtained by the *Escherichia coli* expression system, purified by affinity columns and quantified by the Bradford method. The purified proteins were applied on ELISA plates and assessed for recognition by positive sera from humans and dogs infected with *L. infantum*, sera from other related diseases and sera from healthy individuals. Data analysis and statistical tests were performed by Medcalc and Prism3 programs. The search for new GP63 paralogs was performed using the hidden Markov model and PCR assays. The construction of phylogenetic trees was performed from alignments produced in MAFFT, edited by Trimal, evaluated for the best evolutionary model by ProTest and built by Phyml. Protein modelling was performed using threading methods and prediction of epitopes were obtained through the AAP12, Bcpred and Bepipred programs. As results, we observed that proteins were identified with high potential for the diagnosis of canine or human disease, and mixtures of the best proteins had better performance in the detection of both forms of the disease, suggesting a single test for the diagnosis. In a second moment it was investigated the main surface antigen of Leishmania species, the protease gp63. We identified many paralogs in *Leishmania* and related species, categorized into distinct groups located on chromosomes 10, 28 and 31. The genes of the latter two groups appear to be closer to genes found in insect or plant parasites, whereas those in the chromosome 10 showed an increase in the number

of paralogs in Leishmania and especially in *L. braziliensis*. These were better studied and presented distinct expression moments, independent expansion in evolution and intragenic recombination events. Sequence variations and regions of antibody prediction were mapped on the outer surface of the proteins. These data suggest a relevant role for the antigenic variation of gp63 in the parasite biology and in the pathogenesis of the disease.

Key words: Leishmaniasis. Diagnosis. Gp63.

LISTA DE ILUSTRAÇÕES

ARTIGO 2

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

LISTA DE ABREVIATURAS E SIGLAS

aa	Aminoácido
DO	Densidade Óptica
ELISA	*Enzyme Linked ImmunoSorbent Assay* – Ensaio imunoenzimático
GPI	*Glycophosphatidylinositol*
HSP	*Heat shock protein*
IFN	Interferon
IL	Interleucina
IPTG	Isopropil-tio-β-D-galactopiranosídeo
Kb	Kilo base
KDa	KiloDalton
KDELMAP	Região de aa conservada nas Gp63
LB	Luria Bertani
LC	Leishmaniose Cutânea
LMC	Leishmaniose mucocutânea
LMW	*Low molecular weight marker* – Marcador de baixo peso molecular
LV	Leishmaniose Visceral
mM	Mili molar
Pb	Pares de base
PCR	*Polymerase Chain Reaction* – Reação em Cadeia da Polimerase
pET	Vetor de expressão comercial
pGEM T- EASY	Vetor de clonagem comercial
pGEX	Vetor de expressão comercial
pRSET	Vetor de expressão comercial
Th1	Células T *Helper* 1
Th2	Células T *Helper* 2
TNF	Fator de necrose tumoral

SUMÁRIO

1 INTRODUÇÃO

A leishmaniose é uma doença infecciosa parasitária causada por protozoários flagelados da ordem Kinetoplastida, família *Trypanosomatidae*, gênero *Leishmania* e que são transmitidos por insetos dípteros flebotomíneos das espécies *Phlebotomus* ou *Lutzomyia*. Atualmente, existem cerca de 30 espécies de *Leishmania* que infectam mamíferos e provocam as formas clínicas clássicas da leishmaniose, cutânea e visceral. Estas espécies são incluídas em dois subgêneros, *Leishmania* e *Viannia*, porém o subgênero *Leishmania* evoluiu principalmente na África, enquanto que o *Viannia* é exclusivo das Américas. Diferenças importantes são observadas entre as espécies desses subgêneros de ordem genética e no local do desenvolvimento no intestino do inseto vetor.

A leishmaniose é considerada uma das principais doenças negligenciadas e acomete milhões de pessoas ao redor do mundo. Para seu controle, é de grande importância a existência de testes diagnósticos rápidos e eficientes para detecção da doença em seus hospedeiros (humano e cão, principalmente). Deste modo, as regiões endêmicas podem ser mapeadas e intervenções podem ser feitas de maneira rápida. Testes sorológicos tem o melhor potencial de produzir resultados satisfatórios, mas o teste utilizado atualmente (composto pelo antígeno rK39) apresenta sensibilidade variável em humanos e é ineficaz para diagnosticar a doença em cães. São então necessários estudos para identificação de novos antígenos ou avaliação dos já existentes quanto a sua capacidade de detecção da leishmaniose.

Devido a necessidade de aprimoramento do diagnóstico da leishmaniose visceral para humanos e cães, estudos que precederam este trabalho levaram a identificação de treze proteínas antigênicas com potencial para uso no diagnóstico sorológico da doença. Proteínas recombinantes derivadas de cinco destas já foram avaliadas e apresentaram sensibilidade e especificidade variáveis frente a soros de cães e humanos, mas nenhuma apresentou a capacidade de detectar tanto a leishmaniose humana quanto a canina. Este estudo se propôs, primeiramente, a contribuir na avaliação dos demais antígenos e investigar alternativas para o desenvolvimento de um sistema único para a detecção de ambas as formas da leishmaniose visceral provocada pela *Leishmania infantum*.

Em continuidade, reconhecendo que medidas de controle dependem de um maior conhecimento a respeito da biologia do parasito, e que no caso da *Leishmania* este conhecimento depende de uma melhor caracterização de suas principais proteínas antigênicas, este estudo se propôs também a estudar uma proteína descrita como principal antígeno de superfície de espécies de *Leishmania*. Neste trabalho, com base em análises *in silico* e experimentais, buscou-se também avaliar o potencial antigênico e a diversidade da proteína de virulência gp63 em diferentes espécies de *Leishmania*, buscando entender o seu possível papel para a biologia do parasito ou para a relação parasito-hospedeiro, com o foco principal na *L. braziliensis*, principal responsável pela leishmaniose tegumentar no Brasil. Esta proteína, denominada de gp63, é uma protease que demonstra ter papel crítico na biologia de diferentes espécies de *Leishmania*, mas que ainda permanece pouco conhecida.

2 REVISÃO DA LITERATURA

2.1 A LEISHMANIOSE

A leishmaniose é uma doença infecto-parasitária causada por protozoários flagelados pertencentes à família *Trypanosomatidae* e ao gênero *Leishmania*. Estes protozoários apresentam um ciclo de vida heteroxeno, vivendo alternadamente em hospedeiros vertebrados (incluindo o homem e outros mamíferos silvestres e/ou domésticos como o cão) e insetos dípteros vetores, estes últimos pertencendo aos gêneros *Phlebotomus* e *Lutzomyia* (PACE, 2014). Atualmente, existem pouco mais de 50 espécies de *Leishmania* descritas, que foram agrupadas em três subgêneros (*Leishmania, Viannia* e *Sauroleishmania*) e dois complexos (complexo *L. enriettii* e Paraleishmania). Desse total de espécies de *Leishmania*, 30 infectam mamíferos e 20 são conhecidas por infectar o homem (AKHOUNDI et al., 2016).

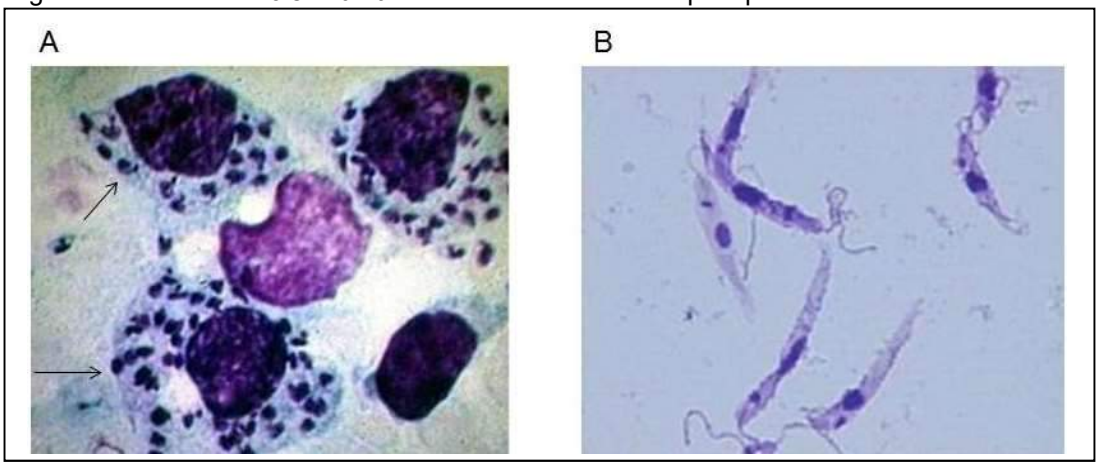
A infecção por *Leishmania* pode causar uma variedade de manifestações clínicas, que depende da interação complexa entre a resposta imune do hospedeiro e as espécies de protozoários. Essas manifestações podem ser desde lesões cutâneas isoladas ou disseminadas, até a forma visceral da doença, que pode ser letal se não tratada (KEVRIC; CAPPEL; KEELING, 2015). De acordo com o modo de transmissão da doença, essa infecção pode ser classificada em duas formas: zoonótica e antroponótica. A forma zoonótica da leishmaniose é caracterizada pela presença de mamíferos selvagens como reservatórios e pela transmissão do parasito presente neles para o humano, através do inseto vetor. A forma antroponótica é definida como a transmissão humano-humano, onde o parasito é

transmitido entre os humanos, sem passar por outras espécies de reservatórios (AKHOUNDI et al., 2016; STEVERDING, 2017).

2.1.1 MORFOLOGIA DO PARASITO

Os protozoários do gênero *Leishmania* possuem um ciclo de vida digenético e uma variedade de diferentes formas celulares que são adaptadas ao hospedeiro ou ao inseto vetor. As duas principais formas são a amastigota (no hospedeiro mamífero) e a promastigota (no vetor) (Figura 1) (SUNTER; GULL, 2017). A forma promastigota é extracelular, possui formato alongado, flagelo longo e pode ser encontrada no trato digestivo do inseto vetor. Já a forma amastigota, apresenta-se com formato oval ou esférico e seu flagelo não é livre, por apresentar apenas uma porção intracitoplasmática, e não é móvel. Ela é encontrada no interior dos macrófagos dos hospedeiros mamíferos (SUNTER; GULL, 2017; TORRES-GUERRERO et al., 2017).

Figura 1. Formas de *Leishmania* observadas ao microscópio óptico.



(A) Forma amastigota dentro de macrófago, indicada pelas setas. (B) Forma promastigota. Fonte: http://fcfrp.usp.br/dactb/Parasitologia/Arquivos/Genero_Leishmania.htm

2.1.2 VETOR

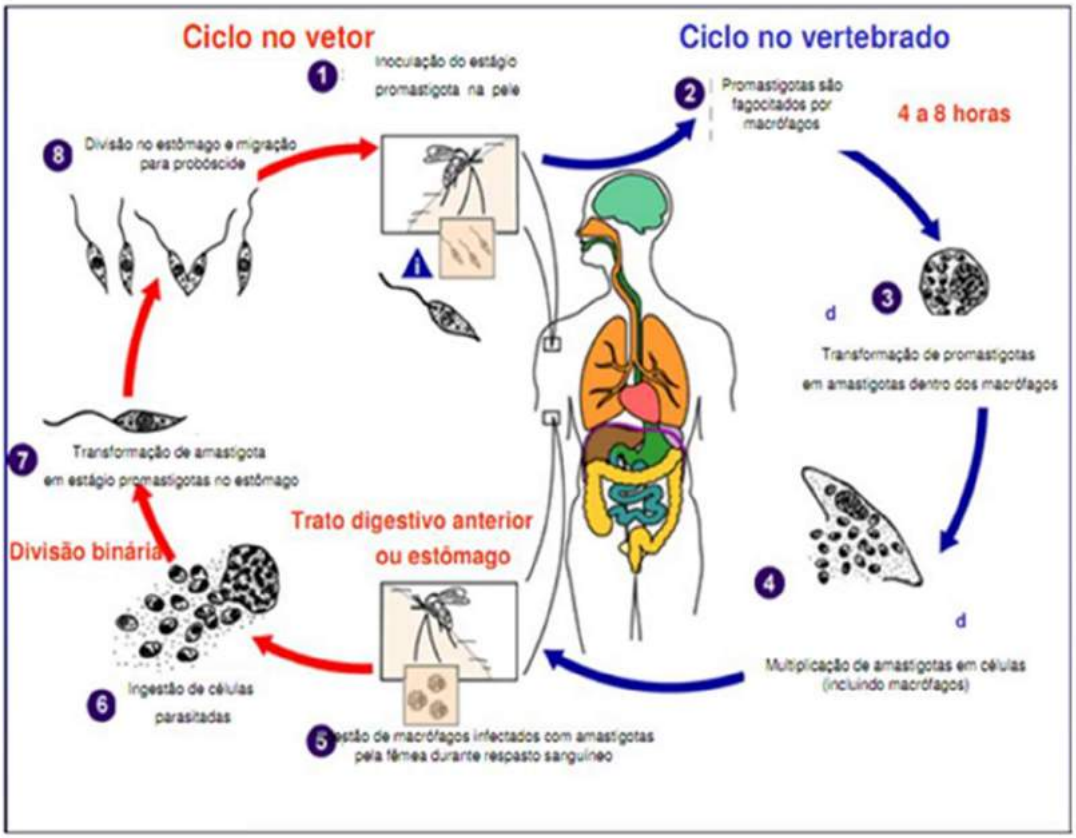
A leishmaniose tem como vetores insetos flebotomíneos pertencentes ao filo Arthropoda, classe Insecta, ordem Díptera, família *Psychodidae*, subfamília *Phlebotominae*. Dois gêneros são conhecidos de vetores da leishmaniose, *Phlebotomus* (presente no Velho Mundo) e *Lutzomyia* (presente no Novo Mundo) (AKHOUNDI et al., 2016; READY, 2013). Há uma estimativa de aproximadamente 500 espécies deste grupo de insetos, mas apenas cerca de 30 foram associados com a transmissão da leishmaniose. As fêmeas desses insetos adquirem o parasita por meio de repasto sanguíneo, onde se desenvolvem por um período de 4 a 25 dias, até serem inoculados de volta em hospedeiros mamíferos durante a alimentação, completando o ciclo (WHO, 2017).

2.1.3 CICLO DE VIDA

O ciclo de vida do parasita *Leishmania* é dividido em dois momentos, o primeiro no hospedeiro vertebrado (mamífero) e o segundo no hospedeiro invertebrado (flebotomíneo). Este ciclo começa quando a fêmea do flebotomíneo infectado ingere sangue do hospedeiro vertebrado (cão, humano ou animal silvestre). Enquanto ingere o sangue, o inseto regurgita a forma promastigota metacíclica do parasita (forma infectante, localizada na válvula estomadeal do vetor), juntamente com seus componentes salivares. O parasita é então fagocitado por diferentes tipos de células do sistema imune encontrados no local da inoculação onde se diferencia na forma amastigota. Este ciclo se completa quando os macrófagos infectados por amastigotas são ingeridos por outro flebotomíneo, onde

passarão por vários estágios de desenvolvimento até voltar à forma metacíclica. Cada um desses estágios é caracterizado por mudanças morfológicas e funcionais com o objetivo de assegurar a sobrevivência dentro do vetor. Após as etapas de diferenciação, a forma promastigota metacíclica migrará para a válvula estomadeal para ser inoculada em outro hospedeiro vertebrado e dar continuidade ao ciclo (KAMHAWI, 2006; KAYE; SCOTT, 2011).

Figura 2. Ciclo de vida das espécies de *Leishmania*.

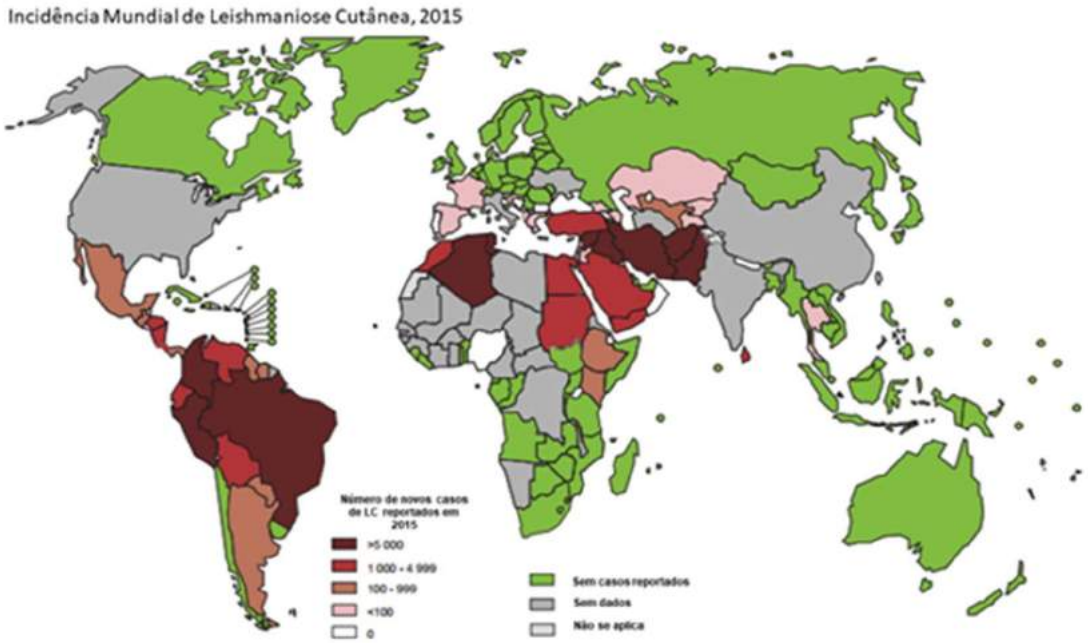


Mosquitos flebotomíneos regurgitam a forma promastigota metacíclica no hospedeiro vertebrado, onde serão fagocitadas por células do sistema imune (macrófagos) e diferenciadas na forma amastigota. Os macrófagos infectados com amastigotas são ingeridos por outros mosquitos vetores. Dentro do mosquito, a forma amastigota passará por várias diferenciações até voltar à forma promastigota metacíclica, que se localizará na válvula estomadeal do inseto e infectará novos hospedeiros. Fonte: Modificado de https://www.cdc.gov/parasites/leishmaniasis/biology.html

2.1.4 DISTRIBUIÇÃO GEOGRÁFICA

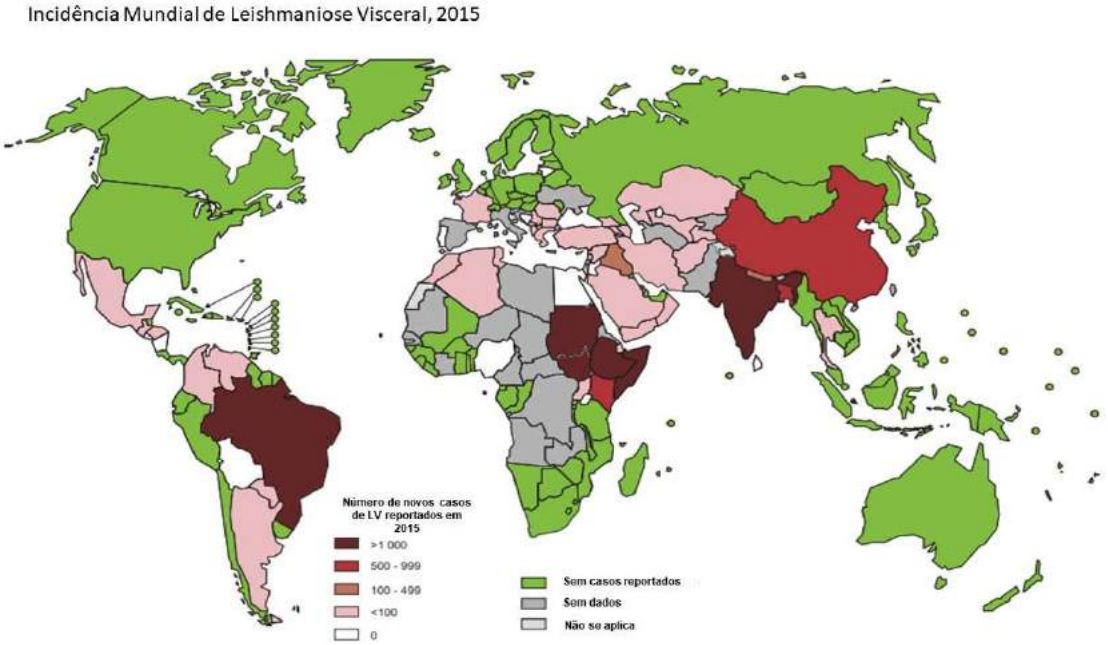
A leishmaniose é considerada como uma das principais doenças negligenciadas, afetando principalmente indivíduos de países em desenvolvimento. A prevalência ocorre em regiões tropicais e subtropicais da África, Ásia, Mediterrâneo, Sudoeste da Europa e Américas Central e do Sul. Esta doença é endêmica em 98 países sendo 72 desses países em desenvolvimento. A doença apresenta uma incidência mundial de 700 mil a 1,2 milhões de novos casos de leishmaniose cutânea (LC) e 200 mil a 400 mil casos de leishmaniose visceral (LV) ao ano (ALVAR et al., 2012). A grande maioria dos casos de leishmaniose cutânea (90%) ocorre no Afeganistão, Brasil, Bolívia, Colômbia, Irã, Peru, Arábia Saudita, Nicarágua e Síria (Figura 3). Em relação à leishmaniose visceral, 90% dos seus casos são provenientes dos seguintes países: Etiópia, Bangladesh, Brasil, Índia, Nepal e Sudão (Figura 4) (THEEL; PRITT, 2016).

Figura 3. Distribuição dos casos de leishmaniose cutânea mundialmente no ano de 2015.



Fonte: Adaptado de WHO, 2015. http://www.who.int/leishmaniasis/burden/en/

Figura 4. Distribuição dos casos de leishmaniose visceral mundialmente no ano de 2015.



Fonte: Adaptado de WHO, 2015. http://www.who.int/leishmaniasis/burden/en/

No Brasil, a LC é o tipo mais frequente de Leishmaniose e tem como principal causador a *Leishmania (Viannia) braziliensis* (COSTA-SILVA et al., 2014). A LC registra aproximadamente 20 mil casos por ano, podendo chegar a uma estimativa de pelo menos 100 mil casos anuais (ALVAR et al., 2012). Em relação à leishmaniose visceral, no Brasil ela é associada à infecção por *L. infantum*, e afeta principalmente crianças, com 55% dos casos ocorrendo na região Nordeste e uma incidência anual de 2,2 mil casos registrados (DOS REIS et al., 2017).

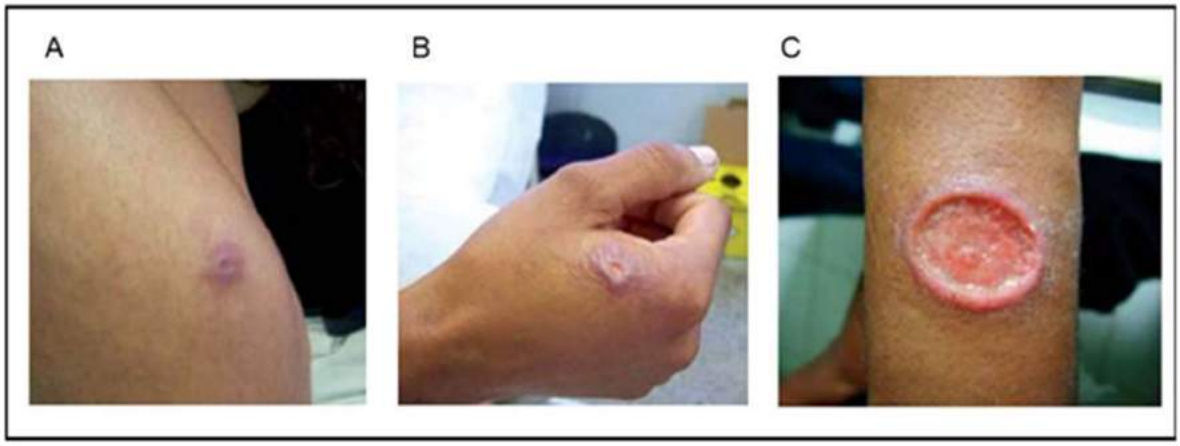
2.1.5 MANIFESTAÇÕES CLÍNICAS

2.1.5.1 LEISHMANIOSE CUTÂNEA

A LC é caracterizada pelo aparecimento de uma lesão ulcerativa ou nodular, que se desenvolve nas proximidades ou no próprio local da inoculação do parasito.

Esta lesão geralmente é encontrada em áreas descobertas do corpo, susceptíveis a picada do vetor, como rosto, antebraço e parte inferior das pernas. As lesões da LC têm um formato arredondado ou oval e podem medir desde alguns milímetros até centímetros. Elas têm uma base eritematosa, bordas bem delimitadas e elevadas, infiltradas (com o parasita), com fundo avermelhado e granulado (Figura 5). A área com ulceração pode ser susceptível a infecções bacterianas secundárias que, quando ocorrem, podem causar dor local, produzir exsudato e cobrir total ou parcialmente o fundo da mesma (BRASIL, 2017). A LC pode ser causada pelas espécies: *Leishmania (L.) major, L. (L.) tropica* (Velho Mundo), *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) guyanensis*, *L. (V.) panamensis* e *L. (V.) braziliensis* (Novo Mundo) (MCGWIRE; SATOSKAR, 2014).

Figura 5. Imagens retratando lesões da Leishmaniose Tegumentar.



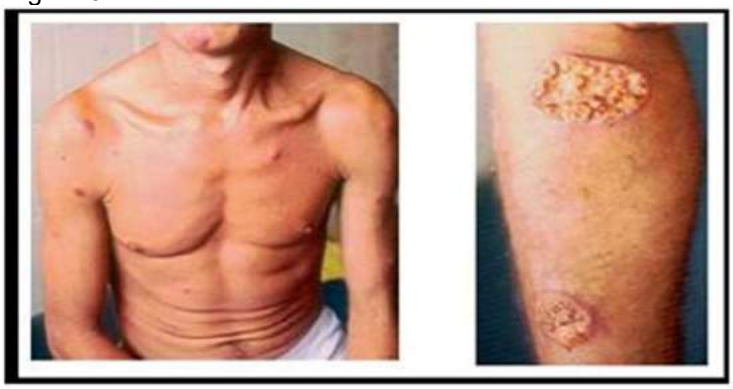
A – Lesão no período inicial de ulceração, com bordas elevadas e infiltração parasitária. B – Lesão já ulcerada e com fundo granuloso. C – Lesão arredondada com ulceração elevada. Fonte: (BRASIL, 2017).

2.1.5.2 LEISHMANIOSE CUTÂNEA DIFUSA

Na leishmaniose cutânea difusa, existem várias lesões nodulares que aparecem distantes do local de inoculação do parasita pela picada do inseto (Figura

6). Ela é causada principalmente pelas *L. (V.) braziliensis* e a *L. (L.) amazonensis* (BRASIL, 2017; MCGWIRE; SATOSKAR, 2014).

Figura 6. Lesões de leishmaniose cutânea difusa.



Lesões múltiplas presentes no mesmo paciente, algumas com ulceração superficial. Fonte: (BRASIL, 2017).

2.1.5.3 LEISHMANIOSE MUCOCUTÂNEA

A forma mucocutânea da leishmaniose é causada pela *Leishmania (V.) braziliensis*. Ela pode ser originada a partir de uma leishmaniose cutânea convencional, visto que de 3 a 5% dos pacientes desenvolvem a forma mucocutânea, ou pode ser proveniente da picada do parasita em áreas de tecido mucoso, como o nariz, lábios ou olhos. O parasita *Leishmania* é levado, por via linfática e hematológica, para os principais locais acometidos por essa forma da doença (mucosas nasal, orofaringe, palatos, lábios, língua, laringe) (Figura 7) (BRASIL, 2017; KEVRIC; CAPPEL; KEELING, 2015; MCGWIRE; SATOSKAR, 2014).

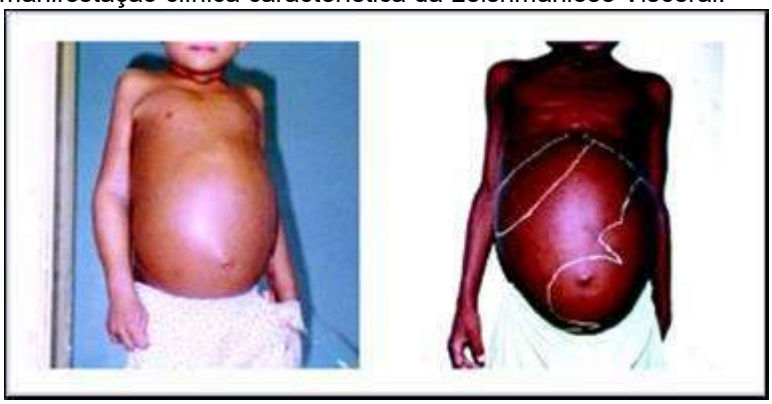
Figura 7. Imagens de pacientes com lesões características de leishmaniose mucocutânea, nos lábios, nariz e palato.

Fontes: (CHAPPUIS et al., 2007; REITHINGER et al., 2007) .

2.1.5.4 LEISHMANIOSE VISCERAL

Assim como na leishmaniose cutânea, a LV se inicia quando o parasita, na forma promastigota, é inoculado pelo vetor, sendo então fagocitado por macrófagos, onde se diferencia em amastigota. Nessa forma da doença a *Leishmania* se dissemina através de células do sistema monocítico fagocitário, que leva à infiltração do baço, medula óssea, fígado e linfonodos (KEVRIC; CAPPEL; KEELING, 2015). O tempo de incubação da LV varia de 3 a 8 meses, mas também pode levar desde poucas semanas a anos para se manifestar. Os indivíduos começam apresentando uma febre alta, anorexia, fadiga, fraqueza, perda de peso e aumento no volume de órgãos (fígado, baço, linfonodos). À medida que a doença progride, pode haver um aumento da esplenomegalia, causando distensão abdominal e dor, que pode aumentar devido a uma concomitante hepatomegalia (Figura 8) (CHAPPUIS et al., 2007; KEVRIC; CAPPEL; KEELING, 2015; MCGWIRE; SATOSKAR, 2014; TORRES-GUERRERO et al., 2017).

Figura 8. Pacientes apresentando hepatoesplenomegalia, manifestação clínica característica da Leishmaniose Visceral.



Fonte: (MURRAY et al., 2005).

No velho mundo, a LV é causada pela *L. donovani* (principalmente em regiões da Índia, Paquistão, China e África) e pela *L. infantum* (na região do Mediterrâneo). No novo mundo, essa forma da doença é causada pela *L. infantum*, com maior número de acometidos no Brasil (MCGWIRE; SATOSKAR, 2014).

2.1.5.5 LEISHMANIOSE VISCERAL CANINA

Além dos humanos, os cães são um dos principais hospedeiros mamíferos acometidos pelo parasita *Leishmania*, sendo vítimas da leishmaniose visceral canina (LVC). Dados obtidos no Brasil demonstram uma prevalência variável dessa forma da doença em áreas endêmicas (0,7% em Salvador, 51,6% em São Luiz do Maranhão) e sugerem sua provável participação na urbanização da LV, através de processos de migração de cães assintomáticos (MELO et al., 2016).

Além da participação no ciclo de transmissão zoonótico para a população humana, os cães acometidos pela LVC podem desenvolver um quadro grave, caracterizada pela evolução crônica de sinais viscero-cutâneos que se manifestam

vários meses após a exposição ao parasita (GRADONI, 2015). Geralmente a infecção pode se apresentar na forma assintomática, mas no caso da infecção sintomática pode levar o animal à morte. Os sintomas clínicos comuns são: fadiga, linfoadenopatia, diarréia crônica, sangramento nasal, edema nas patas, atrofia muscular, hepatoesplenomegalia e inanição. Além do acometimento sistêmico, a LVC apresenta também lesões na pele, como dermatite esfoliativa, pápulas e pequenos nódulos, úlceras indolentes, crostas, hipotricose e distúrbios disqueratóticos generalizados (KRAUSPENHAR et al., 2007; SARIDOMICHELAKIS, 2009).

2.2 DIAGNÓSTICO

O padrão ouro para a confirmação do diagnóstico da leishmaniose é a identificação do parasito *Leishmania* em material retirado por escarificação de lesões na pele ou por biópsia de órgãos internos, como a medula óssea e o baço. Esse método, contudo, é pouco sensível, laborioso e requer procedimentos invasivos (AKHOUNDI et al., 2017; SRIVASTAVA et al., 2011). Testes moleculares foram recentemente desenvolvidos, baseados em variações na técnica de PCR e, embora possam apresentar alta sensibilidade e especificidade, com um mínimo de invasividade, não são aplicáveis no campo (DE RUITER et al., 2014; MOREIRA; YADON; CUPOLILLO, 2017).

A utilização de técnicas sorológicas para detecção de anticorpos anti-*Leishmania* também são amplamente empregadas. Elas são raramente empregadas para detecção de LC ou LMC e mais comumente usadas para o

diagnóstico de LV. Esse tipo de diagnóstico pode ser realizado por vários métodos, que apresentam sensibilidade e especificidade variáveis (SAKKAS; GARTZONIKA; LEVIDIOTOU, 2016; SAVOIA, 2015). Entre essas técnicas se destacam as reações de: RIFI (imunofluorescência indireta), DAT (teste de aglutinação direta), ELISA (ensaio imunoenzimático) e teste rápido. A RIFI apresenta baixa sensibilidade, porém boa especificidade, onde se observam reações cruzadas com outras doenças como a Doença de Chagas, malária, tuberculose, brucelose e febre tifóide. Sua aplicação requer alto nível de habilidade, experiência e também equipamento especializado e de alto custo (SAKKAS; GARTZONIKA; LEVIDIOTOU, 2016; SRIVASTAVA et al., 2011). O DAT é baseado na detecção da aglutinação direta de antígenos de *Leishmania* que reagem com anticorpos anti-*Leishmania* no soro, resultando na aglutinação. Ele pode ser facilmente realizado em laboratórios equipados e com pessoal treinado, mas apresenta algumas limitações, etapas múltiplas, reprodutibilidade e incubações longas (SUNDAR; RAI, 2002). O ELISA é uma técnica amplamente utilizada para a detecção de várias doenças infecciosas. De maneira geral, ela é baseada na captura de anticorpos contra antígenos específicos, que atuam como o reagente inicial da técnica. A sensibilidade e a especificidade do ELISA dependerão do antígeno usado. Comumente esses antígenos são constituídos do extrato proteico total do parasita ou de proteínas recombinantes. Esses dois tipos de antígenos podem ser utilizados para detecção da leishmaniose visceral, mas o melhor desempenho é observado com a proteína recombinante rK39 (SAKKAS; GARTZONIKA; LEVIDIOTOU, 2016).

O antígeno rK39, obtido a partir de *L. infantum*, consiste em 39 aminoácidos repetitivos e é derivado da porção N-terminal de uma proteína de 230 kDa pertencente à família das cinesinas (BURNS et al., 1993; CHAPPUIS et al., 2007).

Após a comprovação da sua eficácia para detecção da leishmaniose visceral humana através do método de ELISA, essa proteína foi utilizada para a fabricação de testes rápidos imunocromatográficos para aplicação em campo. Quando essa plataforma foi testada em campo, foi observado uma sensibilidade e especificidade geralmente altas, mas isso pode variar dependendo da região em que está sendo testada, diminuindo a eficácia do antígeno (BOELAERT et al., 2014; MAIA et al., 2012; VAN GRIENSVEN; DIRO, 2012). Este antígeno também se mostrou ineficaz em diagnosticar cães assintomáticos, o que torna ele limitado para uso em estudos epidemiológicos ou programas de controle de infecção em áreas endêmicas (QUINNELL et al., 2013).

Para o diagnóstico da LVC, são utilizadas plataformas como testes rápidos (DPP), ELISA e diagnósticos moleculares (qPCR). Um estudo que comparou esses métodos demonstrou que a PCR em tempo real se mostrou mais promissora, com boa performance na detecção de cães assintomáticos, quando comparadas as outras plataformas sorológicas (Carvalho et al. 2018). Esses dados reforçam a necessidade do desenvolvimento de um eficiente teste sorológico para a detecção dessa forma da doença em áreas endêmicas, levando em conta que os testes atuais ainda apresentam sensibilidades inferiores ao método molecular (não aplicável para áreas endêmicas), ou não apresentam sensibilidade alta para ser aplicado em campo (Nogueira et al. 2018).

2.3 IMUNIDADE E IMUNOPATOGÊNESE NA LEISHMANIOSE

Na leishmaniose, a partir do momento em que o parasita na forma promastigota metacíclica entra na corrente sanguínea, eles são fagocitados

principalmente por macrófagos e neutrófilos. Após a fagocitose, são internalizados em fagossomos, seguidos pela fusão com lisossomos. Em seguida, vários tipos de células do hospedeiro (neutrófilos, monócitos ou macrófagos, células *natural killer* e células dendríticas) interagem com o do parasita *Leishmania* (LAUTHIER; KORENAGA, 2018; VON STEBUT; TENZER, 2017). Ao mesmo tempo, o sistema imune também inicia outras ações da imunidade inata para a proteção contra a infecção. Dentre essas ações, a ativação do sistema complemento é uma das primeiras linhas de defesa. A partir de uma reação em cascata, proteínas se agruparão na superfície do patógeno, formando o Complexo de Ataque a Membrana (CAM), que é responsável pela lise da sua membrana celular (GUPTA; OGHUMU; SATOSKAR, 2014).

Além da imunidade inata, também se nota ações de grupos celulares da imunidade adaptativa, mediada por linfócitos B, T e células dendríticas (KEDZIERSKI, 2010). O envolvimento de alguns grupos dessas células e sua citocinas associadas estariam relacionadas às condições de resistência e susceptibilidade a infecção. Estudos em modelos murinos (SELVAPANDIYAN et al., 2006) e de infecção de macrófagos *in vitro* demonstraram que os tipos de resposta mediado através das células T-helper 1 (Th1), e citocinas associadas (IFN-γ e IL-2), estariam ligadas a indivíduos resistentes a infecção, por ser uma resposta montada contra agentes infecciosos intracelulares. Já em infecções não controladas, em indivíduos susceptíveis à doença, ocorre uma proliferação da resposta Th2, juntamente com a produção de IL 4, IL5 e IL10, associadas a respostas humorais e alérgicas (SAHA; MUKHOPADHYAY; CHATTERJEE, 2011).

Nos casos de leishmaniose humana, a resposta imunológica não se apresenta tão polarizada, tendo em vista que indivíduos infectados já foram

identificados com altos níveis de citocinas características da resposta Th1 e Th2, nos momentos iniciais da infecção. Estes níveis de citocinas sofrem diminuição nos momentos de regressão da doença, durante o tratamento (SAHA; MUKHOPADHYAY; CHATTERJEE, 2011). Esses dados demonstram que além da resposta protetora, a resposta Th1 também pode estar relacionada a imunopatologia da doença. Em casos de leishmaniose cutânea, a produção exacerbada de citocinas Th1, como as TNF-α e IL-1, que promovem a inflamação induzindo a expressão e adesão de moléculas na superfície endotelial, podem induzir danos nos tecidos (COSTA et al., 2011; LAUTHIER; KORENAGA, 2018). Além disso, estudos também demonstraram que indivíduos assintomáticos infectados com *L. braziliensis*, produziram menos citocinas Th1 do que pacientes com lesões cutâneas (COSTA et al., 2011).

2.3.1 IMUNIDADE NA LEISHMANIOSE CUTÂNEA

A imunidade na leishmaniose cutânea varia de acordo com a espécie que infectanda o hospedeiro, mas de modo geral a infecção cutânea é caracterizada por uma infiltração de células apresentadoras de antígenos ativadas (CAA) e a produção de respostas Th1 associadas ao IFN-γ e a IL-12. Geralmente, a produção da IL-12 pode ser aumentada por outras citocinas (IL-1α, IL-18, IL-23 e IL-27 como mecanismo inato), o que gera uma diferenciação e proliferação de células Th1. Essas células Th1 produzirão IFN-γ, que atuará na mediação da ativação de macrófagos para produção de óxido nítrico (ON), um dos principais compostos que leva a destruição do parasita *Leishmania*. Durante o curso da infecção, células T CD8+ da imunidade celular podem desempenhar as funções de proteção contra o

parasita através da produção de IFN-γ. Da mesma forma, podem contribuir para a evolução da patologia quando exercem sua função citolítica. Nas lesões cutâneas, a produção de IFN-γ pode levar a uma resposta inflamatória exagerada, que promove dano tecidual, e patologia exacerbada. Outro fator que também pode contribuir para a progressão desta forma da leishmaniose é a ausência ou baixos níveis de citocinas anti-inflamatórias como as IL-4, IL-10, IL-13 e TGF-β, que quando associadas a níveis elevados de IFN-γ, e dependendo da localização da lesão no corpo, podem fazer com que a LC progrida para uma LMC (LAUTHIER; KORENAGA, 2018; VON STEBUT; TENZER, 2017).

2.3.2 IMUNIDADE NA LEISHMANIOSE VISCERAL

A imunidade na leishmaniose visceral também se iniciará com a ativação do sistema imune inato e o recrutamento de células (neutrófilos, macrófagos, células dendríticas e células *natural killer*) para o local da infecção. A resistência ou suscetibilidade também vão estar relacionadas as respostas Th1 e Th2, respectivamente, embora assim como já relatado anteriormente as duas respostas podem estar presentes em indivíduos sintomáticos. Quando há proteção, ela está associada a produção de IFN-γ por células T CD4+ e/ou TCD8+ e ao desenvolvimento de moléculas efetoras anti-*Leishmania*, como espécies reativas de oxigênio e nitrogênio. Já os casos de susceptibilidade são reforçados pela produção de IL-10 e TGF-β, que em altos níveis levarão, durante o curso da infecção, a persistência do parasita e a cronicidade da doença (KUMAR; NYLÉN, 2012; LAUTHIER; KORENAGA, 2018).

2.4 MECANISMOS DE VIRULÊNCIA E O ESCAPE DO SISTEMA IMUNE

Ao longo da evolução, as espécies de *Leishmania* desenvolveram métodos de escape da imunidade inata (sistema complemento), além de outras ações de imunidade celular do hospedeiro. Estes mecanismos de defesa são mediados por fatores de virulência, que tem como principais representantes conhecidos os lipofosfoglicanos (LPG) e a proteína gp63. Através de suas ações, e da modulação de funções biológicas dos hospedeiros, estes garantem que o parasita consiga invadir o tecido alvo, sobreviva, se diferencie e prolifere (CUERVO et al., 2008; YAO; DONELSON; WILSON, 2003).

2.4.1 LIPOFOSFOGLICANOS (LPG)

O LPG é o glicoconjugado mais abundante da superfície das células de *Leishmania*, na sua forma promastigota. Esta é uma macromolécula composta de quatro domínios distintos: uma âncora GPI, um núcleo de glicano (porção de carboidrato), uma cadeia linear de fosfoglicano e um *cap* de oligossacarídeo na parte final. Sua estrutura e quantidade variam de acordo com a espécie e estágio do ciclo de vida do parasita, com maior número de moléculas presente na fase promastigota da *Leishmania* e com grande redução na amastigota (ATAYDE et al., 2016; FORESTIER; GAO; BOONS, 2015).

O LPG parece desempenhar ações tanto no hospedeiro invertebrado, quanto no vertebrado, nas primeiras horas de infecção. Em relação ao hospedeiro invertebrado, foi sugerido que o LPG proteja as formas promastigotas de *Leishmania* da degradação por enzimas proteolíticas destinadas a digestão do

sangue ingerido pelos insetos. Além disso, também foi relatado que o LPG tem funções de adesão nas paredes do intestino do inseto, para evitar a excreção do patógeno (ATAYDE et al., 2016).

Quando o parasita *Leishmania* é inoculada no hospedeiro vertebrado, na sua fase promastigota metacíclica, o LPG desempenha papéis de proteção do parasita contra o sistema imune. Por estar na membrana, esta molécula é um dos primeiros alvos do sistema imune e age como uma barreira que protege o parasita contra as ações iniciais deste. Também foi demonstrado que o LPG, através de impedimento alostérico, é capaz de inibir a ligação de moléculas do complemento na superfície do parasita. Sua interação com receptores do tipo *Toll* interfere nas respostas pró inflamatórias do hospedeiro e favorece a sobrevivência intracelular do parasita, modulando a produção de espécies reativas de oxigênio e óxido nítrico (ATAYDE et al., 2016; FORESTIER; GAO; BOONS, 2015).

2.4.2 GP63

A proteína gp63 foi descoberta em 1980, quando foi descrita como o principal antígeno de superfície presente em várias espécies de *Leishmania* e, mais tarde, foi identificada como um importante fator de virulência (MATLASHEWSKI, 2001; OLIVIER et al., 2012). Esta é uma metaloproteinase dependente de zinco, pertencente a classe da metzincina e a família de peptidases M8. Esta classe de proteínas inclui características como o motivo conservado HEXXHXXGXXH, âncora GPI e um pro-peptídeo localizado na região N-terminal da proteína. O pro-peptídeo é removido durante a maturação e ativação da proteína e sua função é tornar a proenzima inativa durante a tradução (YAO; DONELSON; WILSON, 2003). O

mecanismo de ação deste pro-petídeo está associado a um resíduo de cisteína em sua sequência que é conservado em homólogos de gp63 nas diferentes espécies de *Leishmania*. Este resíduo contribui com um mecanismo de troca de cisteína para realização de atividades de protease, que tem como objetivo auxiliar o átomo de zinco a se ligar no sítio ativo e inibir a atividade enzimática, o que protege a célula da autodestruição (MACDONALD; MORRISON; MCMASTER, 1995). Além das características mencionadas, algumas gp63 também contêm um peptídeo sinal em sua extremidade N-terminal, que os dirige para o retículo endoplasmático após a sua síntese e para a via secretora da *Leishmania*, para que elas possam cumprir sua função (YAO et al., 2002).

2.4.2.1 FUNÇÃO NO HOSPEDEIRO MAMÍFERO

Do ponto de vista funcional, algumas funções têm sido atribuídas a gp63 que a implicam como importante fator de virulência na infecção por diferentes espécies de *Leishmania*. Assim, nos hospedeiros mamíferos, no ambiente extracelular, a gp63 atua na inativação da cascata do complemento, inibindo o fator C3b. Esta inativação impede a formação do complexo de ataque de membrana e permite a opsonização do parasita *Leishmania*, facilitando sua fagocitose, além de inibir a produção de IL-12, o que favorecerá o crescimento celular do parasita (GÓMEZ; OLIVIER, 2010; GUPTA; OGHUMU; SATOSKAR, 2014). Estudos mostraram que a gp63 pode facilitar a ligação do parasita ao macrófago através de receptores de fibronectina, além de sua atividade de protease ser capaz de clivar proteínas da matriz extracelular do hospedeiro, fornecendo a *Leishmania* uma entrada mais rápida no macrófago (OLIVIER et al., 2012; SHIO; OLIVIER, 2010). Também foi

observado que a falta de gp63 reduz drasticamente a capacidade da *Leishmania* de estabelecer e manter uma infecção, uma vez que os hospedeiros são mais propensos a induzir respostas inflamatórias da imunidade inata (OLIVIER; HASSANI, 2010). Entre suas funções, a gp63 atua na redução da produção de TNF, IL-12 e óxido nítrico, o que contribui para a proteção e sobrevivência do parasita (PODINOVSKAIA; DESCOTEAUX, 2015).

Outro conjunto de funções atribuídas a gp63 diz respeito a sua atuação diretamente sobre o metabolismo interno dos macrófagos do hospedeiro mamífero. Assim, estudos mostraram que a gp63 pode ser liberada através de exossomos para o meio extracelular. Isso pode facilitar a sua absorção pelo macrófago, mesmo antes de internalizar a *Leishmania*, gerando reações inibitórias internas na célula. Entre essas reações pode ser citada a ação onde a gp63 cliva o fator transcricional AP-1, que regula a produção de citocinas pró-inflamatórias e óxido nítrico, impedindo sua produção (ATAYDE et al., 2016; CONTRERAS et al., 2010). Esta ação de clivagem proteica também foi mostrada em outro estudo onde a gp63 foi associada à inativação da quinase mTOR, uma enzima necessária para a fosforilação da proteína inibidora da tradução 4E-BP1 e cujo modo de ação é conhecido em detalhes. A 4E-BP1 é um regulador da tradução que se liga diretamente à proteína de ligação ao *cap*, nucleotídeo modificado presente na extremidade 5’ dos mRNAs, também conhecida como eIF4E. Quando ligada ao eIF4E, a 4E-BP1 inibe a tradução e sua fosforilação impede esse efeito inibitório. Quando o mTOR é clivado, ele perde a capacidade de fosforilar a 4E-BP1, o que potencializa o efeito inibitório dessa proteína e leva ao bloqueio da formação de um complexo de iniciação da tradução funcional. Todo esse processo permite a inibição da síntese proteica no macrófago, proporcionando um ambiente ideal para a

proliferação do patógeno (JARAMILLO et al., 2011).

2.4.2.2 FUNÇÃO NO INSETO VETOR

Além das funções propostas na defesa contra a resposta imune nos hospedeiros mamíferos, a gp63 também desempenha funções críticas nos insetos vetores. Esta proteína mostrou um potencial envolvimento na degradação de componentes proteicos no epitélio intestinal do inseto que atuando na adesão do parasita (D'AVILA-LEVY et al., 2006; PEREIRA et al., 2009). É possível ainda que, devido a ampla especificidade de substratos que a gp63 pode interagir, ela também pode desempenhar um papel nutricional para o parasita, atuando como uma endopeptidase (D'AVILA-LEVY et al., 2014; SANTOS; BRANQUINHA; D'AVILA-LEVY, 2006). Alternativamente é possível que a gp63 possa ainda atuar na proteção da *Leishmania* contra as defesas dos insetos (D'AVILA-LEVY et al., 2014).

2.4.2.3 ORGANIZAÇÃO GENÔMICA E EXPRESSÃO DA GP63

A organização genômica observadas para os genes codificantes de gp63 demonstra uma variação considerável no número de genes que codificam essas proteínas entre diferentes espécies de *Leishmania*. Em *L. major* foi observado que esses genes estão presentes em mais de um cromossomo ao longo do genoma e várias cópias foram detectadas dispostas em sequência (em *tandem*) (IVENS et al., 2005). O mesmo arranjo de múltiplas cópias gênicas foi encontrado nos genomas de *L. infantum* e *L. braziliensis* (PEACOCK et al., 2007). Contudo foi identificado uma diferença significativa no número de genes codificantes de gp63 entre

espécies pertencentes aos dois principais subgêneros de *Leishmania* (*Leishmania* e *Viannia*) (STEINKRAUS et al., 1993; VICTOIR et al., 1995; VOTH et al., 1998). Esses dados foram originalmente obtidos em estudos iniciais de hibridização, mas posteriormente também se confirmaram nos sequenciamentos mais recentes realizados com espécies de *Leishmania* pertencentes a estes dois subgêneros (GONZÁLEZ-DE LA FUENTE et al., 2017; LLANES et al., 2015).

Em relação à expressão dos genes de gp63, estudos em *L. donovani, L. mexicana* e *L. major* demonstraram que alguns grupos desses genes são diferencialmente expressos durante as fases do ciclo de vida do parasita (amastigota, fase logarítmica da forma promastigota e fase estacionária) (YAO; DONELSON; WILSON, 2003). Essa regulação diferencial não parece estar relacionada a sua organização e estudos têm demonstrado que o fator determinante para esta regulação são sequências características presentes nas regiões intergênicas e nas extremidades 3' não traduzidas dos seus mRNAs (KELLY; NELSON; MCMASTER, 2001; YAO; DONELSON; WILSON, 2003).

2.5 RESULTADOS ANTERIORES E EMBASAMENTO DESTE TRABALHO

Os principais desafios para o controle da leishmaniose são a criação de um diagnóstico sorológico eficaz para utilização em áreas endêmicas e um maior conhecimento sobre a biologia do parasito e sua interação com o hospedeiro, que pode gerar conhecimento para novos alvos de drogas ou vacinas. O presente trabalho se propôs a fazer contribuições em ambos a partir do estudo de diferentes proteínas antigênicas de espécies de *Leishmania*.

No que concerne ao diagnóstico da leishmaniose, apesar dos avanços

científicos e tecnológicos realizados até o momento para esta doença, não existe um diagnóstico sorológico único capaz de identificar ambas as formas, humana e canina, dessa patologia. Nesse sentido, trabalhos prévios levaram a identificação de possíveis novos antígenos de *L. infantum* a partir da triagem de bibliotecas de expressão de cDNA e genômica. Como resultado destas triagens, foram obtidas 13 proteínas, denominadas Lci1 a Lci13. As Lci1 a Lci5 foram expressas em *Escherichia coli* e submetidas a análises de sensibilidade e especificidade através de técnicas de ELISA, também com soros de humanos e cães com leishmaniose, positivos no teste parasitológico. Essas proteínas analisadas individualmente apresentaram sensibilidade de 67,4–93% e 36,4–97,2% frente a soros de cães e humanos, respectivamente. Mas nenhuma proteína sozinha foi capaz de diagnosticar satisfatoriamente concomitantemente a leishmaniose humana e canina (OLIVEIRA et al., 2011). Nesse trabalho buscamos avaliar algumas das mais promissoras entre as demais proteínas originalmente identificadas, Lci6 a Lci13, de forma individual e em misturas proteicas.

Dentro do esforço de compreender melhor a patogênese das leishmanioses, este trabalho buscou contribuir também no entendimento sobre o principal antígeno de superfície de *Leishmania sp.*, a proteína gp63. Para isso optou-se por investigar suas características gênicas e proteicas e avaliar sua importância para a biologia do parasito e na interação com seus hospedeiros, gerando assim subsídios para o desenvolvimento de novas alternativas de controle dos diferentes tipos de leishmaniose.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar e caracterizar proteínas antigênicas e de superfície de *Leishmania sp.* quanto a seu potencial uso no diagnóstico da leishmaniose e na patogênese do parasita.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar a antigenicidade de proteínas selecionadas de uma biblioteca genômica frente a soros de cães e humanos acometidos com leishmaniose visceral quanto ao seu potencial diagnóstico
- Buscar novos parálogos, possivelmente não anotados, codificantes para a proteína de virulência gp63 de *L. braziliensis*
- Analisar a evolução dos genes de gp63 de *Leishmania sp.* e outros cinetoplastídeos avaliando as diferenças e semelhanças e suas implicações para a função destas proteínas
- Caracterizar os ortólogos e parálogos de gp63 de *Leishmania sp*., quanto a inferência do seu momento de expressão, características proteicas e localização subcelular
- Avaliar o potencial de peptídeos de gp63 estimularem a produção de anticorpos, *in silico*, sua localização na proteína modelada e a influência dessa localização no escape do sistema imune

4 ARTIGO 1

Avaliação de novos antígenos de *Leishmania infantum* quanto ao seu potencial para o diagnóstico da leishmaniose visceral

De treze proteínas antigênicas de *L. infantum* inicialmente identificadas em trabalhos que precederam a presente Tese de Doutorado, denominadas de Lci1 a Lci13, cinco já haviam sido devidamente avaliadas, as proteínas Lci1 a Lci5 (Oliveira et al. 2011). Neste trabalho, demos continuidade ao estudo dessas proteínas, com foco nas Lci6 a Lci13. Destas, a Lci13, uma HSP70 mitocondrial, já havia sido previamente caracterizada (CAMPOS et al., 2008). A Lci1, que já demonstrou resultados razoáveis no estudo prévio Oliveira et al. 2011, foi também incluída nos nossos ensaios para compor as análises de misturas proteicas.

Com a exceção da Lci8, fragmentos de todas as proteínas recombinantes dos genes citados foram obtidas através de expressão em *Escherichia coli* e purificadas por cromatografia de afinidade em coluna de níquel. Para avaliar o potencial antigênico das proteínas recombinantes selecionadas para este estudo, realizamos ensaios de ELISA com soro de humanos infectados com *L. infantum*, e com diagnóstico de LV confirmado através de testes parasitológicos, e soros controle de indivíduos saudáveis provenientes de área endêmica. Os resultados obtidos através dos ensaios de ELISA demonstraram que quando testadas frente a soros de humanos infectados, as proteínas apresentaram uma sensibilidade considerada baixa para o diagnóstico. Seus valores variaram entre 26 e 72%, que é consideravelmente inferior ao desempenho observado com o lisado total de

parasitas (LAg - 96 %) ou o antígeno recombinante comercial rK39 (84%).

Embora tenha apresentado uma baixa sensibilidade, as proteínas apresentaram alta especificidade, quando avaliadas com soros de humanos sadios (98 a 100%). Em relação a especificidade soros de humanos acometidos com LC e Doença de Chagas, houve uma queda na especificidade de algumas proteínas (60 a 100%), embora outros antígenos ainda apresentem bons resultados.

A partir da análise individual dos soros nos ensaios de ELISA com o soro humano, foi possível observar que cada soro reconheceu pelo menos um dos antígenos estudados (dados não apresentados). Então, optamos por avaliar uma mistura (“Mix”) das melhores proteínas antigênicas com o intuito de aprimorar as sensibilidades dos testes. Este “Mix” foi composto pelas proteínas Lci1, Lci12 e Lci13 e foi avaliado frente a soros de humanos e cães infectados com *L. infantum*. Quando testado frente a soros humanos, o “Mix” apresentou uma melhora significativa na sensibilidade (84%), quando comparado as proteínas individuais e que se mostrou equivalente a obtida com o sistema comercial de diagnóstico e baseado na proteína recombinante rK39.

Os soros de cão apresentaram uma sensibilidade de 88%, que foi menor que a sensibilidade apresentada individualmente pelas proteínas componentes do “Mix” mas ainda assim bastante superior a observada pelo sistema baseado na rK39 (68%). Em relação a especificidade, o “Mix” demonstrou bons resultados frente aos soros humanos e de cão, com valores de 98 a 100%, respectivamente. A conclusão desse conjunto de experimentos é que a utilização de um sistema baseado em múltiplas proteínas antigênicas é viável e pode ser a solução para o desenvolvimento de um sistema único de diagnóstico da leishmaniose visceral. Os resultados obtidos dentro deste objetivo foram incluídos em artigo publicado em

2017 onde o autor desta tese divide a primeira autoria (MAGALHÃES et al., 2017).

PLOS ONE

RESEARCH ARTICLE

Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis

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Citation: Magalhães FB, Castro Neto AL, Nascimento MB, Santos WJT, Medeiros ZM, Lima Neto AS, et al. (2017) Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis. PLoS ONE 12(9): e0184867. https://doi.org/10.1371/journal.pone.0184867

Editor: Henk D. F. H. Schallig, Academic Medical Centre, NETHERLANDS

Received: April 5, 2017

Accepted: September 3, 2017

Published: September 28, 2017

Data Availability Statement: The L. infantum and Leishmania major genomes are available from the TriTrypDB (http://tritrypdb.org/tritrypdb/) and GeneDB (www.genedb.org) databases. The final sequence has been deposited to GenBank and received the accession number KX018626.

Funding: The work in Dr. de Melo Neto's laboratory was more recently funded with grants provided by the Brazilian funding agency CNPq and the Brazilian Ministry of Health (Grant MCTI/CNPq/MS-SCTIE

Abstract

Current strategies for the control of zoonotic visceral leishmaniasis (VL) rely on its efficient diagnosis in both human and canine hosts. The most promising and cost effective approach is based on serologic assays with recombinant proteins. However, no single antigen has been found so far which can be effectively used to detect the disease in both dogs and humans. In previous works, we identified *Leishmania infantum* antigens with potential for the serodiagnosis of VL. Here, we aimed to expand the panel of the available antigens for VL diagnosis through another screening of a genomic expression library. Seven different protein-coding gene fragments were identified, five of which encoding proteins which have not been previously studied in *Leishmania* and rich in repetitive motifs. Poly-histidine tagged polypeptides were generated from six genes and evaluated for their potential for diagnosis of VL by ELISA (Enzyme Linked ImmunoSorbent Assay) with sera from infected humans and dogs. None of those was valid for the detection of human VL (26–52% sensitivity) although their performance was increased in the canine sera (48–91% sensitivity), with one polypeptide useful for the diagnosis of canine leishmaniasis. Next, we assayed a mixture of three antigens, found to be best for human or canine VL, among 13 identified through different screenings. This "Mix" resulted in similar levels of sensitivity for both human (84%) and canine (88%) sera. With improvements, this validates the use of multiple proteins, including antigens identified here, as components of a single system for the diagnosis of both forms of leishmaniasis.

No.: 404259/2012). The work in Dr. C. H. N. Costa's lab was funded by CNPq (Grant 554797). Fellowships and studentships for the graduate students (FBM, ALCN, MBN, WJTS and ASLN) were provided by CNPq, FACEPE or CAPES. The work carried out at Fiocruz-BA was funded by the Brazilian Ministry of Science and Technology (Program RENORBIO). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Visceral leishmaniasis (VL) is a systemic and chronic disease caused by an intracellular protozoan parasite of the genus *Leishmania* and transmitted via sand flies. It is usually characterized by long duration fever, weight loss, weakness, lethargy, splenomegaly, and pancytopenia, among other manifestations [1–4]. In epidemiological terms, VL is classified into anthroponotic and zoonotic types, with the predominant causal agent for the anthroponotic type being *Leishmania donovani* and for the zoonotic type being *Leishmania infantum*, previously known as *Leishmania chagasi* in Latin America [1,5]. In Brazil, this disease is a public health problem with high transmission and case fatality rates [6,7].

The domestic dog is considered to be the main reservoir of *L. infantum*, whereas the possibility remains for wild animals such as foxes and marsupials acting as sylvatic reservoirs [8,9]. In general, the clinical signs of the canine leishmaniasis and its diagnosis in sick dogs are relatively clear, although the same cannot be said of animals displaying few signs or those which are apparently healthy [10]. Afflicted animals can display signs of the disease soon after infection or remain subclinically infected for significant lengths of time, but both are infectious to the sandfly vector [11].

Detection of the parasite through direct microscopic visualization or culturing from biological samples derived from infected individual is the gold standard of VL diagnosis but this is not practical on a large scale. Early serological methods used for VL serodiagnosis (e.g. the direct agglutination test or DAT and immunofluorescence-based tests), relied on the use of whole extracts and fixed parasites. Diagnostic tests based on the detection of the parasite DNA by PCR have also been implemented and they have the advantage of being able to discriminate an active infection from those in which the parasite has been eliminated. All, however, have cost related issues, require a more complex infrastructure and cannot be implemented in the field [11–15].

An alternative for improving the serological techniques used for VL diagnosis is the use of specific molecules that are readily recognized by most sera from infected individuals. For this purpose several recombinant antigens have been tested and the most promising antigen for VL diagnosis so far, is the rK39 [15,16]. This antigen is based on a 39 amino acid repeat derived from a *L. infantum* kinesin-related polypeptide [17] and early studies indicated its potential for the diagnosis of human VL and the evaluation of its progression [18]. Extensive field tests have been carried out since then using this antigen in different formats and an rK39 strip test, readily applicable in the field, has been shown to be a sensitive and a reliable indicator of VL in human patients. In a meta-analysis study evaluating the data from independent studies using this rK39 strip test, the results were overall seen to be quite uniform with very high sensitivity and specificity [19]. In Eastern Africa, however, its performance was seen to be inferior to what was observed elsewhere [16–21] and in general it was seen to be positive in a significant proportion of healthy individuals from endemic regions and for extended periods after cure of the disease [14,22]. Another relevant aspect is that tests made in the field focusing on the diagnosis of VL in dogs did not show equivalent results to those seen with human samples and suggest that a rK39-based test may not be suitable for the identification of infected dogs on its own [23–25].

The humoral immune responses generated during an infection by *L. infantum* in infected dogs and humans are quite distinct and the antigens most suitable for the diagnosis of VL in humans are not necessarily valid to use with canine samples [26]. In a previous study our group identified and evaluated five antigens obtained through the screening of a *L. infantum*

cDNA library for their potential for the diagnosis of both human and canine VL. Although some of the antigens displayed high sensitivity and specificity to one or the other species, none of them were effective for the serodiagnosis in both humans and dogs [23]. Within this context, we aimed at expanding the panel of available antigens for the diagnosis of VL through the evaluation of newly selected antigens, as well as a mixture of some of the best among those evaluated, for the diagnosis of both canine and human forms of the disease.

Materials and methods

Parasites

Leishmania infantum (MHOM/BR2000/Merivaldo2, 2240) promastigotes were maintained at 26˚C in modified LIT medium, pH 7.2 [0.2% sucrose (w/v), 0.36% liver broth (w/v), 0.1% triptose (w/v), 0.002% haemin (w/v)], or Schneider medium, pH 7.2 (Sigma-Aldrich), containing 10% fetal bovine serum, ampicilin (10 U/ml) and streptomycin (10 μg/ml). Parasites were kept on log phase growth by passaging to new culture media every 3 to 4 days. Total parasite lysate (LAg) was obtained by sonication of log-phase parasites. The protein content of the lysate was quantified by the Bradford method.

Sera

The human sera panel was composed of: 50 clinically and parasitologically diagnosed VL patients obtained from an endemic area in Piaui State, Brazil, independently of sero-reactivity; 50 negative sera samples obtained from healthy individuals of various ages from Pernambuco State, also endemic for VL; 26 parasitologically confirmed cutaneous leishmaniasis patients; 40 serologically confirmed patients with chronic Chagas' disease. The canine serum samples were obtained from: 46 dogs with parasitologically confirmed VL, from the endemic area of Jequié (Bahia state, Brazil); 15 healthy young dogs of various ages and breeds from non-endemic areas; and sera from 31 dogs afflicted with other infectious diseases (4 with babesiosis, 20 with erhlichiosis, and 7 with demodicosis) were also used.

Ethical approval

All dogs were handled in agreement with the Oswaldo Cruz Foundation guidelines for experimentation on animals and the collection of the sera used in this study was approved by the ethics committee for the use of animals in research (CPqGM-FIOCRUZ, Ceua, license N.040/2005). All human sera were collected after approval of their use by the appropriate ethics committees, as follows: use of the sera from VL patients was approved by the ethics committee from the Federal University of Piaui (0116/2005); the negative control sera were included in the study approved by the ethics committee of the Brazilian Ministry of Health (25000.119007/2002-03); sera from patients with cutaneous leishmaniasis were included in the project CAEE 0014.0.095.000–05, approved by the ethics committee from CPqAM-FIOCRUZ (03/08/2008); and the samples from chagasic patients used in this study were obtained from the serum bank at the Reference Laboratory for Chagas Disease at CPqAM-FIOCRUZ. Written informed consent was obtained from all adults or legal guardians of children before blood was collected.

Leishmania infantum genomic library and serological screening

A *L. infantum* genomic library was constructed with reagents from the Stratagene Corporation (La Jolla, USA) using total *L. infantum* DNA partially digested with Tsp5091 and ligated into the λ-ZAP Express bacteriophage, previously digested with EcoRI. Library amplification and screening was carried out as previously described [27], using a pool of six sera from patients with VL, at a 1:1000 dilution, to screen approximately 30.000 clones. The inserts were sequenced and compared with sequences from the *L. infantum* and *Leishmania major*

genomes available at the TriTrypDB (http://tritrypdb.org/tritrypdb/) and GeneDB (www.genedb.org) databases. Sequences obtained from the ends of each insert were then used for BLAST searches against genomic nucleotide sequences of *L. infantum* available at TriTrypDB. For the 5' ends of nearly all selected clones (except Lci9), sequences identical in nearly 100% of the nucleotides were found within regions predicted as protein coding regions whereas the corresponding 3' ends matched nearby sequences within the same open reading frame or within the neighboring intergenic regions. For Lci9, not found within the available *L. infantum* databases, the sequence for the whole 2.4 kb insert was generated by direct sequencing. The final sequence has been deposited to GenBank and received the accession number **KX018626**.

Subcloning strategies

For recombinant protein expression, a distinct strategy was used for each insert, depending on the occurrence of internal restriction sites compatible with the subcloning strategy into the expression plasmids of the pRSET series (A, B or C—Invitrogen), as follows: the Lci6 insert (3.8 Kb) was recovered after digestion with BamHI/KpnI and the insert ligated into the same sites of pRSET C; Lci7 (2.4 Kb) was recovered using BamHI/SalI and the insert was ligated into the BamHI/XhoI sites of the pRSET B; Lci9 (2.4 Kb) was recovered with BamHI/XhoI and ligated into the same sites of pRSET B; Lci10 (0.9 Kb) was also recovered with BamHI/XhoI but the insert was ligated into the same sites of pRSET C; Lci11 (1.9 Kb) was first recovered using the enzymes BamHI/NotI and subcloned into the same sites of the vector pET21a (Novagen), followed by a second subcloning event where the Lci11 fragment was recovered from the resulting plasmid using BamHI/XhoI and the insert ligated into the same sites of pRSET A; Lci12 (2.8 Kb) was subcloned in pRSET B after digestion with the enzymes BamHI/PstI. In all cases, the resulting constructs encode for fusion proteins containing a common 32 amino acid segment at their N-terminuses, derived from the pRSET vector and which includes a polyhistidine tract (MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKD) plus, eventually, a few further amino acids encoded by the vector's multiple cloning sites immediately before and after the antigen coding segment. For Lci13 it was generated after two sets of subcloning reactions, where first a 1 Kb PstI fragment from the previously described Lc2.2 clone [28] was subcloned into the PstI site of the pTZ18R vector, with its 5' end facing the vector´s T7 promoter. For the second subcloning reaction, the insert was recovered from the pTZ18R vector through digestion with BamH I/Hind III and subcloned into the same sites of pRSET A. For Lci1, the plasmid used for its expression has been described before [23].

Expression and purification of recombinant proteins

For the expression of His-tagged recombinant proteins, *Escherichia coli* BL21(DE3) pLysS (Invitrogen) bacteria were transformed with the pRSET derived plasmids, grown in LB medium and expression induced by IPTG. Induced cells were harvested, resuspended in 0.15 M phosphate buffered saline, pH 7.2 (PBS) and lysed by sonication. Protein purification was performed with Ni-NTA Agarose (Qiagen). Protein products were analysed by 15% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), followed by staining of the proteins with Coomassie blue R-250. For estimation of the recombinant proteins concentrations, the densities of their stained bands in Coomassie blue stained gels were compared with those of known concentrations of bovine serum albumin (BSA).

ELISA

The ELISA assays were essentially carried out as previously described at [23]. Briefly, ~400 ηg of the individual recombinant proteins or mixes of three proteins consisting of 300 ηg of each

protein were added to each well of ELISA plates. The wells were then incubated with the selected sera at a dilution of 1:200 (canine sera) or 1:900 (human sera), followed by incubation with the secondary antibody, namely peroxidase-conjugated goat anti-dog IgG (1:1200) or anti-human IgG (1:10000), depending on the tested sera. For the rK39 ELISA assays, the commercial recombinant rK39 antigen was purchased from Rekom Biotech (Granada, Spain) and the assays were carried out following the manufacturer's specifications.

Statistical analysis

The cutoff values for the ELISAs were defined as means of results obtained with serum samples from 50 healthy donors plus three standard deviations. The ROC curves and the sensitivity and specificity values were generated with the Medcalc Software version 15.8. The graphs were generated by the GraphPad Prism 3.

Results

Serological screening and identification of novel *Leishmania infantum* antigens

A total of 60 positive clones from a genomic *L. infantum* expression library were identified after an immunoscreening with a pool of six sera from Brazilian VL patients. Inserts from 50 clones were sequenced and seven different protein-coding gene fragments were identified and their protein products named as Lci6, Lci7, Lci8, Lci9, Lci10, Lci11, and Lci12, to avoid confusion with the five antigens previously described by us from *L. infantum* [23]. Thirty-three clones were found to contain fragments of the Lci6 gene, three encoded Lci7, two encoded Lci8 and the remaining genes (encoding Lci9, Lci10, Lci11 and Lci12) were represented by one clone each. When compared with *L. infantum* and *L. major* sequences, five of the identified antigens (Lci6, Lci8, Lci9, Lci10 and Lci12) are either annotated as hypothetical or have not been properly studied in *Leishmania*. Lci11 has been previously described from *Leishmania amazonensis* as a phosphoprotein which binds specifically to a homologue of the translation initiation factor eIF4E, named as Leish4E-IP (for 4E interacting protein) [29]. This is a hydrophilic protein conserved in *L. infantum* and *L. major* but with limited conservation in *Trypanosoma* spp and which is very rich in the amino acids proline, glutamine, alanine and serine. The seventh polypeptide, Lci7, is the *L. infantum* orthologue of the stress-inducible protein sti1, originally described in *L. major* [30]. Table 1 lists the *L. infantum* accession numbers from TriTrypDB for the genes encoding most of the identified proteins. For Lci9, its gene hasn't been properly annotated within the *L. infantum* genomic sequences, although sequences resembling parts of this gene can be found split within two distinct segments of chromosome 28, suggesting an assemblage error perhaps due to the shotgun nature of the sequencing of this

Table 1. TriTrypDb accession numbers for the newly identified antigenic proteins.

Protein	Accession number in TriTrypDB
Lci6	LinJ.26.1950
Lci7	LinJ.08.1020
Lci8	LinJ.32.2420
Lci9	LmjF.28.3010
Lci10	LinJ.34.2360
Lci11	LinJ.35.4030
Lci12	LinJ.29.0110

https://doi.org/10.1371/journal.pone.0184867.t001

genome [31]. An orthologue for the Lci9 gene is clearly identifiable in *L. major*, however, and its accession number is also listed in Table 1.

Sequence analysis of the novel *L. infantum* antigens

All five novel *Leishmania* antigens identified in the *L. infantum* screening (Lci6, Lci8, Lci9, Lci10 and Lci12) have in common the presence of tracts of *in tandem* repetitive motifs (Fig 1). Most of these (Lci6, Lci8, Lci10 and Lci12) are predicted to be large proteins. The length of their repeats varies significantly, from only 8 (Lci12) to more than 50 (Lci6 and Lci10) amino acid residues, but little similarity in sequence between the repeats is observed, with the exception of those from Lci8 and Lci12, which seem to be related. Lci6 is composed by a non-repetitive N-terminal region (=450 residues long), followed by 21 non-identical repeats of variable length (varying from 95 to 135 residues) and a very short C-terminus. It is the *L. infantum* orthologue of a microtubule-associated protein, described from *Trypanosoma brucei* as GB4 [32]. Lci8 consists of 61 identical repeats of 10 amino acid residues, flanked by short N and C-terminal regions of 331 and 242 residues in length, respectively. It is an orthologue of a *T. brucei* membrane associated protein possibly involved with vesicular transport, Tb-291 [33]. Based upon the gene sequence of its *L. major* orthologue, the full-length Lci9 is shorter than the other antigens discussed here and consists of two sets of related repeats of 25 (14 copies) and 34 (11 copies) residues, flanked by very short N and C-terminal regions (169 and 97 residues long, respectively). This is the orthologue of the protein named nucleoporin (TbNup140) [34]. Lci10 encodes a hypothetical protein that has orthologues in other *Leishmania* species but is absent from *Trypanosoma*, although it might be related to a protein found within the flagellar attachment zone in *T. brucei*. The sequence available from the *L. infantum* genome appears to be incomplete but the Lci10 clone contains multiple related repetitive motifs of different sizes (varying from 68 to 198 residues) which follow a non-repetitive N-terminal region. Lci12, also defined as a hypothetical protein, is the *Leishmania* orthologue of the membrane-associated protein Tb-292 from *T. brucei*, related to the Lci8 orthologue Tb-291 [33]. The *L. infantum* protein was also identified in a bioinformatic screening for proteins with tandem repeat domains [35]. It is composed by an N-terminal region containing approximately 160 amino acids, followed by a region containing 30 repeats of an 8 amino acids-long motif and a carboxi-terminal region containing the *trans*-membrane segments.

Recombinant antigen expression

With the exception of Lci8, all the other antigens identified here were efficiently expressed in *E. coli* and purified by affinity chromatography on nickel columns. Results of representative analysis by SDS-PAGE of the various recombinant proteins are shown in Fig 2a. For Lci6, based on the subcloned fragment, the recombinant protein would encompass residues 246 to 1548 of the original polypeptide with a predicted molecular weight of 286 kDa. However, a single band of ~40 kDa (a likely result of internal proteolytic cleavage) is seen after expression and purification; it includes the His-tag at the N-terminus and approximately the first one-third of the protein. The Lci7 subcloned fragment encodes for a polypeptide consisting of most of the protein (residues 69 to 547), generating a 54 kDa recombinant protein. For Lci9, the recombinant polypeptide consists of 799 residues and includes all elements identified within its *L. major* orthologue, expressed as a 71 kDa band. Lci10 was only expressed as a polypeptide encompassing residues 404 to 717 of the full-length protein and a predicted molecular weight of ~35 kDa (Fig 1d). The recombinant protein, nevertheless, migrates with an apparent molecular weight greater than 50 kDa. Recombinant Lci11 encompasses residues 47 to 688 of the original protein with a predicted molecular weight of 63 kDa, but also migrates in gel with

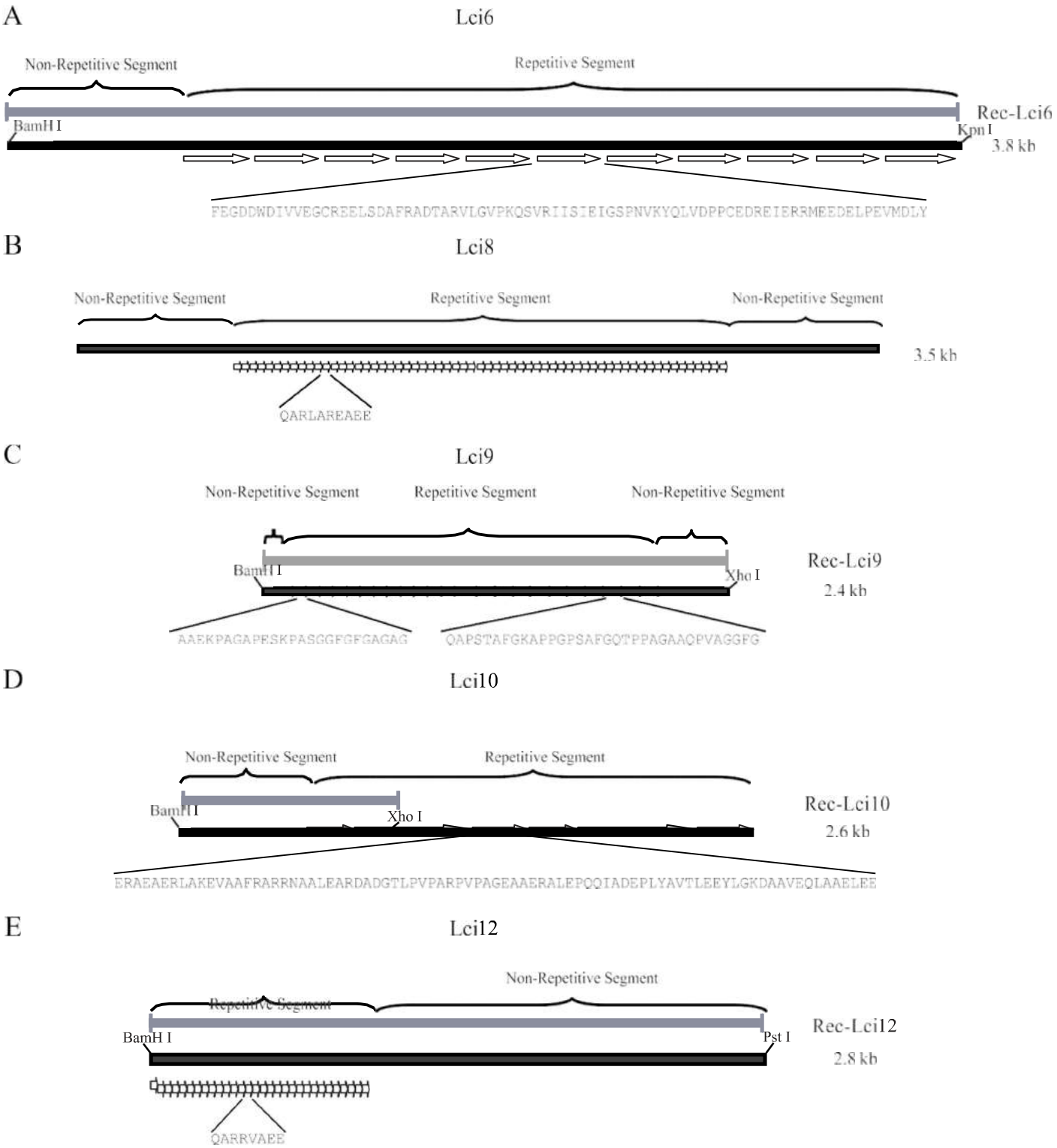

Fig 1. Schematic representation of the various gene fragments and corresponding deduced recombinant proteins evaluated in this study. The maps were derived from the sequences produced after direct sequencing or from the coding genomic sequences available at TriTrypDb.

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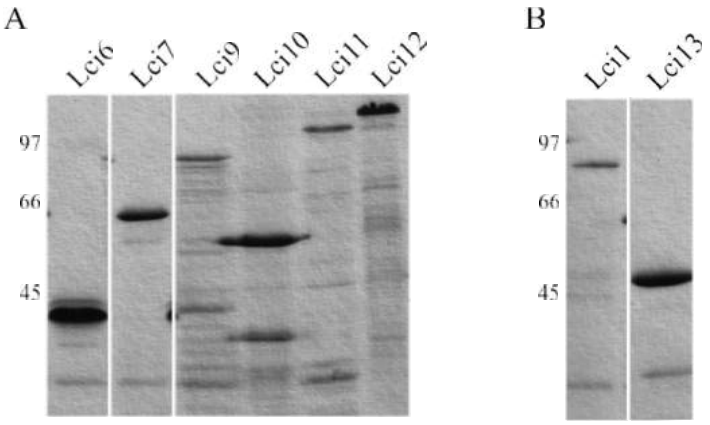

Fig 2. Polyacrilamide gel electrophoresis showing the affinity purified polypeptides evaluated in this study. (A) His-tagged, recombinant fragments of Lci6, Lci7, Lci9, Lci10, Lci11 and Lci12 in denaturing 15% SDS-PAGE stained with Coomassie Blue. All lanes shown are from a single gel but selected regions were removed for clarity. For some of these polypeptides, especially the larger ones, bands of lower molecular weight can be generally seen, but these are likely degradation products due to proteolysis within the bacteria that vary in intensity between different batches of purification. (B) The same for Lci1 and Lci13. The numbers on the left indicate the sizes of molecular weight markers.

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an apparent molecular weight higher than predicted, ~100 kDa. For Lci12, the recombinant polypeptide expressed comprises residues 153 to 1081 of the full-length protein, with a predicted size of ~106 kDa and a compatible migration in gel.

Two other proteins obtained in previous works were added to this study for comparative purposes and shown in Fig 2b. Lci1 encodes a *L. infantum* homologue of the cytoplasmic heat shock protein HSP70 [23] and the recombinant protein migrates with an apparent molecular weight of ~80 kDa. The second protein, named Lci13 here for clarity, encodes part of the *L. infantum* mitochondrial HSP70. The recombinant fragment evaluated was described before [28] and migrates in gel with an apparent molecular weight of ~45 kDa. Despite the fact that both Lci1 and Lci13 belong to the family of HSP70 proteins, the identity between the two in terms of amino acid sequence is less than 50% and a rabbit polyclonal serum produced against recombinant Lci13 does not recognize Lci1 (unpublished data).

Recognition of the *L. infantum* recombinant proteins by human sera

To evaluate the antigenicity of the recombinant antigens selected for this study, we performed ELISA assays with serum from humans infected with *L. infantum* and with VL diagnosis confirmed through parasitological tests (Fig 3 and Table 2). With the exception of Lci1, previously tested [23], none of the others recombinant polypeptides had been evaluated before in similar assays. The different antigens produced ELISA reactions with variable intensities but the sensitivity values for the novel antigens were low, varying between 26 and 48%, and much inferior to the performance seen with Lci1 (72%), also insufficient, or with either the total parasite lysate (LAg– 96%) or the commercial recombinant rK39 antigen (84%). As before [23], and in order to minimize the possibility of false positive results and increase specificity, for these experiments we opted to define a cutoff based on the mean plus three standard deviations of the results generated with control sera from healthy individuals. Indeed, most of the antigens did not produce false positive reactions and the specificity values calculated based on these sera were equal to or very close to 100% (the data also summarized in Fig 3 and Table 2). The various antigens were also tested with sera from patients with cutaneous leishmaniasis and

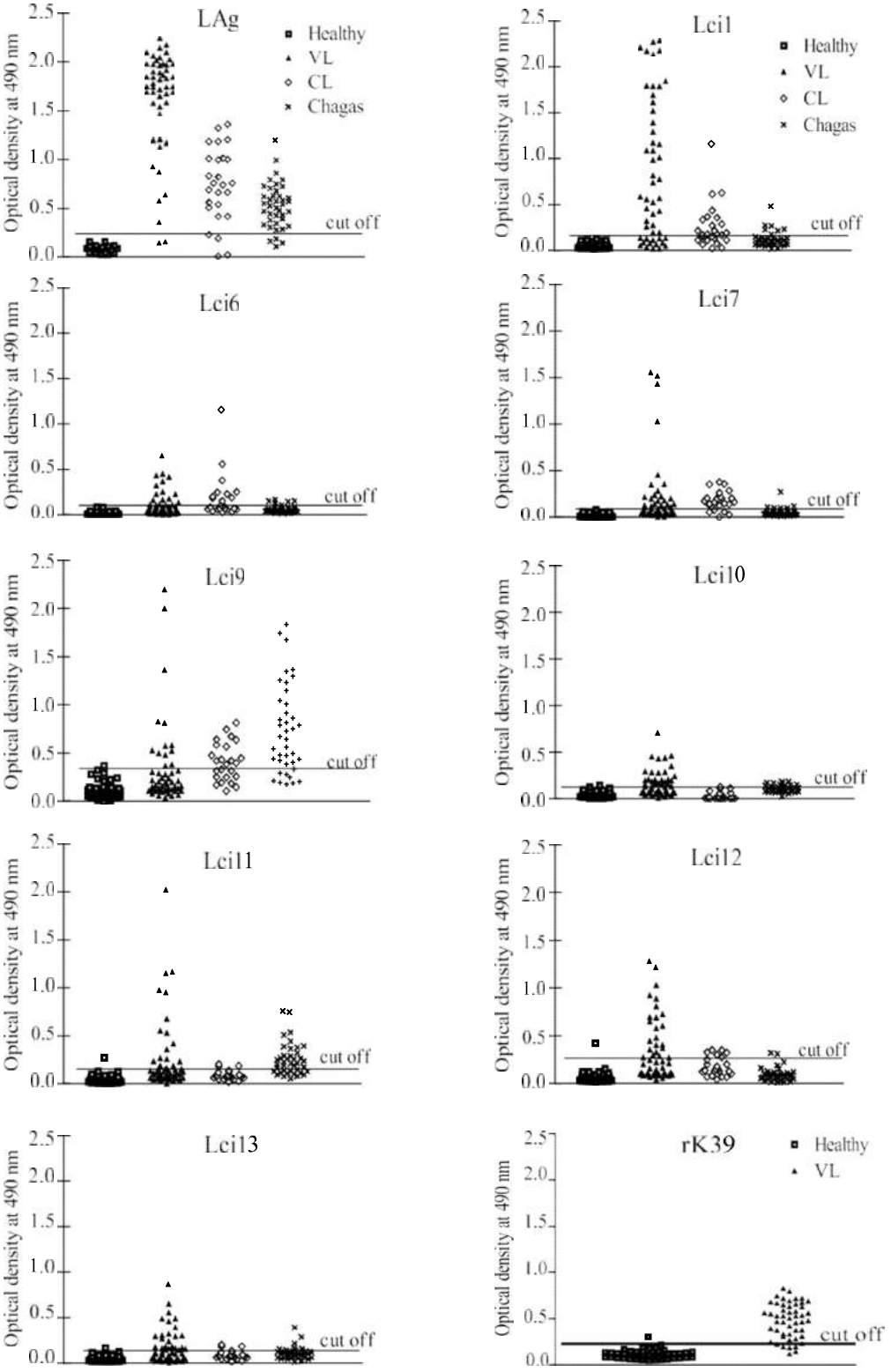

Fig 3. Evaluation of the novel *Leishmania infantum* recombinant antigens for the diagnosis of visceral leishmaniasis in humans. A panel of human sera derived from individuals with confirmed visceral leishmaniasis was tested through an Enzyme-Linked ImmunoSorbent Assay (ELISA) with the recombinant

antigens produced in this study, the rK39 and the total *L*. *infantum* lysate (LAg). The panel was composed of 50 serum samples from individuals with visceral leishmaniasis (VL), 50 serum samples from healthy individuals (Healthy), 23 serum samples from individuals with cutaneous leishmaniasis (CL) and 40 serum samples from individuals with Chagas disease (Chagas). Each symbol corresponds to the result obtained with an individual serum. The horizontal lines indicate cutoff values, calculated by the means of results obtained with serum samples from healthy donors plus three standard deviations.

https://doi.org/10.1371/journal.pone.0184867.g003

Chagas' disease in order to evaluate their cross-reactivity for VL diagnosis. Remarkably, Lci9 produced very strong cross-reactions with sera from Chagas' disease patients and only Lci10 and Lci12 did not display cross-reactions to sera from either cutaneous leishmaniasis or Chagas' disease individuals. With the exception of Lci9 (60%), the specificity values calculated based on these sera were all above 95%. Overall, among the new recombinant polypeptides tested, antigens Lci1 and Lci7 produced a better performance (greater sensitivity with very high specificity), but their low sensitivity compromises their use as single antigens for the diagnosis of human VL (Table 2).

Recognition of the *L*. *infantum* recombinant proteins by canine sera

Next, we assayed the same set of antigens for their potential to identify positive sera from dogs with parasitologically confirmed leishmaniasis. Again, only Lci1 had been previously been tested in similar assays [23]. In general, these antigens had a performance with the canine sera better than that seen for the positive human samples. The ELISA's sensitivity varied between 49 and 91%, for the novel antigens described here, and 93 and 97%, for Lci1 and Lci13,

Table 2. Summary of the ELISA assays carried out with human sera in order to evaluate the performance of the recombinant antigens for the identification of positive cases of human VL. LAg represents the total *L*. *infantum* lysate used as positive control. C.I. stands for confidence interval. NE—Not Evaluated.

	Sera from confirmed VL infected humans		Sera from related diseases (% of cross-reactive sera with positive results)		
Recombinant antigens	**% of sensitivity (99% C.I.)**	**% of specificity/ healthy sera (99% C.I.)**	**Cutaneous Leishmaniasis**	**Chagas' disease**	**% of specificity/ related diseases (99% C.I.)**
LAg	96% (86.3%–99.5%)	100% (92.9%–100%)	52%	20%	74% (62.7%–82.6%)
Lci1	72% (57.5%–83.8%)	100% (92.9%–100%)	26%	2.5%	94% (87.65%–97.47%)
Lci6	48% (33.7%–62.6%)	100% (92.9%–100%)	13%	0%	97% (92.4%–99.4%)
Lci7	52% (37.4%–66.3%)	100% (92.9%–100%)	17%	2.5%	96% (89.98%–98.55%)
Lci9	26% (14.6%–40.3%)	98% (89.2%–100%)	56%	77.5%	60% (50.4%–69%)
Lci10	48% (33.7%–62.6%)	100% (92.89%–100%)	0%	0%	100% (92.9%–100%)
Lci11	32% (19.5% -46.7%)	98% (89.2%–100%)	25%	7.5%	90% (82.5%–94.5%)
Lci12	46% (31.8% -60.7%)	100% (92.9%–100%)	0%	0%	99% (95.2%–100%)
Lci13	44% (30% -58.8%)	98% (89.4%–100%)	12.5%	12.5%	93% (86.5%–96.9%)
Mix (Lci1, Lci12 and Lci13)	84% (70.9%–92.8%)	98% (86.8%–99.9)	NE	NE	NE
rK39	84% (70.9%–92.8%)	100% (92.89%–100%)	NE	NE	NE

https://doi.org/10.1371/journal.pone.0184867.t002

respectively, comparable to that from the total parasite lysate (LAg– 93%). In contrast, the sensitivity for the commercial rK39 was 68%. Again no false positive results were seen when sera from healthy control dogs were evaluated, with the specificity values calculated based on these equal to 100% for the different polypeptides tested. When sera from dogs afflicted with other infectious diseases (erhlichiosis, babesiosis or demodicosis) were evaluated, however, some positive cross-reactions, with moderate intensity were observed, leading to reduced specificity values calculated with these sera (76%–100%) (Fig 4 and Table 3). Lci13 displayed the best performance between the various antigens tested, with 97% sensitivity and very high specificity. Its performance was even superior to the one seen with the assays using total parasite lysate (Lag), which displayed some false negative results as well as strong cross-reactions with the sera from dogs with other infectious diseases. Other antigens also produced strong reactions with the positive dogs' sera (Lci1 and the novel antigen Lci12), but their performance was inferior to Lci13. Nevertheless, with the dog sera at least, all three antigens performed much better than the commercial recombinant rK39 and can be potentially useful as part of novel tests for the diagnosis of canine leishmaniasis.

Evaluation of a mix of three recombinant proteins against both human and canine sera

When the results from the ELISA assays with the VL positive human sera were analyzed in more detail, all were seen to recognize at least one of the recombinant antigens evaluated (data not shown). This led us to test in similar assays, with the VL positive sera from humans and dogs, a mix of recombinant proteins with complementary reactivities (Lci1, Lci12, Lci13). This "Mix" consists of the best three proteins evaluated here in terms of sensitivity and specificity with human and/or canine sera. The results from the assays with the human sera are shown in Fig 5a. The "Mix" produced a strong reaction with the positive sera, displaying a greater sensitivity when compared with the individual antigens (84% sensitivity—also shown in Table 2), and an overall performance more similar to the total parasite lysate or to recombinant rK39 when the results were analyzed through a ROC curve (Fig 5b). A great number of cross-reactive reactions, however, were seen with the sera from Chagas' disease and cutaneous leishmaniasis individuals. The assays using the canine sera also resulted in a significant proportion of positive results for the animals with confirmed leishmaniasis (88% sensitivity—Fig 5c and Table 2). The "Mix", however, produced a lower performance than the one observed for Lci13 and the total parasite lysate, although no significant difference was seen when these results were analyzed through a ROC curve (Fig 5d), with all three samples showing strong sensitivity and specificity for the diagnosis of the canine leishmaniasis. In comparison with rK39, however, the "Mix" still behaved much better than the commercially produced recombinant protein when tested with the dog sera.

Discussion

An early and accurate diagnosis of VL is of great importance to the administration of an effective treatment, screening of endemic areas and consequent interruption of the parasite life cycle [15,16]. In this study, a panel of novel *L. infantum* antigens was evaluated for their potential use for the diagnosis of both human and canine forms of VL. ELISA was chosen for this evaluation since it is a preferred choice for the serodiagnosis of the disease in the laboratory [36] and it has been shown to be more reliable and sensitive than other rapid tests [37]. Several of the antigens tested showed very good potential for the canine form, with one of them (Lci13), demonstrating a better capacity to detect the canine leishmaniasis than the total parasite lysate. This protein is a promising antigen, since some recent studies did not find a single

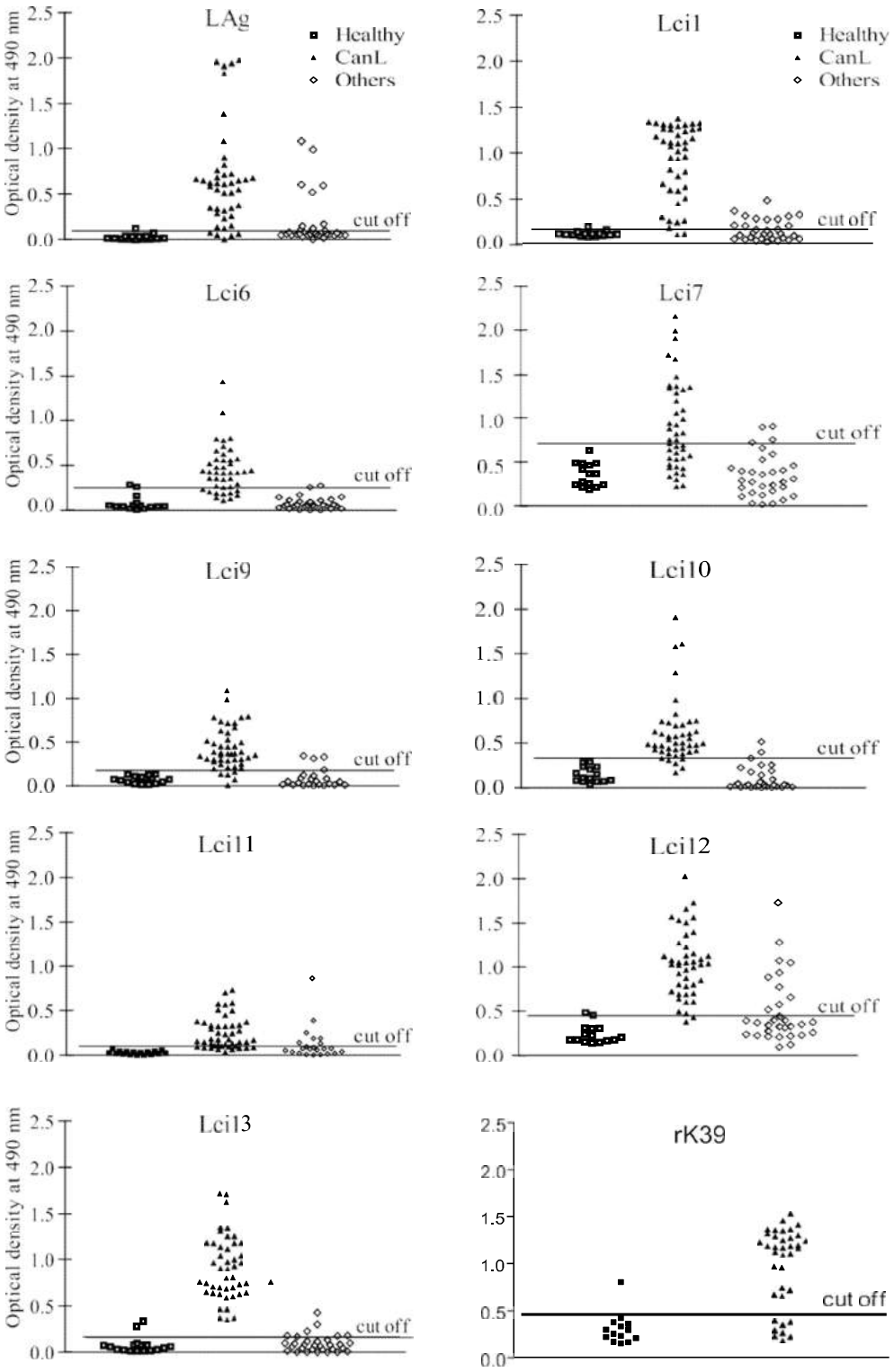

Fig 4. Evaluation of the novel *Leishmania infantum* recombinant antigens for the diagnosis of canine visceral leishmaniasis. The ELISA assay described in the previous Figure was performed also with a panel of canine sera from animals confirmed with visceral leishmaniasis. Serum samples from 46 dogs with

leishmaniasis confirmed through parasitological tests (CanL—canine leishmaniasis), 31 dogs with other infections (4 with babesiosis, 20 with erhlichiosis, and 7 with demodicosis—Others) and 15 healthy control animals (Healthy) were assayed with the recombinant antigens, the rK39 and the total *L. infantum* lysate (LAg). Each symbol corresponds to the result obtained with an individual serum. The horizontal lines indicate cutoff values, calculated as described in Materials and Methods.

https://doi.org/10.1371/journal.pone.0184867.g004

protein capable of such diagnostic efficiency in dogs [38,39]. In contrast, none of the antigens tested were as effective for human VL, highlighting the differences in immune response between the two different disease targets, as highlighted previously [23,26]. In the assays described here, the commercial rK39 antigen was included in order to facilitate the comparison of the newly identified antigens with other previously described and also to evaluate the immune response level from the sera selected for this study. The results for the rK39 were indeed consistent with what has been reported in the literature and confirming that it is effective for the diagnosis of human VL but performs much less satisfactorily with the canine form of the disease [19,24].

Most of the antigens tested here were proteins bearing repetitive motifs. Such proteins have been reported in the literature to be present in various organisms, from viruses to humans, and are characterized by the presence of at least two or more copies of an amino acid sequence. Several studies show that they are particularly antigenic and it is believed that this occurs due to stimulation of B cells by binding these repetitive antigens, by a route independent of the T-lymphocyte stimulation [40–43]. For these reasons, these proteins are strong candidates for the development of serological assays and vaccine targets. Here, the evaluated proteins bearing repetitive motifs did not show good sensitivity in humans. However, when tested against dog

Table 3. Performance of the recombinant antigens with dog sera. LAg represents the total *L. infantum* lysate used as positive control. C.I. stands for confidence interval. NE—Not Evaluated.

	Sera from dogs with confirmed leishmaniasis		Sera from related diseases (% of cross-reactive sera with positive results)			
Recombinant antigens	**% of sensitivity (99% C.I.)**	**% of specificity/ healthy sera (99% C.I.)**	**Ehrlichia**	**Babesiosis**	**Demodicosis**	**% of specificity/ related diseases (99% C.I.)**
LAg	93% (80.9%–98.5%)	100% (76.8%–100%)	15%	50%	0%	89% (76.4%–96.4%)
Lci1	93% (80.9%–98.5%)	100% (76.8%–100%)	50%	0%	14%	76% (61.2%–87.4%)
Lci6	67% (51.5%–80.9%)	100% (76.8%–100%)	0%	0%	0%	100% (76.8%–100%)
Lci7	49% (33.3%–64.6%)	100% (76.8%–100%)	15%	0%	0%	94% (82.1%–98.6%)
Lci9	85% (71.1%–93.7%)	100% (76.8%–100%)	10%	0%	14%	92% (78.6%–98.3%)
Lci10	77% (61.4%–88.2%)	100% (76.8%–100%)	15%	0%	14%	91% (79.2%–97.6%)
Lci11	83% (68.6%–92.2%)	100% (76.8%–100%)	30%	0%	28%	79% (62.7%–90.4%)
Lci12	91% (77.9% -97.4%)	100% (76.8%–100%)	35%	50%	0%	80% (66.1%–90.6%)
Lci13	97% (85.8%–99.9%)	100% (76.8%–100%)	5%	0%	0%	98% (88.5%–99.9%)
Mix (Lci1, Lci12 and Lci13)	88% (72.6%–96.7%)	100% (76.8%–100%)	NE	NE	NE	NE
rK39	68% (50.2%–82%)	100% (78.2%–100%)	NE	NE	NE	NE

https://doi.org/10.1371/journal.pone.0184867.t003

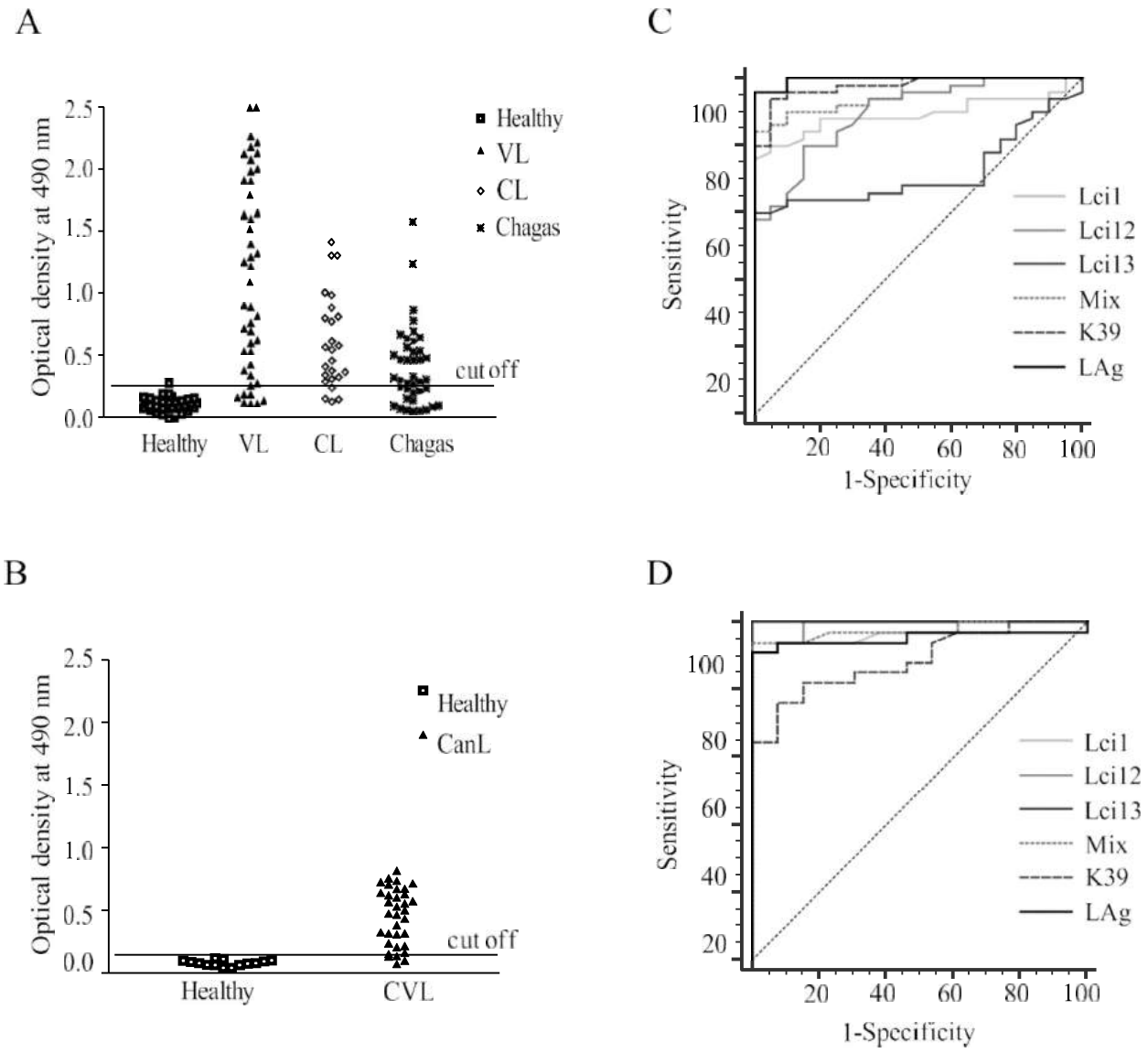

Fig 5. Reactivity of an antigenic mix with human and canine sera positive for visceral leishmaniasis. (A) Reactivity of an antigenic mix with human sera from individuals with confirmed visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), Chagas' disease (Chagas) and healthy controls (Healthy). (B) ROC curves displaying the performance with the human sera of the protein "Mix", the individual recombinant proteins that were included in the "Mix", rK39 and the total *L. infantum* lysate (LAg). (C) Serum reactivity of dogs with canine leishmaniasis (CanL) and healthy control animals (Healthy) with the same protein "Mix". (D) SROC curve showing the performance with the canine sera of the protein "Mix", the individual recombinant proteins that were included in the "Mix", rK39 and the total *L. infantum* lysate.

https://doi.org/10.1371/journal.pone.0184867.g005

sera, they displayed high sensitivity, corroborating previously published data where a repetitive protein showed higher sensitivity than non-repetitive proteins with canine samples [44].

The tested recombinant antigens displayed major differences in sensitivity when tested in human and dog sera, as previously seen by us and others using different recombinant polypeptides in the same type of assay [23,26]. This variance may be due to the differentiated way that the vertebrate hosts react to the parasite, mainly regarding the recognition and presentation of the distinct antigens studied to the immune system or, as has been proposed [26], this response

may be due to different mechanisms for parasite survival in each host (man and dog). On the other hand, since it has been shown that variations in sensitivity may be due to the symptomatic and asymptomatic phases of the disease, the symptomatic phase displaying the best sensitivity performance in the serological tests [45], these differences may, to some extent, reflect the stage of the disease in the individuals from whom the sera were collected. In general, the differential recognition of the studied proteins by the sera from the two hosts highlights the differences in the immune responses elicited by the parasite and the need to optimize the current serological tests.

In order to improve the diagnosis of VL in humans, we proposed the evaluation of an antigen "Mix", composed by antigens which already had produced good performance with the dog samples. These antigens were chosen in order to combine the high sensitivity and specificity found in dog sera and improve the tests made with the individual proteins to detect the disease in dog and humans, resulting in a promising serological test. Recombinant Lci1 had been evaluated before with a good performance for VL immunodiagnosis in dogs [23] and it was selected as one of the three proteins (with Lci12 and Lci13) included in the "Mix". The use of this "Mix" led to a substantial increase in the sensitivity for the human disease, with a minor decrease in performance for the canine leishmaniasis, when compared with Lci13 alone and total *Leishmania* lysate, with no false positive results seen when assayed against the healthy control sera. The "Mix" was therefore a significant improvement as a tool to detect the disease in both humans and dogs, although further optimization would still be required in antigen representation in other to increase sensitivity prior to any considerations regarding a commercial test. Nevertheless, these results contrast with a recent report where a similar mix of three antigens did not lead to an increase in their diagnostic performance when it was compared with the individual proteins alone [39]. Lack of significant improvement attempted by a mixture of proteins may be explained by the decrease of the antigenicity of each individual protein, due to the presence of the antigenic peptide in lower concentration in the solid phase. An alternative serological method that could be used to deal with the limitations presented by the protein mixture is the development of chimeric proteins, containing the regions of the proteins that presented the best performance in serological evaluations. Some studies in this area have already been done and they showed a significant improvement in the sensitivity of the serological test [46–49].

In summary, the recombinant antigens tested individually in this study in human and dog sera, displayed different sensitivities for the serodiagnosis of VL, with a better performance in dogs. In fact, Lci13 displayed a sensitivity for the dog sera higher than the current field tests, which demonstrates the potential for this recombinant antigen to detect CVL on its own. The goal of identifying a single natural antigen valid for the diagnosis of both forms of the disease does not seem viable. Recently, the use of chimeric proteins based on multiple antigenic peptides for the diagnosis of the canine leishmaniasis has been tried with promising results. The performance seen with the protein mix tested here indicates that, with improvement, the inclusion of selected epitopes from these antigens into novel chimeric proteins could be a good option to obtain serological tests with higher sensitivity and which would simultaneously be effective for both species.

Acknowledgments

We thank members of Dr. O. P. de Melo Neto's laboratory for helpful discussions and support. We also would like to thank Dr. Marli T. Cordeiro for the human sera used as negative control, Dr. Maria Edileuza F. Brito for the sera from patients with cutaneous leishmaniasis and Dr. Yara M. Gomes for the sera from chagasic patients. The authors thank the Program for

Technical Development of Health Inputs-PDTIS-FIOCRUZ for the use of its facility, the automatic sequencing facility RPT01C, at the Fiocruz-PE.

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5 ARTIGO 2

Caracterização *in silico* de múltiplos genes codificadores da proteína de virulência gp63 de *Leishmania braziliensis*: identificação de fontes de variação e prováveis ações na evasão imune

A gp63, principal proteína de virulência de *Leishmania* sp., tem sido amplamente estudada quanto a suas funções de protease para o escape do sistema imune, no hospedeiro vertebrado, e para a manutenção do parasita, no hospedeiro invertebrado (SANTOS et al. 2006; OLIVIER et al. 2012). Contudo, apesar de anos de estudo, pouco se sabe sobre a base molecular do surgimento dos múltiplos genes de gp63 presentes em diferentes espécies de *Leishmania* e em especial qual a razão do seu grande número de cópias encontradas no subgênero *Viannia*, em comparação às espécies do subgênero *Leishmania*. Momentos de expressão gênica, variação de sequência e sua provável influência na relação parasita hospedeiro são então todas questões levantadas e que nortearam este estudo.

Primeiramente, observamos em bancos de dados que de fato existe uma grande variação na quantidade de genes codificantes de gp63 entre as sequências depositadas pertencentes a espécies dos dois principais subgêneros de *Leishmania* (*Viannia* e *Leishmania*). Enquanto que 39 genes foram anotados para a *L.* (*V.*) *braziliensis*, apenas seis e oito genes foram identificados em *L.* (*L.*) *major* e *L.* (*L.*) *infantum*, respectivamente. A partir desses dados, buscamos investigar a importância dessa quantidade de genes para a célula de *Leishmania* e suas possíveis implicações na interação com o sistema imune.

O próximo passo foi realizar uma busca por possíveis parálogos ainda não anotados no genoma de *L. braziliensis*. Para isso, realizamos uma busca através da metodologia de modelos ocultos de Markov (*Hidden Markov Models* - HMMs), criados a partir de grupos de ortólogos de diferentes espécies de *Leishmania*. Nesta análise foram utilizados os dados da cepa 2904, que é a cepa descrita com a maior quantidade de genes de gp63 e melhor qualidade do genoma anotado. Após a busca, não foram encontrados novos parálogos de gp63 nos bancos de dados e todas as sequências de gp63 anotadas foram identificadas, com exceção de um único gene que não parecer realmente ser de gp63. Ainda com o objetivo de identificar o maior número de genes de gp63 possível, decidimos realizar amplificações desses genes utilizando oligonucleotideos construídos para regiões conservadas em suas sequências. Após amplificação, clonagem e sequenciamento, foram obtidos da *L. braziliensis* 2904 um total de 40 fragmentos de genes de gp63 diferentes. Desses 32 se mostraram distintos dos descritos nos bancos de dados, ressaltando uma diversidade ainda maior de genes de gp63 nessa espécie do que a predita apenas baseado nos dados genômicos.

Para esclarecer a relação entre os múltiplos genes gp63 em *Leishmania*, optamos então por rever a sua organização cromossômica nas suas principais linhagens patogênicas e novamente tomando como base os dados genômicos de cepas de referência. Para *L. major*, que possui o melhor e mais estudado genoma entre as espécies de *Leishmania*, três conjuntos de genes de gp63 foram encontrados distribuídos nos cromossomos 10, 28 e 31, com uma organização genômica semelhante também foi observada para *L. infantum* e *L. mexicana*. Para *L. braziliensis*, contudo observou-se uma grande expansão dos genes no cromossomo 10, uma menor expansão daqueles encontrados no cromossomo 31

e, diferentemente das outras espécies, nenhum gene foi anotado no cromossomo 28. A grande diferença encontrada no número de genes pertencentes ao cromossomo 10, em relação ao cromossomo 31, é consistente com nossos dados de PCR. Em geral, esses resultados são consistentes com a expansão do número de genes de gp63 em *L. braziliensis* e em outras espécies pertencentes ao subgênero *Viannia*, direcionadas principalmente aos genes do cromossomo 10. É importante ressaltar que uma expansão semelhante foi observada nos genes do cromossomo 10 de *L. tarentolae*, um parasita de lagarto.

Em seguida buscamos entender um pouco mais sobre os grupos de genes relacionados aos cromossomos, 10, 28 e 31. Como os genes de gp63 estão presentes em outros tripanossomatídeos e cinetoplastídeos mais distantes evolutivamente, investigamos se existiria alguma relação entre eles e os genes de *Leishmania* e se alguma função pode ser inferida com base nos genes encontrados em cada organismo. Diferentes quantidades de genes de gp63 foram descritas variando ao longo das várias linhagens de cinetoplastideos e reforçando os múltiplos papéis que esta proteína possui, independentemente do organismo envolvido. Comparamos então, através de análise filogenética, as sequências mais divergentes e representativas dos três principais grupos de genes de gp63 de *Leishmania* (dos cromossomos 10, 28 e 31), com genes de organismos mais evolutivamente distantes. Esta análise pôde demonstrar a clara divergência de genes de gp63 mapeados no cromossomo 10 de *Leishmania*, dos genes mapeados nos cromossomos 28 e 31. Também observamos que os genes nos cromossomos 28 e 31 da *Leishmania* sp. provavelmente surgiram antes dos cinetoplastídeos colonizarem vertebrados e podem não estar envolvidos na sua patogênese neles. O fato de que alguns genes de *L. pyrrhocoris* e *C. fasciculata*, parasitas de insetos,

estão relacionados aos genes de gp63 no cromossomo 10 de *L. braziliensis*, pode ainda indicar a existência de genes que tenham atividades relacionadas às ações dessas proteínas no inseto. Avaliamos também a multiplicação dos genes ocorrida no cromossomo 10 em *Leishmania*, investigando se este era um evento independente, dentro de cada espécie ou linhagem e evolutivamente mais recente, ou se ocorreu antes da separação destas linhagens. A análise demonstrou uma expansão independente do número de genes de gp63 em diferentes espécies de *Leishmania*, especialmente dos genes presentes no cromossomo 10 de *L. braziliensis*.

Após essas análises, decidimos nos aprofundar no estudo de caracterização dos genes do cromossomo 10 de *L. braziliensis.* A partir de dados da literatura e de comparação entre sequências na extremidade C-terminal das proteínas e das regiões 3' não traduzidas (3' UTR) preditas dos seus mRNAs, foi possível correlacionar alguns dados de expressão para algumas das sequências e desta forma agrupá-las em classes distintas. Para *L. major* e *L. infantum* três grupos foram identificados no total, com possíveis diferenças de expressão e diferenças na sua região C-terminal compatíveis com alterações funcionais ligadas a sua localização na membrana ou secreção. Em *L. braziliensis* esta análise revelou seis grupos de genes de gp63 no cromossomo 10.

Em seguida, direcionamos nosso estudo a entender os motivos da expansão dos genes de gp63 encontrados no cromossomo 10 de *L. braziliensis*, através de pesquisa de eventos de recombinação. Nestas análises, observou-se que os genes de gp63 presentes no cromossomo 31, utilizados como parâmetro de comparação, não apresentaram eventos de recombinação detectáveis. Já em relação aos genes do cromossomo 10 (obtidos por PCR e presentes nas bases de dados), de um total

de 67 sequências analisadas, 40 deles (60% do total) foram relatados como genes recombinantes. A maioria dos eventos de recombinação ocorre na região do terminal N e/ou C-terminal do gene com apenas um único caso de recombinação na área central da proteína.

Investigamos, então, se as variações encontradas nas diferentes gp63 codificadas no cromossomo 10 eram suficientes para alterar significativamente a estrutura da proteína. Para isso foi feita a modelagem de sequências selecionadas com base na estrutura descrita para uma gp63 da membrana de células promastigota de *L. major*. A partir das proteínas modeladas demonstramos que algumas tinham estruturas muito similares entre si e outras eram mais divergentes. Mapeamos em seguida as regiões variáveis identificadas pelos alinhamentos múltiplos anteriores. Esta análise mostrou que a maioria das regiões variáveis se posicionavam externamente nas estruturas proteicas.

Como último ponto de investigação, buscamos avaliar se as regiões variáveis nas proteínas estariam envolvidas na sua interação com células B do sistema imunológico e seriam potencialmente capazes de gerar anticorpos. Assim, realizamos predições de epítopos de células B lineares e conformacionais das proteínas modeladas. De uma forma geral a análise revelou que boa parte dos epítopos foram mapeados para as regiões externas das proteínas e coincidiam com motivos que exibem variação de sequência. Este conjunto de resultados, relatados de forma mais detalhada no manuscrito a seguir, em fase final de redação para publicação, constituem uma contribuição significativa no entendimento da diversidade dos genes de gp63 em *Leishmania*, e geram pontos a se investigar que dizem respeito ao papel desta proteína ao longo do ciclo de vida do parasita e na sua patogenia.

IN SILICO CHARACTERIZATION OF MULTIPLE GENES ENCODING THE GP63 VIRULENCE PROTEIN FROM *LEISHMANIA BRAZILIENSIS*: IDENTIFICATION OF SOURCES OF VARIATION AND PUTATIVE ROLES IN IMMUNE EVASION.

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Abstract

The leishmaniasis are parasitic diseases caused by protozoans of the genus *Leishmania*. The parasites of this genus produce proteases that aid the *Leishmania* against the immune response from their host during early stages of infection. The main representative protein from this group is gp63, a membrane protein able to evade actions of the host immune system that would lead to destruction of *Leishmania*. Concerning their gene organization, there is a noticeable difference in the number of *gp63* genes throughout the *Leishmania* species. This study sought to identify and characterize gp63 orthologs and paralogs *in silico*, evaluating their differences and similarities and the implications for the protein's function. Through our analysis we could identify a large number of gp63 gene paralogs in *Leishmania* and related species, categorized into distinct groups and localized on chromosomes 10, 28 and 31. The genes of the latter two groups appear to be more related to genes found in insect or plant parasites, whereas those in chromosome 10 have experienced an expansion in the number of paralogs in *Leishmania* and especially in *L. braziliensis*. These were better studied and presented distinct expression moments, independent expansion in evolution and intragenic recombination events. Sequence variations and regions predicted to bind antibodies were mapped on the external surface of the proteins. These data suggest a relevant role for the antigenic variation of gp63 in the parasite biology and in the pathogenesis of the disease.

Key Words: Leishmania braziliensis, Gp63, virulence proteins

Introduction

The leishmaniasis are parasitic infectious diseases caused by flagellated protozoa of the genus *Leishmania* and which are transmitted by sandflies of the *Phlebotomus* or *Lutzomyia* genera (David and Craft, 2009). These diseases are found as three main clinical forms, named as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (CML) and visceral leishmaniasis (VL) or kala-azar (Salotra et al., 2003; Selvapandiyan et al., 2012), with an annual incidence of 0.7 to 1.2 and 0.2 to 0.4 million new cases for CL and VL, respectively (Alvar et al., 2012). The *Leishmania* species have developed effective mechanisms to escape the mammalian host immune response during the early stages of infection. Some of these evasion mechanisms are made by proteases, which help ensure that the parasite can invade the mammalian tissue, survive, proliferate and differentiate (Silva-Almeida et al., 2012).

The gp63 protease was first discovered in 1980, when it was described as the major surface antigen of the promastigote form in many species of *Leishmania* and later it was identified as an important virulence factor (Matlashewski, 2001; Olivier et al., 2012). This is a zinc-dependent metalloproteinase, which belongs to the metzincin class and the peptidase family M8. This class of proteins include features such as the conserved motif HEXXHXXGXXH, a GPI anchor and a pro-peptide located in the protein's N-terminal region that renders the proenzyme inactive during translation and is removed during its maturation and activation (Yao et al., 2003). The sequence that encodes the pro-peptide contains a cysteine residue conserved in the gp63 across the different *Leishmania* species. This residue has been shown to contribute to a cysteine switch mechanism of protease activity, that aids the zinc atom to bind at the active site and inhibit enzyme activity, which

protects the cell from self-destruction (Macdonald et al., 1995). In addition to the mentioned characteristics, the gp63 proteins also contain a signal sequence at their N-terminus, which directs them to the endoplasmic reticulum after its synthesis and to the *Leishmania* secretory pathway (Yao et al., 2002).

In mammals, within the extracellular environment, gp63 acts in the inactivation of the complement cascade, by inhibiting the factor C3b. This inactivation will prevent the formation of the membrane attack complex (MAC) and will opsonize the *Leishmania*, facilitating its phagocytosis, besides it will inhibit the IL-12 production, which will favour cell growth (Gómez and Olivier, 2010; Gupta et al., 2014). Studies have also shown that gp63 can facilitate the binding of the parasite to the macrophage through fibronectin receptors, cleaving proteins from the host's extracellular matrix, and providing *Leishmania* with a faster entry into the macrophage through the activation of a host tyrosine phosphatase (Olivier et al., 2012; Shio and Olivier, 2010). It has also been observed that lack of gp63 drastically reduces the *Leishmania*'s ability to establish and maintain an infection, since the hosts are more likely to induce an innate immunity inflammatory responses (Olivier and Hassani, 2010). Gp63 also acts to reduce the production of TNF, IL-12 and nitric oxide, which contributes to the protection and survival of the parasite (Podinovskaia and Descoteaux, 2015).

Gp63 has also been shown to be released through exosomes into the extracellular medium and this may facilitate its uptake by the macrophage, even before it internalizes the *Leishmania*, generating internal cellular inhibitory changes. In fact, macrophage studies have shown that gp63 can cleave the transcriptional factor AP-1, which regulates the production of pro-inflammatory cytokines and nitric oxide (Atayde et al., 2016; Contreras et al., 2010). This protein cleavage action, was

also shown in another study where gp63 was associated with the inactivation of the mTOR kinase, an enzyme required for the phosphorylation of the 4E-BP1 inhibitory protein, a regulator of translation that binds to the cap binding protein, eIF4E, and prevents its recruitment of the translation machinery. When mTOR is cleaved, it does not phosphorylate 4E-BP1 and this lack of 4E-BP1 phosphorylation prevents it from blocking the formation of a functional eIF4F translation initiation complex. This gp63 action allows the inhibition of protein synthesis in the macrophage, providing an ideal environment for the proliferation of the pathogen (Jaramillo et al., 2011).

In addition to the immune evasion in the mammalian hosts, the gp63 also perform functions in the insect vector. A potential involvement in the degradation of protein components that would lead to the adhesion of the parasite in the insect gut epithelium has been shown (D'Avila-Levy et al., 2006; Pereira et al., 2009). Due to the gp63 wide substrate specificity, it can also perform a nutritional role for the parasite, acting as an endopeptidase (D'Avila-Levy et al., 2014; Santos et al., 2006) or even protecting the *Leishmania* against insect defences (D'Avila-Levy et al., 2014).

Concerning the gp63 gene organization, there is a noticeable variation in the number of gene copies encoding these proteins among different *Leishmania* species. In *L, major* these genes are present in more than one chromosome and multiple copies have been detected arranged in tandem (Ivens et al., 2005) and the same multi-copy arrangement was found in the *L. infantum* and *L. braziliensis* genomes (Peacock et al., 2007). Indeed, in early hybridization studies a remarkable difference in gene numbers have been reported between species belonging to the two major *Leishmania* subgenus, *Leishmania* and *Viannia* (Steinkraus et al., 1993;

Victoir et al., 1995; Voth et al., 1998). This was confirmed by recent sequencing performed with *Leishmania* belonging to the *Viannia* subgenus (Llanes et al., 2015), although there is no consensus to the number of genes among them. Regarding the expression of these genes, it was observed that some clusters of gp63 are differently regulated during the parasite life cycle stages (amastigote, logarithmic promastigote, and stationary stage) in *L. donovani*, *L. mexicana* e *L. major* (Yao et al., 2003). The regulation of the differential expression of these genes does not seem to be related to the organization of these genes in tandem. Studies have shown that the determining factor for this regulation are characteristic sequences present in the intragenic regions and 3 'UTR of the genes (Kelly et al., 2001; Yao et al., 2003).

Despite all scientific data and studies regarding the gp63, little is known about the need for a high copy number of the genes encoding these proteins in *L. braziliensis*. Therefore, to assess the importance of this group of proteins in the parasite-host interaction and generate a greater understanding about the biology of the parasite, as there is a noticeable difference in the number of genes encoding these proteins throughout the *Leishmania* species, this study sought to identify and characterize in *L. braziliensis* orthologs and paralogs of gp63 *in silico* and through genes obtained by PCR assays, evaluating their differences and similarities and the implications for the protein's function.

Methods

Parasites and culture conditions

In this study, we used *Leishmania (Viannia) braziliensis*

(MHOM/BR/75/M2904) in the promastigote form. The cells were cultured at 26º C in Schneider (Sigma) pH 7.2 supplemented with 20% fetal bovine serum (FBS), antibiotics (Streptomycin / Penicillin 0.1%) and 0.1% Hemin.

PCR, cloning and sequencing

Approximately, 10^8 *L. braziliensis* promastigotes were used for total genomic DNA extraction using DNAzol (Invitrogen) and standard procedures. PCR reactions for the amplification of the gp63 sequences were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs), following the manufacturer's protocol and with oligonucleotides used as primers listed in the Supplementary Table 1. After amplification, cloning and sequencing of the PCR products, a nomenclature was created for the newly generated sequences in order to identify from which set of primers they were derived, whether from those encoding the KDELMAP or GPI region and defining which gp63 gene it most closely resembles and to be used in other analysis.

Search for new gp63 paralogs through Hidden Markov Models

First, the predicted proteomes of the following organisms were downloaded from TritrypDB in August 25, 2014: *L. braziliensis* strain 2903 [taxid: 1295825], *L. braziliensis* strain 2904 [taxid: 420245], *L. infantum* [taxid: 435258]*, L. major* [taxid: 347515]*, L. donovani* [taxid: 981087]*, L. mexicana* [taxid: 929439] and *L. tarentolae* [taxid: 5689]. For this analysis, only genes annotated as gp63, with more than 30 amino acids and no more than one stop codon per sequence were considered.

All of the protein sequences derived from genes that met the inclusion criteria described earlier were submitted to the analysis of the OrthoMCL program (Li et al.,

2003), which uses the MCL (Markov Cluster) algorithm to create groups of homologous proteins based on sequence similarity (Enright et al., 2002). The protein sequences included in each group were used as input for a multiple alignment by the MAFFT software (default settings) (Katoh et al., 2002). The proteins which displayed a low alignment quality were excluded from the analysis. Finally, a multiple alignment of each group was used as input to hmmbuild, a tool from the HMMER package, version 3.0 (Eddy, 1998). This tool was used to build Hidden Markov Models (HMMs) based on the multiple alignments. The HMMs obtained were used to search for new paralogs within the *L. braziliensis* strain 2904 proteome utilizing the hmmsearch tool (part of the HMMER package). A cutoff of 0.001 for hit significance (e-value < = 0.001) was applied.

Gp63 phylogenetic analysis and detection of recombination events

A phylogenetic tree was built from all gp63 protein sequences used in the previous steps, the ones obtained by PCR and gp63 sequences from *Leishmania guyanensis* [taxid: 5670] *Leishmania panamensis* [taxid: 5679], *Bodo saltans* [taxid: 75058], *Trypanossoma cruzi* [taxid: 353153] and *Trypanossoma brucei* [taxid: 185431], that started with a methionine and had no stop codons in the middle of their sequence, were selected and aligned by MAFFT (default settings). The alignment was automatically edited by Trimal (Capella-Gutiérrez et al., 2009) to keep just phylogenetically informative sites. The tool called ProtTest (Abascal et al., 2005) was used to predict the best evolutionary model which was then used as a setting to build the phylogenetic tree with PhyML tool, applying the Maximum Likelihood method (Guindon et al., 2010). The branch support for each tree was given by non-parametric bootstrap analysis using 1000 replicates. After analysis in

196 the tree results, we attempted to make an inference of the gp63 gene expression
197 profile, using 500 pb of the 3 'UTR regions of the gp63 genes of *L. major, L. infantum*
198 *and L. braziliensis*. The UTRs were aligned, analyzed and used for the construction
199 of phylogenetic trees in the same parameters as mentioned above. Together with
200 the UTRS analysis, the C-terminal region of the same genes were also analyzed.
201 To try to identify some common or differentiated function between the genes on
202 chromosome 10, 28 and 31, we constructed a phylogenetic tree with gp63
203 sequences of diverse *Leishmania* species and other species of Kinetoplastidae
204 protozoans, like Phytomonas sp. isolate Hart11 [taxid: 134014] *Crithidia fasciculata*
205 [taxid: 5656] and *Leptomonas pyrrhocoris* [taxid: 157538]. The aligned nucleotide
206 sequences from *L. braziliensis,* obtained by the TriTryp database and PCR assays,
207 were analyzed for recombination in the RDP4 program (Martin et al., 2015).
208
209 **Modelling of gp63 homologs and searches for non-conserved regions**
210 Based on multiple alignments and the groups of proteins found by the
211 phylogenetic tree analysis, the sequences of the 10 most divergent gp63 homologs
212 were chosen for the three-dimensional modelling. The modelling was performed for
213 the amino acid sequences previously obtained from *TritrypDB* using a threading
214 method implemented by the I-TASSER (Roy et al., 2011) and Phyre2 (Kelly et al.,
215 2015) tools. When the models were completed, the results were compared
216 assessing their qualities through Procheck (Laskowski et al., 1996). Only the models
217 with the best scores were chosen to be used in subsequent assessments. Specific
218 regions of the protein models were then evaluated using the initial alignment
219 information, highlighting the non-conserved regions which were characterized by
220 amino acid exchanging or by indels.

B-cell epitope prediction

Linear B-cell epitope predictions were performed for the protein sequences used in the 3D modeling step. The predictions were carried out using the following programs: AAP12 (Chen et al., 2007), BCPred12 (EL-Manzalawy et al., 2008) and BepiPred (Larsen et al., 2006). Only epitopes predicted by at least two programs, with lengths equal to or greater than 10 amino acids and with scores greater than 0.8 were considered as positive predictions on AAP12 and BCpred12, and epitopes with scores over 0.5 obtained by BepiPred were included in the analysis.

In addition to the linear prediction, a conformational prediction of epitopes was also performed to evaluate if the protein structures were also able to generate interaction with the immune system. The conformational epitopes were predicted by the CBTOPE web server (Ansari and Raghava, 2010), where only epitopes that had more than 10 amino acids and had a score above 4 were considered for this study. After the prediction, an assessment was performed to map the localization of all the epitopes on the modeled proteins (internal or external).

Results

Search for new *L. braziliensis* gp63 paralogs

Early studies based on hybridization assays (Llanes et al., 2015; Steinkraus et al., 1993; Victoir et al., 1995) have suggested that the total number of gp63 genes found within *Leishmania* species belonging to the *Viannia* subgenus is greater than the number of genes available at the TriTrypDB database and identified after the *L. braziliensis* genome sequencing and annotation. Here, to begin to understand the true diversity of the *L. braziliensis* gp63 genes, we first sought to reevaluate the

246 available *L. braziliensis* gp63 gene sequences considering that the automatic
247 annotation methods might have missed further genes. We therefore performed a
248 reanalysis of the *L. braziliensis* genome sequences and searched for possible new
249 gp63 paralogs that might not have been annotated. To do this we performed a
250 search in the *L. braziliensis* genome using the Hidden Markov Models (HMMs)
251 methodology, carried out after a grouping of the entire proteome set from different
252 *Leishmania* species (described in methods). Nine subsets of gp63 sequences were
253 created, as shown in Supplementary Table 2, with the number of genes in each
254 subset varying in size from 56 to only two. Despite this difference in size, all nine
255 subsets were used to build HMMs and these were then applied for the search of
256 new paralogs in the predicted proteome from *L. braziliensis* 2904. In general, all
257 HMMs were able to find the gp63 sequences assigned to each subset, however no
258 new paralogs were found during the search. The genome of *L. braziliensis* strain
259 2904 deposited on Tritrypdb lists 39 gp63 genes and, in total, the HMMs identified
260 the presence of 38 related sequences. A single gene (LbrM.10.1720) was not
261 recovered using these models and indeed its coding sequence did not provide an
262 alignment with a score high enough to be considered as a gp63. The results of the
263 search for each HMM are summarized in Table 1 and confirm the lower number of
264 gp63 genes derived from the *L. braziliensis* genome sequencing.

265 Next, we considered that the shot gun nature of the sequencing strategy used
266 for the assembly of the best genome available from a *Viannia* species, from the *L.*
267 *braziliensis* 2904, might have led to the grouping of similar gp63 genes together,
268 causing in turn a reduction in the number of genes found. In order to obtain as many
269 natural gp63 sequences as possible, and therefore have a clearer idea of the true
270 number of genes present in the *L. braziliensis* genome, we opted to amplify these

genes using primers directed to conserved regions of representative genes identified in the genome analysis. The PCR strategy used to amplify the gp63 genes can be seen in Figure 1, superimposed on a schematic representation of a typical gp63. The scheme highlights conserved elements found on all gp63 sequences, such as the zinc-binding motif, multiple cysteine residues, the GPI anchor site and a nearly universally conserved motif of seven consecutive amino acids we named KDELMAP. Six oligonucleotides annealing to sequences encoding the N-terminal ends of the gp63 sequences were used as 5' primers, considering the variation previously observed within the N-terminus of the various gp63 genes and in order to maximize the number of genes amplified. As 3' primers, two sets of two oligonucleotides annealing to the more conserved KDELMAP or the GPI anchor site motifs were alternatively used. After amplification, cloning and sequencing a total of 40 different gp63 gene fragments were obtained, with thirty-two of those distinct from the ones described in the databases. The new gp63 obtained by PCR are listed in Supplementary Table 3, which also includes the set of oligonucleotides used to amplify each sequence. The new sequences have all been submitted to GenBank and were compared with the already known gp63 genes from *L. braziliensis* and these analyses will be discussed further below. Nevertheless, they are consistent with a higher copy number for the *L. braziliensis* gp63 genes than predicted based on the genome sequencing alone and more in agreements with the original estimates based on Southern-blots.

Genomic analysis of known *Leishmania* gp63 genes

To clarify the relationship between the multiple gp63 genes in *Leishmania*, we opted to review their chromosomal organization within the major lineages of

296 pathogenic *Leishmania*. For *L. major*, the best studied of the available *Leishmania*
297 genome sequences, three sets of gp63 genes were found distributed in
298 chromosomes 28 (one gene), 31 (one gene) and 10 (four genes), (Ivens et al.,
299 2005). Based on the sequences available at the Tritryp database, a similar
300 organization is also observed for *L. infantum* (summarized in Figure 2) and *L.*
301 *mexicana* (not shown), represented by two and one genes for chromosome 28 in *L.*
302 *infantum* and *L. mexicana*, respectively, and one gene gene for chromosomes 31 in
303 both species, considering that the *L. mexicana* chromosome 30 is equivalent to the
304 *L. major* chromosome 31. Five gp63 genes are also found in chromosome 10 for
305 both species and in agreement with previously reported data for *L. infantum*
306 (Peacock et al., 2007).

307 In *L. braziliensis*, based on the available genomic data for the 2904 strain,
308 major differences in the organization of the gp63 genes are observed when they are
309 compared with those found in species from the *Leishmania* subgenus. First, no gp63
310 gene is found on chromosome 28 despite the presence of orthologues to the same
311 genes flanking the single gp63 sequence from *L. major* and *L. infantum*. In contrast,
312 six gp63 genes or gene fragments are found on chromosome 31, again generally
313 flanked by orthologues to the same genes found flanking the gp63 gene found in
314 the *L. major* and *L. infantum* chromosome 31. Even more noteworthy, however, are
315 the 33 gp63 genes found clustered on chromosome 10. Again these are localized
316 to the same region seen harboring the other *Leishmania* chromosome 10 genes, as
317 confirmed by the presence of neighboring sequences encoding orthologues to those
318 found flanking the *L. major* and *L. infantum* gp63 genes from chromosome 10.
319 However, the precise gene organization cannot be properly defined and many of the
320 genes sequenced are assembled in relatively short contigs, as indicated in the

scheme from Figure 2C. Again, this might be due to the high similarity between the gene sequences and the nature of the sequencing strategy which might have prevented a proper assembly of repeated sequences.

The significantly greater number of *L. braziliensis* gp63 genes from chromosome 10 is supported by our PCR data where primers sets directed to the chromosome 10 genes were able to amplify more genes than the ones originally used for their synthesis. For example, a primer pair designed to amplify the gene LbrM.10.0470 allowed the amplification of eight different gene fragments (G0510B2; G0560B1; G0560B2; G1610B3; G1610B4; G1610B5; G1610B6; G1620B1) and similarly, the primer pair directed to gene LbrM.10.0540 amplified fragments from six different genes (G0510C1; G0510C2; G0540C1; G0560C4; G1640C1; G1640C2). In contrast, two sets of PCR reactions directed to a single gp63 gene from chromosome 31, using the same 5' primer and two distinct 3' primers, only led to the amplification of the same gene, LbrM.31.2260 (Supplementary Table 3). Overall these results are consistent with the expansion in the number of gp63 genes in *L. braziliensis* and other species belonging to the *Viannia* subgenus being mainly directed to the chromosome 10 genes, with a more reduced expansion in chromosome 31 also observed.

Gp63 evolutionary analyses

Sequences encoding gp63 related genes are also found in other trypanosomatids and more distantly related kinetoplastids and these include multiple genes from *T. brucei*, *T. cruzi*, and others. The number of genes in these parasites is quite variable. *T. cruzi* has over 150 gp63 genes annotated in the TriTrypDB database, but with many pseudogenes among them. *T. brucei* and *C.*

fasciculata have a smaller amount with 10 and 18 genes respectively. This multiplicity of gp63 genes along the various kinetoplastids lineages reinforce the multiple roles this protein has, independent of the life cycle of the organism involved. Here, we next sought to assess how the *Leishmania* gp63 genes are related to those found in more distantly related kinetoplastids and whether some function can be inferred based on which genes are found in each organism. To do this we built a phylogenetic tree comparing the most divergent and representative sequences from the three major sets of *Leishmania* gp63 genes (from chromosomes 10, 28 and 31) with genes from different *Trypanosoma* species (*T. brucei*, *T. cruzi* and *T. theileri*) and more distantly related organisms. These included species that parasitize reptiles (*L. tarentolae*) and plants (*Phytomonas sp.*), have monoxenous life-cycles in insects (*Crithidia fasciculata* and *Leptomonas pyrrhocoris*) and are free living (*Bodo saltans*). As shown in Figure 3, the phylogenetic analysis could separate the gp63 genes mapped to the *Leishmania* chromosome 10 from those genes mapped to chromosomes 28 and 31. We also could observe a clear separation between the *Leishmania* subgenus based on the genes located on chromosome 10. The *T. cruzi* and *T. brucei* gp63 sequences were more closely associated with the gp63 genes from *B. saltans* and *T. theileri*. When we observe the clustering of the genes present in chromosomes 28 and 31 from *Leishmania*, they show a close relation to the genes from *L. pyrrhocoris, C. fasciculata* and *Phytomonas sp*. Nevertheless, one *L. pyrrhocoris* and two *C. fasciculata* genes are more closely related to those from the *L. braziliensis* chromosome 10.

Overall, the gene clusters shown in the tree highlight the higher similarity between the *Leishmania sp.* genes from chromosomes 28 and 31 with the gp63 genes found in organisms that live in insects only or parasitize plants. Indeed, the

number of gp63 genes from *Phytomonas*, related to other *Leishmania* chromosome 28 gp63, is greater than any other vertebrates kinetoplastid genome sequenced so far, with 38 annotated genes. It is then possible to hypothesize that these genes might me more involved in the insect stage of the parasite life cycle. Genes more closely related to the chromosome 10 gp63 genes can be found in the insect parasites *L. pyrrhocoris* and *C. fasciculate*, but in general these genes seem to have suffered a substantial expansion within the *Leishmania* lineage and may be more associated with the vertebrate pathogenesis.

Evaluation of the sequence diversity of the *Leishmania* gp63 genes from chromosome 10

Considering the expansion of the chromosome 10 gp63 genes in the *Leishmania* lineage in general, and even more so in *L. braziliensis* and other *Viannia* species, we then opted to investigate the origins of their diversity further. To do this we compared the full extent of the chromosome 10 gp63 sequences from relevant *Leishmania* species using as outgroups selected genes from chromosomes 28 and 31. For this analysis, we also included sequences from *L. tarentolae*, where a similar expansion in the chromosome 10 gp63 genes was noticed, with 49 genes found in this chromosome while only one gene was found in chromosomes 31 and another in chromosome 33. *L. tarentolae* is currently classified within the *Sauroleishmania* subgenus, but it is likely to be more closely related to the *Leishmania* subgenus than to *Viannia* (Akhoundi et al., 2016; Maurício, 2018). The relevance in including the *L. tarentolae* sequences is due to the fact that it does not parasitize mammals, only lizards, meaning that any potential role in pathogenesis associated with the chromosome 10 gp63 genes is not dependent on their mammalian hosts. We also

included in this analysis the new *L. braziliensis* sequences generated by us through the PCR approach. The phylogenetic tree shown in Figure 4 summarizes the results from these analyses based on alignments using the full length sequences for all proteins (or the full length PCR fragments). For clarity, only the most divergent representative sequences were used to build this tree, with those very similar or nearly identical to the ones shown purportedly removed from the final figure. In the original analysis all chromosome 10 sequences from the selected species were used but with similar results (not shown). Within each of the three *Leishmania* subgenera analysed, all gp63 sequences from chromosome 10 are more closely related to sequences from the same or related species than to sequences found in species belonging to the other subgenera. Even within the *Leishmania* clades, the *L. infantum* genes seemed to be more closely related to each other than to their *L. major* counterparts, although for the two *Viannia* species analysed (*L. braziliensis* and *L. guyanensis*) genes more closely related between the two species were found. These results are in agreement with independent expansions on the number of the chromosome 10 gp63 sequences in each clade, with major expansions occurring for both *Sauroleishmania* and *Viannia* species. For the latter species, at least, the start of this expansion may have preceded the split between *L. braziliensis* and *L. guyanensis* but has subsequently continued and may be an ongoing process. The *Leishmania braziliensis* chromosome 31 has 6 gp63 genes annotated, unlike *L. major* and *L. infantum* chromosomes 31, which has only one gene. We believe that this number of *L. braziliensis* gp63 genes may be related to possible genome assembly errors. From the 6 studied genes, only one (LbrM.31.2260) has gp63-related protein features such as the propeptide domain (HEXXH), and high similarity (85%) with *L. major* and *L. infantum* chromosome 31 genes. The LbrM.31.2230,

LbrM.31.2240 and LbrM.31.2250 genes presented stop codons in the middle of their sequences and in alignments showed identical N-terminal or C-terminal regions from LbrM.31.2260, which may indicate that these genes would be parts of LbrM.31.2260 that were not assembled correctly (data not shown). This hypothesis is consistent with our PCR data, which found only the LbrM.31.2260 gene, unlike the reactions performed for genes on chromosome 10, which showed that a pair of oligos was able to detect more than one gene.

Identification of functional differences between the various *Leishmania* gp63 genes from chromosome 10

So far not much is known regarding possible functional differences between the various gp63 genes found within any specific trypanosomatid, and even less so regarding the reasons for the multiple gene copies identified in different organisms. In *Leishmania*, with the goal of defining functional differences between multiple gp63, and even prior to the completion of the first *Leishmania* genomes, early studies investigated the expression pattern of selected genes attempting to identify differences in expression during the parasite life cell cycle (Mccoy et al., 1998; Ramamoorthy et al., 1992; Yao et al., 2003). The more detailed analysis, using *L. infantum* promastigotes, found three growth stage-specific patterns of expression for the then known gp63 genes, with one gene constitutively expressed, a second gene expressed during the log phase of growth and the third gene expressed only in stationary phase cells Ramamoorthy et al., 1992. Here, by comparing their 3' UTRs with the 3' intergenic regions from the available *L. infantum* genome, we were able to map those genes within chromosome 10 (indicated in Figure 2). LinJ.10.0510 gene is the constitutively expressed gene, characterized by a unique

3’ UTR and 3’ intergenic region that is absent from the remaining *L. infantum* chromosome 10 genes. Two other neighbouring genes, LinJ.10.0490 and LinJ.10.0500, are equivalent to the previously described log phase gene, while both LinJ.10.0520 and LinJ.10.0530 correspond to the stationary phase one. These four genes have nearly identical sequences within the first ~400 nucleotides of their 3’UTRs but these subsequently diverge into two distinct patterns that correlate with the previously published log or stationary phase profiles. The five *L. infantum* chromosome 10 genes can then be divided into three groups according to both 3’UTR and expression profiles with presumably functional differences that yet need to be defined: group 1 - LinJ.10.0510; group 2 - LinJ.10.0490 and LinJ.10.0500; group 3 - LinJ.10.0520 and LinJ.10.0530. We also investigated conserved features within their *L. major* counterparts and found that LmjF.10.0470 has a 3’UTR very similar to the *L. infantum* LinJ.10.0510, while LmjF.10.0460 and LmjF.10.0465 have UTRs more similar to the group 2 genes and LmjF.10.0480 is the sole representative of the 3’UTR from group 3. These genes and their grouping are highlighted by different shades of gray in the scheme from Figure 2, with the same shade of gray indicating genes belonging to the same group. Their conservation implies also a conservation of function between different *Leishmania* species, at least those belonging to the subgenus *Leishmania*.

Next, we attempted to identify conserved and divergent elements within the 3’UTRs available for the *L. braziliensis* chromosome 10 genes, the main focus of this study. Thirty genes were analyzed based on the sequences available from the reference genome sequence and these were classified in six groups according to their 3’UTR, with the first two groups represented by 8 and 15 genes, respectively, while the remaining groups included only one or two genes. We then tried to

compare the various *L. braziliensis* groups with the three different groups identified in *L. infantum* and *L. major* but no clear correlation was possible, possibly due to a large sequence variation between species from the two distinct subgenus.

We opted then to try to find protein features within the chromosome 10 gp63 genes that could be correlated with the 3'UTR groups from all three species. Searches for features such as signal peptide, transmembrane domains and isoelectric points were carried out but we also could not find any correlation between the clustered UTRs and these features. For the *L. infantum* and *L. major* proteins we did find a clear distinction between the C-terminus of the group 1 proteins with those from group 2 and 3, with a near complete lack of homology between these proteins starting near the GPI anchor attachment site. Indeed the longer group 1 C-terminus is absent from the remaining proteins and include several tryptophan, proline, cysteine and lysine residues conserved in both *L. major* and *L. infantum* proteins. In contrast the shorter C-terminus from the group 2 and 3 proteins all end in a 12 residue stretch of mostly hydrophobic residues consisting only of leucine, valine and alanine residues (Figure 5). We then compared the C-terminus from the chromosome 10 gp63 sequences from *L. braziliensis* and could separate the C-terminal regions into six groups (Table 3) related to their sequence similarity. A clear correlation between each 3'UTR group and the C-terminal ends of the proteins is seen, perhaps with the exception of the LbrM.10.0550 gene, but groups 4, 5 and 6 consists of truncated proteins and will not be considered further here. When the C-terminus of each of the remaining *L. braziliensis* group were compared with the *L. infantum* and *L. major* groups, a clear correlation between the two proteins from the *L. braziliensis* group 3 and the *L. major*/*L. infantum* group 1 can be seen, since they share nearly identical C-terminus. The group 1 genes from *L. braziliensis* also share

conserved elements with the *L. major*/*L. infantum* group 2, such as the motif "MRQWRERMTALATVT" found in the *L. braziliensis* sequences which is very similar to the "MQRWNDRMAGLATAA" motif found in *L. infantum* LinJ.10.0510 gene. Only the *L. braziliensis* group 2 genes, characterized by a shorter C-terminus, do not seem to have counterpart in *L. infantum* nor *L. major*. These correlations may indicate that, as observed in *L. infantum*, different groups of *L. braziliensis* chromosome 10 gp63 genes may also be differentially regulated during the parasite growth in culture and this agrees with the different 3'UTRs seen associated with each group.

Gene recombination in gp63 sequences from chromosome 10

Through analyzes of the alignments generated in this study, we identified that specific regions of certain gp63 gene sequences were very similar to equivalent regions from other gp63 genes which otherwise were more divergent. For example, certain small motifs generally seen only on the *L. braziliensis* group 1 genes were also found in one or more of the group 2 genes and vice-versa, an indication of gene recombination. Indeed, the locus for these genes is reported as having high plasticity (Victoir et al., 1995; Victoir and Dujardin, 2002) and the data from the literature shows that this gene family of can be influenced by mosaic or fragmental gene conversion (Mauricio et al., 2007). Here, in order to understand why the expansion of the gp63 genes occurs mainly on chromosome 10, we next performed an *in silico* search for recombination events targeting these genes so as to better evaluate whether their variability was related to intragenic recombination. The software chosen to find the recombination events (RDP4) uses several tools to determine events such as the likely position of recombination breakpoints and the

identity of sequences most closely related to the gene being evaluated. In this study we only considered recombination events that were detected by at least two of the tools tested. Therefore, we decided to perform a gene recombination analysis with all the gp63 genes of *L. braziliensis* present in databases and the ones generated by us through PCR. We first targeted the chromosome 31 gp63 genes, but no recombination events were detected by the software. In contrast, from a total of 67 gp63 sequences analyzed from chromosome 10, generated by PCR or retrieved from the *L. braziliensis* reference genome, 40 of them or 60% were reported as recombinant genes. As shown in Figure 6, most of recombination events occur in the N-terminal and/or C-terminal regions of the genes, with only a single case of recombination predicted to target the area coding the central part of the protein. Gene duplication and recombination events then are possibly the major source of the novel gp63 sequences seen in the chromosome 10 from *L. braziliensis* and closely related sequences.

Protein structural modeling, mapping of variable regions and B-cell epitope prediction

Based on the crystallized structure of a membrane gp63 from *L. major* promastigotes, gp63 was identified as a compact protein consisting predominantly of β sheet secondary structure elements divided into three distinct domains (N-terminus, Central domain and C-terminus) and with features typical of the catalytic modules of zinc proteases (Schlagenhauf et al., 1998). After observing the recombination events and sequence variations observed between the multitudes of *L. braziliensis* gp63 genes, we decided to investigate where these variations were seen along the 3D structure of the protein. Through three-dimensional protein

546 structure predictions using the threading method, we were able to predict the
547 structure of number of gp63 sequences with high modeling scores, as can be seen
548 in Figure 7. After a structural comparison between the generated models, we
549 mapped the most variable motifs identified by the previous multiple alignments.
550 These non-conserved motifs were also highlighted in blue in the structures from
551 Figure 7, where the motifs that showed sequence variations among the various
552 proteins are labeled in blue and the red markings display the predicted site for the
553 GPI anchor. As can be observed, most of the variable regions were positioned
554 externally on the structures.

555 We next sought to evaluate how the various gp63 sequence would be
556 recognized by the B cells from the mammallian immune system and also to predict
557 their ability to induce de production of specific antibodies. Linear and conformational
558 B-cell epitope predictions were carried out using the various chromosome 10 gp63
559 sequences from *L. braziliensis*. The linear epitope predictions returned 100 epitopes
560 from the sequences of the modeled proteins (Supplementary Table 3). Regarding
561 their localization within the various structures, from a total of 100 epitopes 81 were
562 mapped to the proteins' external regions, whereas 19 were localized internally. Out
563 of the 100 epitopes, 59 coincide with motifs that display sequence variation, while
564 22 are found in regions conserved between the different gp63 sequences.
565 Considering only the epitopes that were predicted to localize internally, 14 coincide
566 with variable sequences while 5 are associated with conserved regions. As for the
567 prediction of conformational B-cell epitopes, the analysis returned 54 epitopes, with
568 40 mapped to the proteins' external region. Twenty-seven of those were in motifs
569 with sequence variation, while 13 were in conserved regions. Regarding the 14
570 epitopes localized internally, eight coincided with variable sequence motifs and six

were in conserved motifs. We also identified 30 epitopes that were present in both linear and conformational predictions. Again, with most of the epitopes coincided with variable motifs localized externally, as shown in Table 4.

Discussion

Proteinases are important virulence factors due to their participation in biological processes associated with the start and maintenance of infection by pathogens. The gp63 is a major protease present on the surface of *Leishmania sp.* and other trypanosomes (Silva-Almeida et al., 2012). In this study, orthologs and paralogs of gp63 were identified and characterized in order to evaluate their differences and similarities and their implications for the protein's function. Due to the large number of gp63 genes in *L. braziliensis* 2904 found by our PCR and databases, and to achieve a more complete characterization of these genes, we carried out a search for possible new paralogs not yet annotated. The lack of new paralogs identified in our search is probably due to the shotgun nature of the *L. braziliensis* genome (Peacock et al., 2007).

Phylogenetic analysis with orthologs and paralogs of gp63 is consistent with other published data that demonstrate the phylogenetic relationship among the species of *Leishmania*, where genes from the subgenus *Viania*, *Leishmania* and *Sauroleishmania* are clustered in separate clades, regardless of the genes used to build the tree (Jackson, 2014; Ma et al., 2011; Schonian et al., 2013). The distribution of these genes on chromosome 10 shows their independent expansion throughout the *Leishmania* species, particularly among the *Sauroleishmania* and *Viannia* subgenera. Such expansion of multiple genes arranged in tandem, originated from duplication and recombination events, demonstrates the adaptability

596 of *Leishmania* species to the environment, associated with the evolutionary
597 pressure suffered by these gp63 genes (Rogers et al., 2011). As a result, the
598 presence of these multi-copy arrays may lead to speciation (Lynch and Conery,
599 2003) or indicate the possible need for stage-specific genes, due to the large
600 amount of generated transcripts from the multi-copy genes (Rogers et al., 2011).
601 There is considerable difference in the number of gp63 genes between the
602 *Leishmania* subgenus (*Leishmania, Viannia* and *Sauroleishmania*) (Isnard et al.,
603 2012; Raymond et al., 2012). Among the pathogenic species, the *Leishmania*
604 *Viannia braziliensis* strain 2904 possesses the greatest number of these genes on
605 chromosome 10. This variation may be explained by the evolutionary pressure
606 mentioned above, as it evolved only in the New World. As the other species from
607 the subgenus *Viannia* also have a great number of these genes on chromosome 10
608 (Steinkraus et al., 1993), it is believed that the smaller number of genes on the
609 *Leishmania* subgenus gene is linked to the fact that the event that led to their
610 expansion occurred only after the formation of the subgenus *Viannia* (Valdivia et al.,
611 2015).
612 The genes present in chromosomes 28 and 31 of *Leishmania sp*, were more
613 related to the genes of *C. fasciculata* and *L. pyrrhocoris*, which are monoxenous
614 trypanosomatids that mainly infect insects (Vickerman, 1994). Since these proteins
615 of monoxenous trypanosomatids are more related to the survival within the insect,
616 performing adhesion and nutrition functions (Santos et al., 2006; Vickerman, 1994),
617 we can suggest that the *Leishmania* genes located on chromosomes 28 and 31 are
618 more related to these same functions within the insect. The genes located on
619 chromosome 10, which have a large number of genes in *Leishmania*, possibly would
620 be more involved in actions against the vertebrate immune system. These actions

may vary according to the subcellular location of these proteins, determined by the characteristics found in the C-terminal groups and the presence or absence of the GPI anchor. C-terminal group 2 presented a more hydrophilic region when compared to the other groups and the absence of the GPI anchor signal peptide, which is consistent with proteins that are directly secreted into the extracellular medium, as reported in a *L. mexicana* Gp63 study from Ellis et al., 2002. This release into the extracellular environment contributes at the early stages of infection, due to the ability of Gp63 to digest the extracellular matrix proteins, which facilitates parasite mobility and invasion (Ellis et al., 2002; Mcgwire et al., 2003). Although group 1 possesses a characteristic GPI anchor attachment C-terminal, with a hydrophilic region followed by a hydrophobic region, we did not find a binding domain as clear as that of group 3. But we cannot rule out that group 1 proteins can be bound to membrane, as group 3 are.

After this analysis, we focused on understanding the great expansion of genes on chromosome 10 that may be caused by a great genetic variability, commonly associated with events of mosaicism (Mauricio et al., 2007). In studies performed with *L. guyanensis*, it was suggested that the genes may be generated by events of mosaicism between the combination of 5' and 3' UTR and protein coding regions (Steinkraus et al., 1993). The data obtained by us corroborate with other gp63 recombination studies that showed this event mainly related to the N-terminus and C-terminus regions of the gene (Mauricio et al., 2007; Victoir and Dujardin, 2002).

A study by Ma et al., (2011) evaluated the structure variability of the zinc binding site in gp63 and found great variety in this region. This contrasts with what was reported by Nocua et al., (2014), which evaluated structures from *L. braziliensis*

646 homologous proteins that were involved in DNA replication processes and showed
647 well preserved structures. This conservation can be justified by the fact that the
648 proteins studied by Nocua et al., (2014) were associated with key structural
649 processes required for the cell duplication, unlike to the gp63, which the main focus
650 is the interaction with the host and escape the immune system. In a study by Sutter
651 et al., 2017, it was suggested that this structural difference between multiple gp63
652 sequences may be related to the parasite's ability to interact with several substrates,
653 favoring the processes of infection and interaction with the host immune system. A
654 differentiated protein structure also would help the parasite cell in this interaction,
655 mainly because the host would need to produce different antibodies to neutralize a
656 single group of proteins. To reinforce this idea, we performed epitope predictions for
657 B cells and observed that most of the epitopes were found in the protein's external
658 region and coincided with motifs having sequence variation. This result was also
659 corroborated by Victoir and Dujardin, 2002, that analyzed the crystallized structure
660 of *L. major* gp63 and also identified regions with sequence variation in the surface
661 of the protein and suggested that it is related to structural flexibility, which may have
662 implications for the host-parasite relationship. The same study also evaluated the
663 presence of B cell epitopes in gp63 proteins from *L. major* and *L. infantum*. As our
664 results showed in *L. braziliensis*, they also found a great variety of epitopes. The
665 large amount of proteins and epitopes found in these analyses possibly are due to
666 a selective pressure on these proteins due to a probable evasion of the immune
667 system.

668 Overall, the data presented here suggest that the *Leishmania* gp63 proteins
669 evolved independently from different species generating a wide range of paralogs,
670 which display sequence variations and may be generated by recombination. These

variations might help the parasite to evade the recognition by the host immune system, making it more effective during the early stages of infection, and its maintenance within their vertebrate hosts. However, more studies are needed to better understand the recognition of these different paralogs by the host immune system.

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952 **Supplementary Table 1. Oligonucleotides used for the PCR reactions**
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Oligonucleotide	Sequences
0470-F	5’-ATGTCCCGCGACCGCAGCGT-3’
0540-F	5’-ATGTCCCGCGACCGCAGCAG-3’
0590-F	5’-ATGCCCCTCGACAGCAGCAG-3’
1516-F	5’-ATGTCCCGCGACCGCAGCAGC-3’
0456-F	5’- ATGYCCCKCGACMGCAGCAG-3’
GPI-R	5’-GGCCTGGCACACCTCCACGTA-3’
KDELMAP-R	5’- GGCCTGGCACACCTCCACGTACGGC-3’
31-F	5’- ATGTCGCGCGTACCCGTAGCGT-3’
31-GPI-R	5’- CGCGTAGCACACATCGTCGTAGGGC-3’
31-KDELMAP-R	5’-AGGTGCCATCAGCTCGTCCTTCGC-3’

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955 **Supplementary Table 2. Subsets of Gp63 sequences used to build the Hidden Markov Models**
956 **(HMMs).** Lbr (*Leishmania braziliensis*), Lmj (*L. major*), Lin (*L. infantum*), Ldn (*L. donovani*), Lmx (*L.*
957 *mexicana*), Lta (*L. tarentolae*).
958

Subset	Number of genes	*Leishmania* species (chromosome)
1	56	Lbr (Chr. 10), Lmj (Chr. 10), Lin (Chr. 10), Lmx(Chr. 10), Lta (Chr. 10)
2	5	Lmj (Chr. 28), Lin (Chr. 28), Ldn (Chr. 28), Lmx (Chr. 28)
3	9	Lbr (Chr. 31), Lmj (Chr. 31), Lin (Chr. 31), Ldn (Chr. 31), Lmx (Chr. 30), Lta (Chr. 31)
4	3	Lbr (Chr. 31)
5	2	Lbr (Chr. 10)
6	2	Lta (Chr. 10)
7	2	Lta (Chr. 10)
8	2	Lta (Chr. 10)
9	2	Lta (Chr. 10)

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961 **Table 1.** Table showing the number of gp63 genes identified by each HMM after the search for new
962 paralogs in the proteome from *L. braziliensis* 2904.
963

HMM	Number of Gp63 identified in *L. braziliensis* strain 2904
1	38
2	38
3	38
4	34
5	30
6	31
7	31
8	31
9	31

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Supplementary Table 3. Set of oligonucleotides and respective sequences obtained by PCR.

Oligonucleotide pair	Sequences obtained
A - 10.0540 (oligo forward); KDELMAP (oligo reverse)	K1610A1; K1610A2; K1650A1
B - 10.0470 (oligo forward); GPI (oligo reverse)	G0510B2; G0560B1; G0560B2; G1610B3; G1610B4; G1610B5; G1610B6; G1620B1
C - 10.0540 (oligo forward); GPI (oligo reverse)	G0510C1; G0510C2; G0540C1; G0560C4; G1640C1; G1640C2
D - 10.0590/0610 (oligo forward); GPI (oligo reverse)	G0590D1; G0590D2
F - 10.0406 (oligo forward); KDELMAP (oligo reverse)	K0510F4; K1630F1; K1650F2; K1650F3
G - 10.1516 (oligo forward); GPI (oligo reverse)	G0510G2; G0520G1; G0560G3
H - 10.1516 (oligo forward); KDELMAP (oligo reverse)	K1620H2; K1620H3; K1650H4
I - 10.0470 (oligo forward); KDELMAP (oligo reverse)	K0510I3; K1620I2; K1640I3; K1650I5
J - 31.2260 (oligo forward); KDELMAP (oligo reverse)	K312260J1
K - 31.2260 (oligo forward); GPI (oligo reverse)	G312260K1
L - 10.0590/0610 (oligo forward); KDELMAP (oligo reverse)	K0590L3

ST3. In the nomenclature, the "K" indicates that the sequence is extended to the conserved KDELMAP region, while the "G" indicates that the sequence ends close to the GPI binding area on the protein. The four-digit sequence after "K" or "G" represents the last four numbers referring to the gp63 genes present in the databases, which most closely resembled the obtained sequences. The letters that follow the nomenclature, which represent from which oligo pair the sequence was originated is shown in the oligonucleotide section of the table. The last number of the nomenclature identifies the number of genes already described in the databases, since in this work different sequences with high identity of the same gene were obtained.

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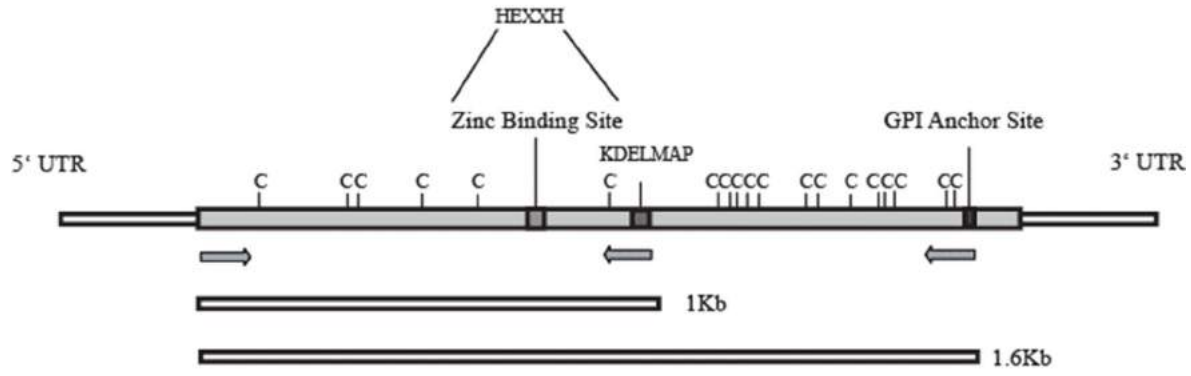
Figure 1



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979 Figure 1. PCR strategy used to amplify the gp63 superimposed on a schematic representation of a
980 typical gp63. The scheme highlights conserved elements found on all gp63 sequences, such as the
981 zinc-binding motif, multiple cysteine residues, the GPI anchor site and a nearly universally conserved
982 motif of seven consecutive amino acids we named KDELMAP.

Figure 2

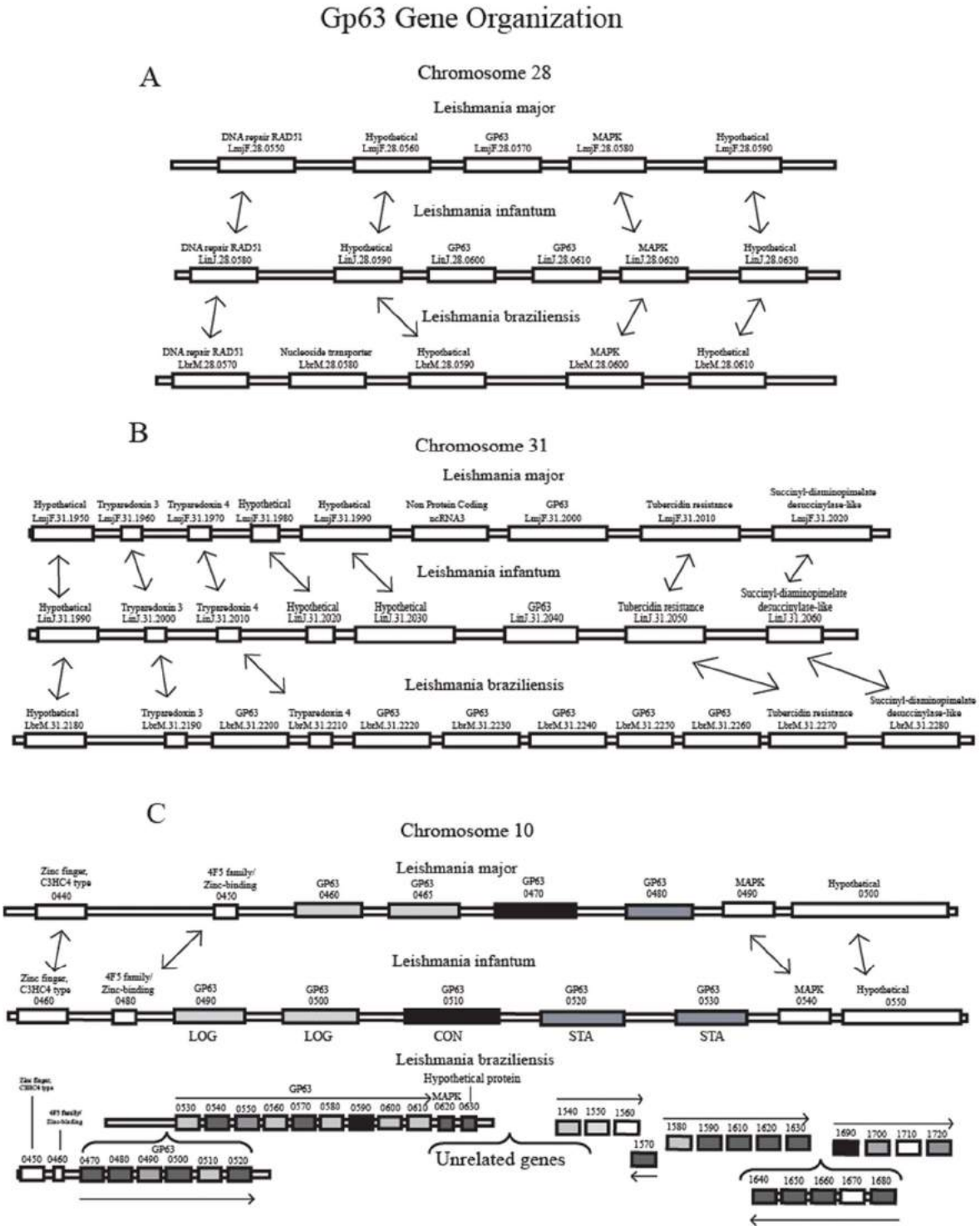

Figure 2. Genomic organization of gp63 genes from Leishmanis sp., showing the general distribution and location of these genes along the chromosomes 10, 28 and 31. The arrows indicate the transcription sense of the genes and the shades of grey show the UTR group that each gene is related.

Figure 3

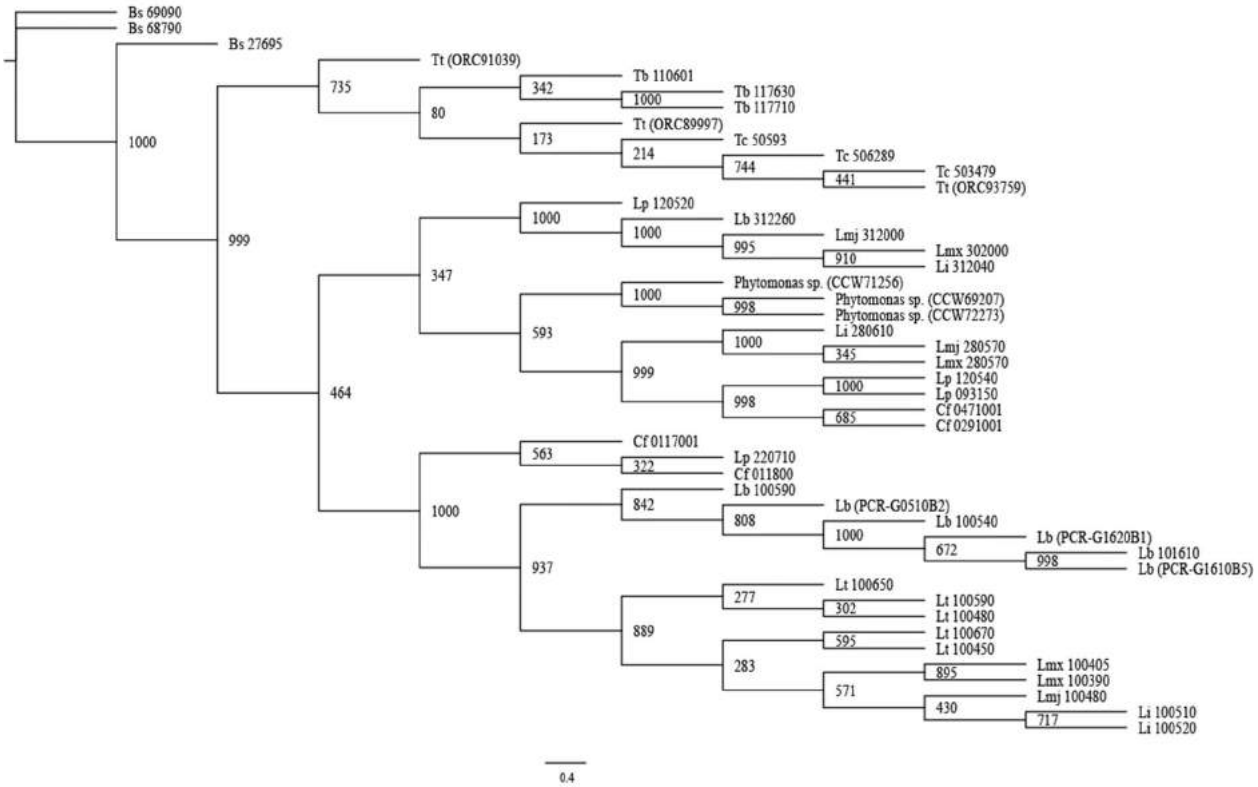

Figure 3. Tree constructed with several Gp63 sequences from Kinetoplastids, demonstrating the close relation of the gp63 genes from *Leishmania* chromosomes 28 and 31, with the monoxenes trypanosomatids genes. Bs: *Bodo saltans*, Tt: *Trypanosoma theileri*, Tb: *Trypanosoma brucei*, Tc: *Trypanosoma cruzi*, Lp: *Leptomonas pyrrhocoris*, Cf: *Crithidia fasciculata*, Lb: *Leishmania braziliensis* strain 2904, Lmj: *Leishmania major*, Lmx: *Leishmania mexicana*, Li: *Leishmania infantum*, Lt: *Leishmania tarentolae*.

Figure 4

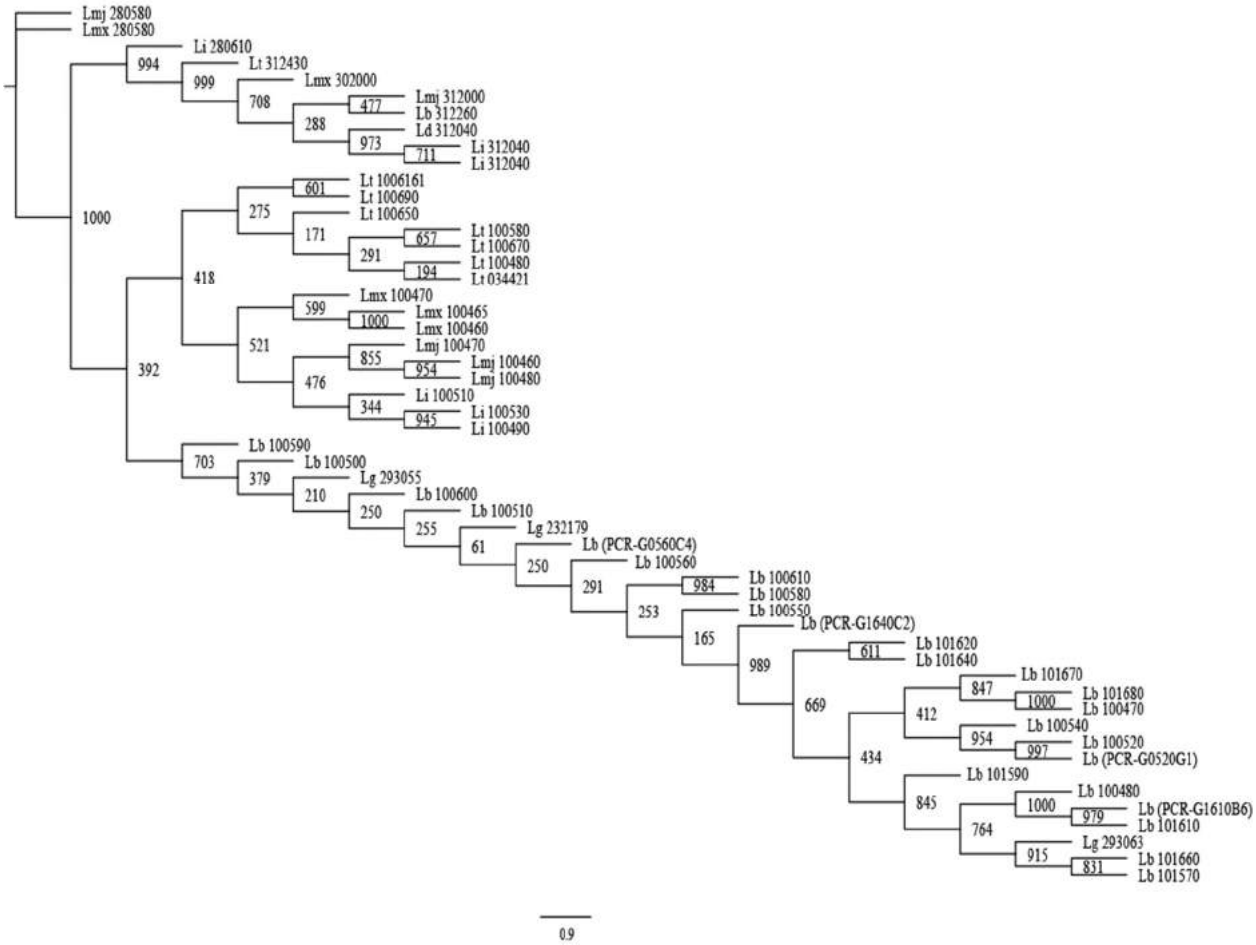

Figure 4. Phylogenetic tree showing the clear separation of gp63 genes on chromosome 10 according to their main subgenera (*Sauroleishmania*, *Leishmania* and *Viannia*). It is also possible to observe the independent expansion of the chromosome 10 genes from the subgenus *Viannia*, when compared to the other subgenus. Lb: *Leishmania braziliensis* strain 2904, Lmj: *Leishmania major*, Lmx: *Leishmania mexicana*, Li: *Leishmania infantum*, Lt: *Leishmania tarentolae*, Lg: *Leishmania guyanensis*.

Table 2. Sets of the 3 'UTR groups among the species of *L. infantum*, *L. major* and *L. braziliensis*, based on their sequence similarity.

Group	*L. braziliensis* 3'UTRs
1	LbrM.10.1540, LbrM.10.0600, LbrM.10.0530, LbrM.10.1580, LbrM.10.0610, LbrM.10.0510, LbrM.10.0560, LbrM.10.0580
2	LbrM.10.1570, LbrM.10.0500, LbrM.10.0540, LbrM.10.1610, LbrM.10.1620, LbrM.10.1630, LbrM.10.0570, LbrM.10.1640, LbrM.10.1650, LbrM.10.0600, LbrM.10.1680, LbrM.10.0470, LbrM.10.0520, LbrM.10.1590, LbrM.10.0480
3	LbrM.10.0590, LbrM.10.1690
4	LbrM.10.1720, LbrM.10.0550
5	LbrM.10.0490, LbrM.10.1700
6	LbrM.10.1550
7	LbrM.31.2200, LbrM.31.2220
8	LbrM.31.2230
9	LbrM.31.2250, LbrM.31.2260

Table 3. Table showing the Gp63 gene groups, based on the sequence similarity of their C-terminus regions.

C-terminus Group	*L. braziliensis* Gp63
1	10.1580
	10.0510
	10.0610
	10.0550
	10.0560
	10.1540
	10.0600
	10.0530
2	10.0540
	10.0500
	10.0520
	10.1570
	10.0570
	10.1630
	10.1620
	10.1640
	10.1650
	10.1610
	10.1590
	10.1660
	10.1680
	10.0470
3	10.1690
	10.0590
4	10.1720
5	10.0490
	10.1700
6	10.1550

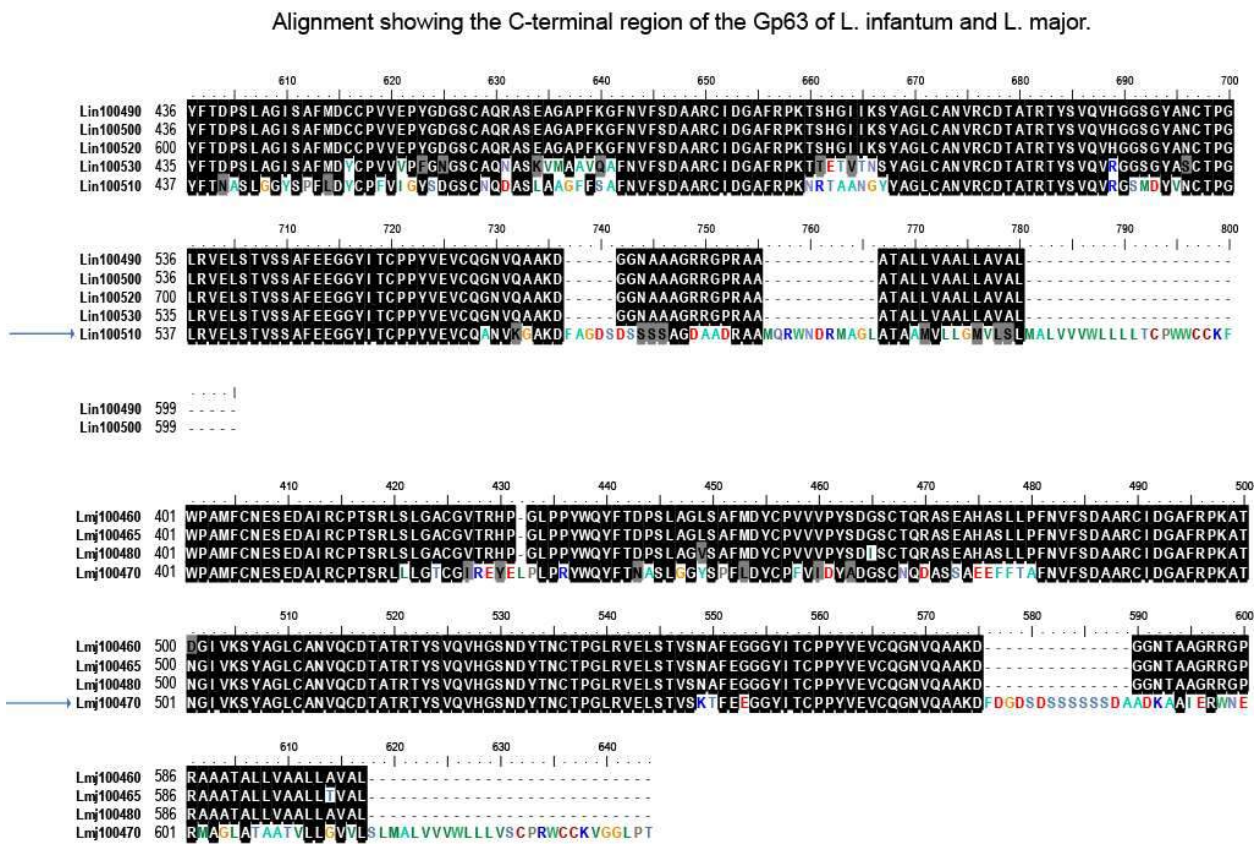

Figure 5. Alignment showing the difference in the C-terminal regions of the sequences of the proteins that present divergent UTRs between the sequences present in chromosome 10.

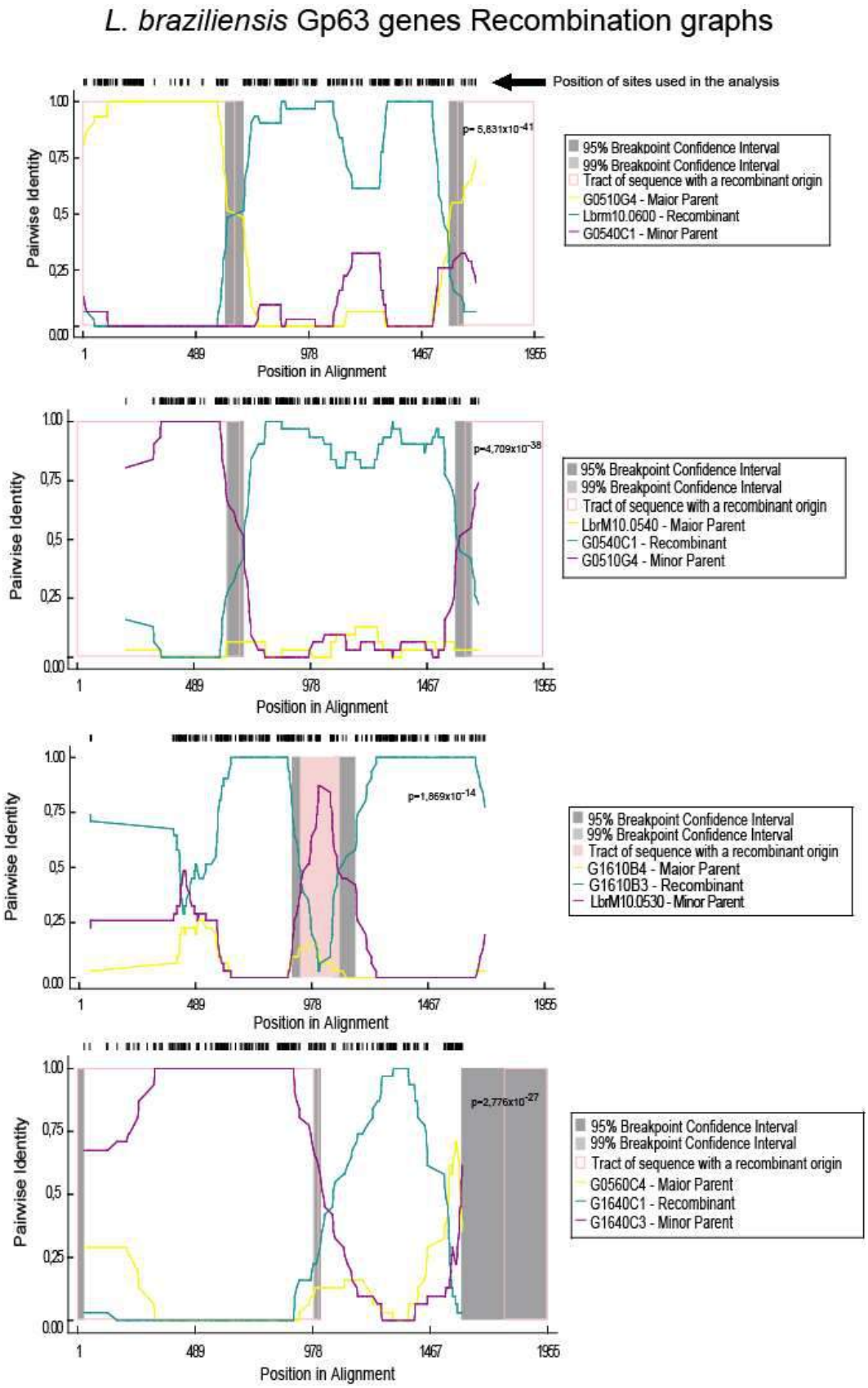

Figure 6. Demonstrative figure of some gp63 genes, which had recombination events, obtained by the RDP4 program. Each graph represents the recombination of a gene. In green, the gene that has recombined is represented. In purple and yellow, genes involved in recombination are represented, classified according to their identity with the recombinant gene, as shown in the graph.

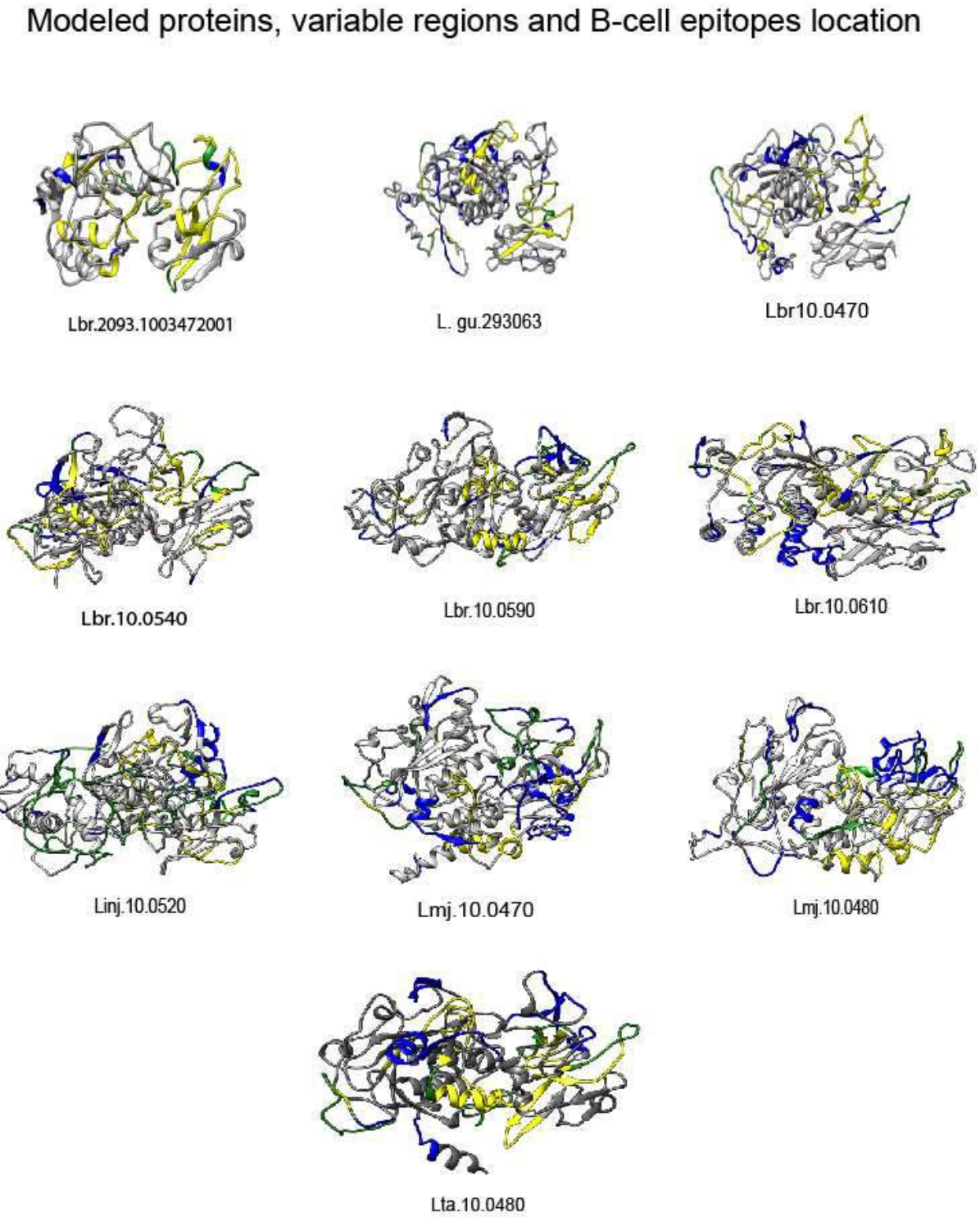

Figure 7. Modeled structures of Gp63 orthologs and paralogs from *Leishmania sp*. The blue labelled regions display the sequence variation region between these proteins, the yellow regions represent the predicted linear B-cell epitopes and the green markings show sequence variation sites among the predicted epitopes.

1121 Table 4. Table showing the distribution of the predicted epitopes in the studied Gp63.

Total number of epitopes	Conserved region	Sequence variation region	Type of epitopes	Protein Region
81	22	59	Linear	External
19	5	14	Linear	Internal
40	13	27	Conformational	External
14	6	8	Conformational	Internal

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6 DISCUSSÃO GERAL

6.1 AVALIAÇÃO DE UM NOVO GRUPO DE ANTÍGENOS RECOMBINANTES PARA O DIAGNÓSTICO SOROLÓGICO DE LEISHMANIOSE VISCERAL HUMANA E CANINA.

As leishmanioses são doenças negligenciadas que acometem milhões de pessoas anualmente. A utilização de testes diagnósticos eficazes e o estudo da patogenia da doença, importante para a identificação de novos alvos terapêuticos ou vacinais, são essenciais para contribuir com o controle da doença (DAVIES et al., 2003; SRIVIDYA et al., 2012).

Neste estudo, primeiramente avaliamos novos alvos antigênicos quanto ao seu potencial diagnóstico, frente a soros humanos e caninos infectados com *L. infantum*. A maioria desses antígenos testados aqui eram proteínas compostas por uma série de motivos repetitivos, caracterizadas pela presença de pelo menos duas ou mais cópias de uma sequência repetida de aminoácidos. Na literatura, elas foram relatadas em vários organismos, de vírus a humanos, e geralmente apresentam alta antigenicidade. Foi sugerido que esta antigenicidade estaria relacionada provavelmente devido a estimulação da produção de anticorpos pelas células B, através da ligação direta destes antígenos repetitivos, por uma via independente das ações padrões de estimulação de linfócitos T (VOS et al., 2000; GOTO et al., 2008; GOTO et al. 2010; VALIENTE-GABIOUD et al., 2011).

Os antígenos testados apresentaram grande variação de antigenicidade entre os cães e humanos, com melhor resultado associado a detecção da doença em cães. Isso também foi encontrado no estudo de FONSECA et al. 2014, que

testou a eficiência de três antígenos em detectar a leishmaniose canina e humana e apresentou melhor resultado em cães. Nos nossos testes, vários antígenos testados mostraram ótimo potencial para o diagnóstico da forma canina da doença, com um deles (Lci13), demonstrando a capacidade de detectar a leishmaniose canina melhor do que o lisado total de parasitas. Esta proteína é um antígeno promissor, uma vez que alguns estudos não encontraram uma única proteína com sensibilidade tão alta para o diagnóstico canino (FRAGA et al., 2014; FONSECA et al., 2014). No entanto, nenhum dos antígenos testados apresentou um resultado de sensibilidade suficiente para diagnosticar satisfatoriamente a LV humana. Isso corrobora os achados de GOTO et al., 2009 e OLIVEIRA et al., 2011, que destacam as diferenças na resposta imune entre esses dois diferentes alvos da doença.

Nos ensaios descritos aqui, o antígeno comercial rK39 foi incluído para facilitar a comparação dos antígenos recentemente identificados com outros descritos anteriormente e também para avaliar o nível de resposta imune dos soros selecionados para este estudo. Os resultados da rK39 se apresentaram consistentes com o que foi relatado na literatura, confirmando que ele é eficaz para o diagnóstico de LV humana, mas não apresenta boa performance com a forma canina da doença (MAIA et al., 2012; QUINNELL et al. 2013).

Os antígenos recombinantes testados apresentaram diferenças importantes na sensibilidade quando testados em soros de humanos e de cães, como já foi visto por nós e outros usando diferentes proteínas recombinantes no mesmo tipo de ensaio (GOTO et al. 2009; OLIVEIRA et al., 2011). Essa variação pode ser devido a maneira diferenciada que os hospedeiros vertebrados reagem ao parasita, principalmente no que diz respeito ao reconhecimento e apresentação dos antígenos distintos estudados ao sistema imunológico ou, como foi proposto

(GOTO et al., 2009), essa resposta pode ser devido a diferentes mecanismos de sobrevivência parasitária em cada hospedeiro (homem e cão). Por outro lado, uma vez que foi demonstrado que as variações na sensibilidade podem ser devido a fases sintomáticas e assintomáticas da doença, com a fase sintomática demostrando o melhor desempenho de sensibilidade nos testes sorológicos (METTLER et al., 2005), essas diferenças podem, até certo ponto, refletir o estágio da doença dos indivíduos de quem os soros foram coletados. Em geral, o reconhecimento diferencial das proteínas estudadas pelos soros dos dois hospedeiros destaca as diferenças nas respostas imunes induzidas pelo parasita e a necessidade de otimizar os testes sorológicos atuais.

Como uma alternativa para melhorar o diagnóstico da LV em seres humanos, propusemos a avaliação de um "Mix" de antígenos, composto por proteínas que já produziram os melhores resultados com as amostras de cães. Eles foram escolhidos para compor o “Mix”, baseado em sua alta sensibilidade e especificidade em soros de cães, com o objetivo de combinar suas melhores características e poder criar um teste sorológico que fosse capaz de detectar a leishmaniose visceral nos dois hospedeiros. Neste “Mix”, foi adicionada a proteína Lci1, já descrita e bem avaliada previamente (OLIVEIRA et al., 2011), juntamente com as Lci12 e Lci13, avaliadas neste estudo. Como os resultados mostraram o “Mix” apresentou uma melhoria significativa para o diagnóstico em humanos, quando comparado a performance das proteínas individuais. Mas em cães, ele não se mostrou tão eficiente quando a proteína Lci13. Apesar disso, nossos resultados ainda apresentaram melhores resultados, quando comparado com outro estudo em que uma mistura semelhante de três antígenos, não levou a um aumento no desempenho do teste diagnóstico, quando comparada com as proteínas individuais

sozinhas (FONSECA et al., 2014). Essa falta de melhoria significativa em uma mistura de proteínas pode ser explicada pela diminuição da antigenicidade de cada proteína individual, devido a uma menor disposição dos peptídeos antigênicos em meio a uma mistura proteica.

Uma alternativa que poderia ser usada para lidar com as limitações apresentadas pela mistura de proteína é o desenvolvimento de proteínas quiméricas, contendo as regiões proteicas que apresentaram o melhor desempenho em avaliações sorológicas. Alguns estudos nesta área já foram realizados e mostraram uma melhora significativa na sensibilidade do teste sorológico (BOARINO et al., 2005; CAMUSSONE et al. 2009; CASTRO-JUNIOR et al., 2014).

Em resumo, os antígenos recombinantes testados individualmente neste estudo em soros humanos e de cães mostraram diferentes sensibilidades para o diagnóstico sorológico da LV, com melhor desempenho em cães. A Lci13 mostrou uma sensibilidade mais alta para o soro canino do que os testes de comerciais atuais e o lisado total de Leishmania, o que demonstra o potencial desse antígeno recombinante para detectar a leishmaniose visceral canina por conta própria. O objetivo de identificar um único antígeno ou uma combinação deles, capaz de diagnosticar ambas as formas da doença não se mostrou viável com nossas proteínas. No entanto, o desempenho observado com a mistura de proteínas testada indica que, com a melhoria, ensaios utilizando proteínas quiméricas derivadas de múltiplos antígenos podem ser uma boa opção para a obtenção de testes sorológicos com alta sensibilidade e que seriam simultaneamente eficazes para ambas as espécies.

6.2 CARACTERIZAÇÃO *IN SILICO* DE MÚLTIPLOS GENES CODIFICANTES DA PROTEÍNA DE VIRULÊNCIA GP63 DE *LEISHMANIA BRAZILIENSIS*: IDENTIFICAÇÃO DE FONTES DE VARIAÇÃO E POSSÍVEIS PAPÉIS NA EVASÃO DO SISTEMA IMUNE.

Os resultados do diagnóstico mostrado por nós e outros estudos citados aqui, reforçam a variabilidade da reposta imune nas leishmanioses. Dentre os vários fatores envolvidos na variação desta resposta, destacam-se as ações modulatórias do sistema imune, pelos fatores de virulência das células de *Leishmania sp.* (ATAYDE et al., 2016; MATLASHEWSKI, 2001). Dentre esses fatores, a protease Gp63 é considerado o principal deles, presente na superfície da *Leishmania sp*. e outros tripanosomatideos (SILVA-ALMEIDA et al., 2012). Então, ortólogos e parálogos de gp63 foram identificados e caracterizados para avaliar suas prováveis implicações para a função da proteína. Devido ao grande número de genes que codificam a gp63 na *L. braziliensis* cepa 2904 encontrados por nossas PCRs e em bancos de dados, e com o objetivo de realizar uma completa caracterização desses genes, realizamos a busca *in silico* de novos parálogos possivelmente ainda não anotados no genoma. A falta de novos parálogos identificados em nossa pesquisa provavelmente deve-se à metodologia *shotgun* aplicada para sequenciar o genoma da *L. braziliensis* (PEACOCK et al., 2007). Nos genomas montados a partir desta metodologia, a quantidade real de genes localizados em arranjos em tandem pode não estar bem determinada. Isso acontece devido à dificuldade de montar *contigs* com um grande número de genes relacionados, com alta similaridade, usando leituras curtas. Portanto, espera-se que

o número anotado desses genes pode ser menor do que o real (KELLY et al., 2017).

A análise filogenética com ortólogos e parálogos de gp63 se mostrou consistente com outros dados publicados que demonstram a relação filogenética entre as espécies de *Leishmania*, onde genes do subgênero *Viania*, *Leishmania* e *Sauroleishmania* estão agrupados em clados separados, independentemente dos genes usados para construir a árvore (JACKSON, 2014; MA et al., 2011; SCHONIAN et al., 2013). A distribuição desses genes no cromossomo 10 mostra sua expansão independente em toda a espécie de *Leishmania*, particularmente entre os subgêneros *Sauroleishmania* e *Viannia*. Essa expansão gênica, disposta em *tandem*, que pode ser originada por eventos de duplicação e/ou recombinação, demonstra a adaptabilidade das espécies de *Leishmania* ao meio ambiente, associada à pressão evolutiva sofrida pelos genes de gp63 (ROGERS et al., 2011). Como consequência, esses eventos podem levar a especiação (LYNCH & CONERY, 2003) ou indicar a possível necessidade de genes estágio-específicos, devido à grande quantidade de transcritos geradas a partir de genes com múltiplas cópias (ROGERS et al., 2011).

Um outro resultado gerado a partir dos eventos mencionados acima, foi a existência da notável variabilidade no número de genes gp63 entre os subgêneros de *Leishmania* (*Leishmania, Viannia e Sauroleishmania*) (ISNARD et al., 2012; RAYMOND et al., 2012). Entre as espécies patogênicas, a cepa 2904 de *Leishmania Viannia braziliensis* possui o maior número desses genes no cromossomo 10. Essa variação pode ser explicada pela pressão evolutiva mencionada acima, pois a evolução da *L. braziliensis* aconteceu apenas no Novo Mundo. Como as outras espécies do subgênero Viannia também possuem um grande número desses genes no cromossomo 10 (STEINKRAUS et al., 1993),

acredita-se que o menor número de genes nas espécies do subgênero *Leishmania* está ligado ao fato de que o evento que levou a sua expansão ocorreu somente após a formação do subgênero *Viannia* (VALDIVIA et al., 2015).

Em relação aos genes de gp63 presentes nos cromossomos 28 e 31 de *Leishmania sp*, identificamos que eles estavam mais relacionados aos genes de *C. fasciculata* e *L. pyrrhocoris*, que são tripanossomatídeos monoxénos que infectam insetos (VICKERMAN, 1994). Uma vez que estas proteínas de tripanossomatídeos monoxénos estão mais relacionadas à sobrevivência dentro do inseto, realizando funções de adesão e nutrição (SANTOS et al., 2006; VICKERMAN, 1994), podemos sugerir que os genes de *Leishmania* localizados nos cromossomos 28 e 31 podem estar mais relacionados com essas mesmas funções dentro do inseto. Os genes localizados no cromossomo 10, que têm um grande número de genes em *Leishmania*, possivelmente estariam mais envolvidos em ações contra o sistema imunológico de vertebrados. Essas ações podem variar de acordo com a localização subcelular dessas proteínas, determinadas pelas características encontradas nos grupos de C-terminais e na presença ou ausência da âncora GPI. O grupo 2 de C-terminal apresentou uma região mais hidrofílica em comparação com os demais grupos e a ausência do peptídeo sinal de âncora GPI, que é consistente com proteínas que são diretamente secretadas no meio extracelular, conforme relatado em um estudo com Gp63 (Ellis et al., 2002). Esta liberação no ambiente extracelular contribui nos estágios iniciais da infecção, devido à capacidade de Gp63 digerir as proteínas da matriz extracelular, o que facilita a mobilidade e a invasão dos parasitas (ELLIS et al., 2002; MCGWIRE et al., 2003). Embora o grupo 1 possua uma C-terminal característica de ligação a âncora de GPI, com uma região hidrofílica seguida por uma região hidrofóbica, não

encontramos um domínio de ligação tão claro como o do grupo 3. Mas não podemos descartar que as proteínas do grupo 1 possam estar ligadas a membrana, como as do grupo 3 estão.

Após esta análise, focamos na compreensão da grande expansão dos genes no cromossomo 10, que pode ser causada por uma grande variabilidade genética, comumente associada a eventos de mosaicismo (MAURICIO et al., 2007). Em estudos realizados com *L. guyanensis*, sugeriu-se que estes genes podem ser gerados por eventos de mosaicismo entre a combinação de regiões 5' e 3' UTR e regiões codificadoras das proteínas (STEINKRAUS et al., 1993). Os dados obtidos por nós corroboram o de outros estudos em que a recombinação dos genes gp63 foi encontrada e está relacionada principalmente às regiões N-terminal e C-terminal do gene (MAURICIO et al., 2007; VICTOIR;DUJARDIN, 2002).

Em análises estruturais, o estudo de MA et al., (2011) avaliou a variabilidade no local de ligação do zinco à gp63 e encontrou grande variedade nessa região. Isso contrasta com o que relatou NOCUA et al. (2014), que avaliou estruturas de proteínas homólogas de *L. braziliensis* que estavam envolvidas em processos de replicação de DNA e apresentaram estruturas bem preservadas. Esta conservação pode ser justificada pelo fato de que as proteínas estudadas por NOCUA et al., (2014) foram associadas aos principais processos estruturais necessários para a duplicação celular, ao contrário do gp63, cujo principal foco é a interação com o hospedeiro e a fuga o sistema imunológico. Em um estudo de SUTTER et al., 2017, sugeriu-se que essa diferença estrutural entre múltiplas sequências de gp63 pode estar relacionada à capacidade do parasita de interagir com vários substratos, favorecendo os processos de infecção e interação com o sistema imune do hospedeiro. Uma estrutura proteica diferenciada também ajudaria a célula do

parasita nessa interação, principalmente porque o hospedeiro precisaria produzir diferentes anticorpos para neutralizar um único grupo de proteínas. Para reforçar essa ideia, realizamos predições de epítopos para células B e observamos que a maioria dos epítopos foram encontrados na região externa da proteína e coincidiu com motivos com variação de sequência. Este resultado também foi corroborado por VICTOIR;DUJARDIN, 2002, que analisaram a estrutura cristalizada de gp63 de *L. major* e também identificaram regiões com variação de sequência na superfície da proteína e sugeriram que ela estaria relacionada à flexibilidade estrutural, o que pode ter implicações para a interação. O mesmo estudo também avaliou a presença de epítopos de células B em proteínas gp63 de *L. major* e *L. infantum*. Como nossos resultados mostraram em *L. braziliensis*, eles também encontraram uma grande variedade de epítopos. A grande quantidade de proteínas e epítopos encontrados nessas análises pode ser devido a uma pressão seletiva sobre essas proteínas devido a prováveis interações com o sistema imune.

Em geral, os dados apresentados aqui sugerem que as proteínas gp63 de *Leishmania* podem ser divididas em classes, baseados em sua localização nos cromossomos. Além de uma evolução independente dos genes do cromossomo 10 em diferentes espécies gerando uma ampla gama de parálogos, que podem apresentar diferentes localizações celulares ou serem excretadas em diferentes momentos de expressão durante o ciclo celular, que apresentam variações de sequência possivelmente geradas por recombinação. Essas variações podem ajudar o parasita a evadir o reconhecimento pelo sistema imune do hospedeiro, tornando-o mais efetivo durante os estágios iniciais e na manutenção da infecção em hospedeiros vertebrados.

7 CONCLUSÕES

- Os antígenos Lci6 a Lci13 apresentaram uma melhor performance na detecção da leishmaniose visceral canina, quando testados de forma individual
- Foi possível encontrar novos genes de gp63 por técnicas moleculares, ao contrário da busca *in silico*
- Genes de gp63 dos cromossomos 28 e 31 se relacionaram a ações exercidas nos insetos vetores, enquanto que os do cromossomo 10 a ações no hospedeiro mamífero
- Grupos de C-terminal das proteínas de gp63 apresentaram-se relacionados com momentos de expressão determinados pelas UTRs e sugerem localização subcelular diferente para cada grupo de proteínas
- Os múltiplos parálogos de Gp63 presentes no cromossomo 10 foram originados por eventos de recombinação, que levam a diversidade gênica desse grupo de proteínas, que estão mais localizados na parte externa da proteína e em sua maioria são regiões preditas como epítopos de células B

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ANEXO A - TABELA DE CARACTERÍSTICAS PROTEICAS DAS GP63 DE *L. BRAZILIENSIS*

Gene	Posição Peptídeo Sinal	Posição Domínio Transmembrana	Ponto Isoelétrico	Sequência peptídeo sinal	Sequência domínio transmembrana
101540	N	221 .. 243	4.78	N	YVLATVTAVLLGIVLAAMAGLVV
101550	1 .. 37	168 .. 190	9.17	MDVSQRGGRRARAPAPWPAVVRFLLATSQRVTLSPAQ	LMRLAAAGLVMAVGAAAVWAQAA
101560	1 .. 38	21 .. 40	6.79	MSRDRSSTHRRRSDAARLMRLAAAGLVMAVGAAAVWA	LAAAGLVMAVGAAAVWAQAA
101570	1 .. 40	21 .. 40	5.97	MSRDRSSTHRRRSVAARLMRLAAAGLVMAVGAAAVWVQAA	LAAAGLVMAVGAAAVWVQAA
101580	1 .. 23	544 .. 566	5.3	RVAQQRSPAVSVSALGLPYVSAG	AVLLGIVLAAMAGLVVGLLVISL
101590	N	141 .. 163	6.91	N	LMRLAAAGLVMAVGAAAVWAQAA
101610	1 .. 40	21 .. 40	6.58	MSRDRSSTHRRRSVAARLMRLAAAGLVMAVGAAAVWVQAA	LAAAGLVMAVGAAAVWVQAA
101620	1 .. 40	21 .. 40	5.91	MSRDRSSTHRRRSVAARLMRLAAAGLVMAVGAAAVWVQAA	LAAAGLVMAVGAAAVWVQAA
101630	1 .. 40	21 .. 40	6.1	MSRDRSSTHRRRSVAARLMRLAAAGLVMAVGAAAVWVQAA	LAAAGLVMAVGAAAVWVQAA
101640	N	145 .. 167	6.93	N	LMRLAAAGLVMAVGAAAVWVQAA
101650	1 .. 40	20 .. 42	5.4	MSRDRSSTHRRRSVAARLMWLAAAGLVMAVGAAAVWVQAA	WLAAAGLVMAVGAAAVWVQAAGH
101660	1 .. 31	13 .. 35	5.28	MSRDRSVTARLMRLAAAGLVMAVGAAAVWAQ	RLAAAGLVMAVGAAAVWAQAAGH
101670	1 .. 58	38 .. 60	6.35	MHTHTAAHKPSSSLPLPLCHPHACWPASLAPWHAPARLMRLAAAGLVMAVVAAAVWAQ	LMRLAAAGLVMAVVAAAVWAQAA
101680	N	N	4.36	N	N
101690	N	N	6.85	N	N
101710	N	N	4.96	N	N
101720	N	N	12.62	N	N
100470	1 .. 31	13 .. 35	4.74	MSRDRSVTARLMRLAAAGLVMAVGAAAVWAQ	RLAAAGLVMAVGAAAVWAQAAGH
100480	1 .. 40	21 .. 40	6.37	MSRDRSSTHRRRSVAARLMRLAAAGLVMAVGAAAVWVQAA	LAAAGLVMAVGAAAVWVQAA
100490	1 .. 42	21 .. 43	8.77	MPLDSSSTHRRRSVAARLVRLAAAGVAAALAVGTAAAWAHAA	LAAAGVAAALAVGTAAAWAHAAA
100510	1 .. 38	21 .. 40 E 597 .. 619	6.5	MPLDSSSTPRRRSVAARLMRLAAAGLVMAVGAAAVWAQ	LAAAGLVMAVGAAAVWAQAA e VTAALLGIVLAAMAGLVVGLLVI
100550	1 .. 37	168 .. 190	9.17	MPLDSSSTHRRRSDAARLMRLAAAGLVMAVGAAAWAQ	RLRVRQVQGSWRVTGMTGPICGD

100560	1 .. 38	21 .. 40	6.79	MPLDSSSTHRRRSDAARLMRLAAAGLVMAVGAAAVWAQ	LAAAGLVMAVGAAAVWAQAA
100570	1 .. 40	21 .. 40	5.97	GAIDICEAKDILTEERRHILINILLPLALQLHVERLKVRQ	INILLPLALQLHVERLKVRQ
100580	1 .. 23	544 .. 566	5.3	MPRDSSSTPRRRSVAARLMRLAAAGLVM	TLSAAFVNGSYITCAPYVEVCQA
101700	1 .. 42	21 .. 43	8.77	MPLDSSSTHRRRSVAARLVRLAAAGVAAALAVGTAAAWAHAA	LAAAGVAAALAVGTAAAWAHAAA
100590	1 .. 42	21 .. 43	6.39	MPLDSSSTHRRRSVAARLVRLAAAGVAAALAVGTAAAWAHAA	LAAAGVAAALAVGTAAAWAHAAA
100600	1 .. 42	21 .. 43 E 586 .. 608	7.28	MPLDSSSTHRRRSVAARLVRLAAAGVAAALAVGTAAAWAHAA	LAAAGVAAALAVGTAAAWAHAAA e VTAALLGIVLAAMAGLAVWLLLI
100610	1 .. 39	21 .. 39 E 598 .. 620	5.94	MPLDSSSTHRRRSDAARLMRLAAAGLVMAVGAAAWAQAA	LAAAGLVMAVGAAAWAQAA e AVLLGIVLAAMAGLVVGLLVISL
100500	N	N	4.37	N	N
100520	1 .. 38	21 .. 40	06.08	MSRDRSSTHRRRSDAARLMRLAAAGLVMAVGAAAVWAQ	LAAAGLVMAVGAAAVWAQAA
100530	N	473 .. 495	5.43	N	LATVTAALLGIVLAAMAGLAVWL
100540	1 .. 20	N	4.63	MRLAAAGLVMAVGAAAVWAQ	N
312200	1 .. 20	N	7.82	MAGTLALVSSLVTSETASAA	N
312220	1 .. 20	N	08.03	MAGTLALVSSLVTSETASAA	N
312250	1 .. 17	N	9.57	MAPVFSLARYSSLSLAA	N
312260	1 .. 28	12 .. 34	**8.13**	MSRVPVASWRLGLWLCVLFMYIAFATAA	GLWLCVLFMYIAFATAAIASSAL

Anexo 1. Tabela demonstrando os genes de Gp63 de L. braziliensis, juntamente com a posição e a sequência de peptídeos sinais e domínios transmembrana, obtidos através do banco de dados TritrypDB.

ANEXO B - TABELA DEMONSTRANDO OS PEPTÍDEOS LINEARES PREDITOS

Lbr10.0610				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
1:12	1,3	MPLDSSSTHRRR	Externo	Não
90:104	1,2	**ADSTSP**SVVRAADWG	Externo	90: 95
180: 192	1,2	VTGM**T**GPICGDFK	Externo	184
249: 265	1,2	SRYDQ**G**TTRTVTHEVAH	Interno	254
319: 332	1,3	VEDQGG**S**GSAGSHL	Externo	325
400: 435	1,2	EMFCNT**TERR**YRCPTDRLKLG**T**CGIRTY**ST**PMPTYF	Externo	406:09; 421; 428:9
461: 473	2,3	**SNG**ACNQDPSMASP	Interno	460: 63
Lbr10.0590				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
1:10	1,3	MPLDSSSTHR	Externo	Não
58:68	1,3	RDSAAHRRMPP	Interno	Não
239:55	1,2	SRYDQGTTRTVTHEVAH	Interno	Não
290:301	1,3	TAVAKAREQYGC	Externo	Não
309:22	1,3	VEDQGGSGSAGSHL	Externo	Não
394:406	1,2	NT**DENA**LRCPTDR	Externo	396:99
445:64	1,3	VVPYDDGSCAQRA**SETSSDM**	Externo	449:64
445:57	1,2,3	VVPY**DDGSCAQRA**	Externo	449:57
475:95	1,2	RCLDGAFRP**TTTREDVTYAG**M	Externo/Interno	484:494
517:27	2,3	GYVACTPGESV	Externo	Não
564:74	2,3	**AAAGRRGPRAA**	Externo	564:74
Lmj10.0480				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
62:72	2,3	A**DHHKA**PGAVS	Externo	63:67
87:98	1,2,3	**AAAADPRPGSAR**	Externo	87:98

281:93	1,3	**RIVASVP**NVRGKN	Externo	281:87
303:13	1,3	TAVAKAREQYG	Externo	Não
322:33	1,3	MEDQGSAGSAGS	Externo/Interno	Não
379:90	1,2	PWGQNAGCAFLT	Externo	Não
426:42	1,2	**GVTRHPGLPP**YWQYFTD	Externo	426:35
491:503	1,2	DGAF**RPKATNGIV**	Externo	495:503
527:42	1,2	HGS**ND**YTNCTPGLRVE	Externo	530:31
576:88	2,3	NTAA**GRRGPRAA**	Externo	581:88
Lmj10.0470				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
62:72	2,3	A**DHHKA**PGAVS	Externo	63:67
87:98	1,2,3	**AAAADPRPGSAR**	Externo	87:98
97:108	1,2	**ARS**VVR**DVNWGA**	Externo/Interno	96:99/103:08
303:13	1,3	TAVAKAREQYG	Externo	Não
322:33	1,2,3	VEDQGGAGSAGS	Externo	Não
379:90	1,2	PWGQNAGCAFLT	Externo	Não
461:73	1,2	**DYADGSCNQDASS**	Externo	461:73
464:74	1,3	**DGSCNQDASSA**	Externo	464:74
492:506	1,2	DGAF**RPKATNGIVKS**	Externo	496:506
527:537	1,2,3	VHGS**ND**YTNCT	Externo/Interno	531:32
527:43	1,2	VHGS**ND**YTNCTPGLRVE	Externo/Interno	531:32
572:84	2,3	**AAKDFDGDSDSSS**	Externo/Interno	572:84
Lta10.0480				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
31:42	1,3	**AAATSAAAEPGK**	Externo	31:42
35:48	1,2	**SAAAEPGKFK**VVRS	Externo	35:44
156:70	1,2	VIYVTSVPSRPGVVA	Interno	Não
244:63	1,2	I**EAN**RVVVRAREQYGCDNLK	Externo	245:48

268:80	1,2	EDQGGEGSVQSHI	Externo	Não
359:70	1,2	RCPTNRLGIGSC	Externo/Interno	Não
402:21	1,3	PVV**EDEGEGSCAQNASQAPA**	Interno/Externo	405:21
439:52	1,2,3	DFRPK**GMQSPAKSH**	Externo	444:52
434:68	1,2	RCIDGDFRPK**GMQSPAKSHAG**LCANVRCDPATRTY	Interno	444:54
511:24	1,2,3	CQGNA**GAVKGVGNG**	Interno	
511:27	2,3	CQGNA**GAVKGVGNGVPS**	Interno	
Linj10.0520				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
1:10	1,3	**MKTAGTQRVS**	Externo	1:10
29:46	1,2	**GHITVRPCTDIPPSQRRG**	Externo	29:46
36:46	1,2,3	**CTDIPPSQRRG**	Externo	36:46
49:59	1,3	**RLTEPHTHLPP**	Externo	49:59
80:97	1,2,3	**SGAYTTPTTSSSFRSPSP**	Interno/Externo	80:97
109:33	1,2,3	**LSPPSPDPPTHPIPLHPPLPRPHAR**	Externo	109:33
149:67	1,2	**LATTPHCPQRPRACRA**MSV	Externo/Interno	149:64
249:66	1,2	**AAADRRPGSAPTVVRAAN**	Interno/Externo	249:66
249:62	1,2,3	**AAADRRPGSAPTVV**	Interno/Externo	249:62
293:304	1,2,3	**ISTHDSGSTT**CT	Externo	293:302
289:310	1,2	VGQR**ISTHDSGSTT**CTAEDILT	Externo	293:302
356:67	1,3	KVPPAHITDGLS	Externo	Não
484:94	1,2,3	EDQGGAGSAGS	Externo	Não
538:50	1,2	VMPW**GRN**AGCAFL	Interno	542:44
619:38	1,2,3	VEPY**GDGSCAQRASEAGAPF**	Externo	623:38
611:38	1,2	AFMDCCPVVEPY**GDGSCAQRASEAGAPF**	Interno/Externo	623:38
648:61	1,2	ARCIDGAF**RPKTSH**	Interno/**Externo**	656:61
688:700	1,2	HGGSGYANCTPGL	Externo	Não
735:748	1,2	**DG**GNAAAG**RRGPRA**	Externo	735:36/743:48
Lbr10.0470				

Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
47:63	1,2	LQSVAQQRRPPGSVSAL	Interno/Externo	Não
84:97	1,2,3	**ADSTSP**SVA**HS**PDW	Externo	84:89/93:4
237:49	1,2	PAA**NIRSP**YDQLM	Interno	240:44
317:28	1,3	**GGGSA**AGSHLKG	Externo/Interno	317:21
412:29	1,2	KIGRCSITTYDDPMPTYF	External	Não
458:69	1,2,3	**CN**QDPSTASPTV	External	458:59
488:98	1,2,3	**PKHNTGPSDHY**	External	488:98
Lbr10.0540				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
37:52	1,2	QSVAQQHRPPGSVSAL	Externo/Interno	Não
63:75	1,2,3	**LADSTS**PSVA**HS**A	Externo	63:8/73:4
63:81	1,2	**LADSTS**PSVA**HS**ADWGTLR	Externo	63:8/73:4
266:78	1,2	**E**VPVLNSPTVVAK	Interno	266
316:27	1,2	MAPVMGAGYYTA	Interno	Não
374:99	1,2	EMFCNS**TESS**YRCPTQRLKIGRCSIA	Externo	380:3
438:52	1,2	CNQDPSTASP**ALME**F	Externo	448:51
465:77	1,2,3	TL**TPKHNTGPYEQ**	Externo	467:77
503:13	2,3	GYVACTPGERV	Externo	Não
Lbr.2093.1003472001				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
6:22	1,2	**M**CQAFPD**DR**PAVGVVNI	Externo	6/13:14
121:35	1,2	LMAPA**SD**AGYYTNLT	Interno	126:127
140:52	1,2	QDFGFYKADFTKA	Externo	Não
180:92	1,2	EMFCNTT**DSR**YRC	Externo	187:189
240:55	2,3	**SDGACN**QDPSTASPSL	Externo	240:245
272:86	1,2,3	FTPKHSTGPPGPYNG	Externo	Não
266:305	1,2	RCLDGAFTPKHSTGPPGPYNGLCANVKCD**RVHH**TYSVQVY	Externo/Interno	295:98

L. gu.293063				
Position	Programas	Sequências	Posição na proteína modelada	Variabilidade de sequência
58:68	1,2,3	A**QQRR**PPGSVS	Externo	59:62
92:102	1,2,3	**DSTSP**SVVRA**G**	Externo	92:96/102
289:305	1,2	GRNY**Y**VPVLNSPTVVAK	Interno/Externo	293
322:33	2,3	DTGGTSTAGSHL	Externo	Não
399:414	1,2	WPWMFCNT**TESS**YRCP	Externo	407:10
461:476	2,3	**SNGACN**QDPS**TASPSL**	Externo	461:66/471:76
493:507	1,2,3	HSTGPPG	Externo	Não
490:514	1,2	DGTFRPKHSTGPPGPYNGLCANVKC	Externo/Interno	Não

Anexo 2. Tabela demonstrando os epítopos de célular B lineares preditos a partir dos programas AAP12(1), BCPred (2), Bepipred (3). A tabela também demonstra a posição dos epítopos nas proteínas, a região do epítopo nas proteínas modeladas e em negrito as variações de sequência dentro dos epítopos. Nesta análise só consideramos como uma sequência variante, os epítopos que apresentaram mais de quatro aminoácidos não conservados.

ANEXO C - TABELA DEMONSTRANDO OS PEPTÍDEOS CONFORMACIONAIS PREDITOS

Lbr 10.0470			
Posição	Sequência	Posição na proteína modelada	Variabilidade de Sequência
47:61	LQSVAQQRRPPGSVS	**Interno**/Externo	Não
112:22	PDCYCSYVGQL	Externo	Sim
330:39	NAKDELMAPV	Interno	Não
396:422	FCNTTEPSYRCTSDRLKIGRCSITTYD	Externo	Sim
475:513	FSDSSRCLDGNFAPKHNTGPSDHYNSLCANVKCDRAHHT	Interno/Externo	Variação de sequência na região externa

Lbr 10.0540			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
131:42	IIPQALQLHTER	Externo	Não
259:69	NLRGKDYEVPV	Externo	Sim
307:23	KRRNAKDELMAPVMGAG	Interno	Não
346:59	AEVMPWAEHASCDF	Externo	Sim
461:70	CFDGTLTPKH	Interno /Externo	Sim
474:93	PYEQYNALCANVMCDRAHHT	Externo / Interno	Sim
Lbr 10.0590			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
114:31	RVGQRVSNHADEIVTCTA	Externo	Sim
290:303	TAVAKAREQYGCPT	Externo	Não
542:56	ITCAPYVEVCQANVQ	Externo	Não
Lbr 10.0610			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
123:35	SYVGQRVSNHAGA	Externo	Sim
300:13	TVVAKAREQYGCPT	Externo	Não
374:92	VMPWGRNASCDFITNKCME	Interno/ Externo	Sim
491:502	VFQPRNSNARSE	Externo	Sim
505:19	NALCANVMCDTAART	Interno	Sim
LinJ 10.0520			

Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
590:609	RHPDLPPYWQYFTDPSLAGI	Externo	Sim
614:25	CCPVVEPYGDG	Interno/ Externo	Variação de sequência na região externa
LmjF 10.0470			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
51:67	DAMQARVRQSVADHHKA	Externo/Interno	Variação de sequência na região externa
95:107	GSARSVVRDVNWG	Externo	Sim
121:29	PAYHCARVG	Externo	Não
293:316	NFDVPVINSSTAVAKAREQYGCDT	Externo	Não
382:95	QNAGCAFLTNKCME	Externo	Não
442:54	TNASLGGYSPFLD	Externo	Sim
458:68	FVIDYADGSCN	Externo	Sim
518:37	TATRTYSVQVHGSNDYTNCT	Interno	Sim
LmjF 10.0480			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
51:67	DAMQARVRQSVADHHKA	Externo	Sim
95:107	GSARSVVRDVNWG	Externo	Sim
289:316	VRGKNFDVPVINSSTAVAKAREQYGCDT	Externo	Não
344:57	LMAAASGAGYYTAL	Interno	Sim
381:95	QNAGCAFLTNKCME	Externo	Não

429:41	RHPGLPPYWQYFT	Externo	Sim
517:36	TATRTYSVQVHGSNDYTNCT	Externo	Sim
563:72	EVCQGNVQAA	Interno	Não
Lbr.2903.10034720010			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
115:28	RNAKDELMAPASDA	Interno	Sim
230:44	LDYCPIIVGSSDGAC	External	Sim
247:56	DPSTASPSLK	External	Sim
283:99	PYNGLCANVKCDRVHHT	Interno/External	Variação de sequência na região externa
335:46	MCAPYVEVCQAN	External	Não
Lta 10.0480			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
251:70	VRAREQYGCDNLKYLELEDQ	External	Não
275:295	SVQSHIKMRNAQDELMAADAS	Interno	Sim
328:340	NTGCAFLSEKCME	External	Não
358:369	FRCPTNRLGIGS	External	Sim
452:67	HAGLCANVRCDPATRT	Interno	Sim
470:81	QVRGSSGYMNC	Interno	Não
L.gu293063			
Posição	Sequências	Posição na proteína modelada	Variabilidade de Sequência
54:68	LQSVAQQRRPPGSVS	External	Sim
336:48	RNAKDELMAPAVT	Interno	Sim

419:32	RLGTCRIVSYDNPM	External	Sim
436:77	FQYFTNSALGGRSSFLDYCPIIVGSSNGACNQDPSTASPSLK	External	Sim
500:21	GPPGPYNGLCANVKCDRDHHRY	External/**Interno**	Não
555:67	TCPSYVEVCQAN	External	Não

Anexo 3. Tabela demonstrativa dos epitopos conformacionais preditos pelo programa CBTOPE. Nesta análise os peptídeos foram escolhidos da mesma forma que a tabela anterior.

ANEXO D - NORMAS DA REVISTA

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Authors should seek advice on data analysis prior to submission. A commentary (Morrison, D.A., 2002. How to improve statistical analysis in parasitology research publications. Int. J. Parasitol. 32, 1065-1070) may also be consulted. Papers may be sent to referees with particular expertise in statistics and modelling.

PREPARATION

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

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It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
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Divide your article into clearly defined and numbered sections. Subsections (only in sections 2 and
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Acknowledgements

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List funding sources in this standard way to facilitate compliance to funder's requirements:

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Combes, C., 2001. Parasitism. The ecology and evolution of intimate interactions. University of Chicago Press, Chicago and London.

Kumar, N., Cha, G., Pineda, F., Maciel, J., Haddad, D., Bhattacharyya, M.K., Nagayasu, E., 2004. Molecular complexity of sexual development and gene regulation in Plasmodium falciparum. Int. J. Parasitol. 34, 1451-1458.

Pettersson, E.U., Ljunggren, E.L., Morrison, D.A., Mattsson, J.G., in press. Functional analysis and localisation of a delta-class glutathione S-transferase from Sarcoptes scabiei. Int. J. Parasitol.

Sangster, N.C., Dobson, R.J., 2002. Anthelmintic resistance. In: Lee, D.L. (Ed.), The biology of nematodes. Taylor and Francis, London and New York, pp. 531-567.

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3. The name and version number of the sequence database used in searches. If a custom-made database is used then complete information on the origin of the sequences and database size should be disclosed. Given the dependence of scoring on database size, the use of a small database, or one excluding contaminants, should be justified. 4. A short description of the methods use to interpret the significance of search results, including any statistical analysis, confidence thresholds and other values specific to judging the certainty of the identification.

5. For large-scale experiments a false-positive determination should be reported. This may be the result of randomized database searches or other approaches.

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ANEXO E - CURRÍCULO LATTES ATUALIZADO (CORRESPONDENTE AO PERÍODO DO CURSO)

Artur Leonel de Castro Neto
Curriculum Vitae

Formação complementar

2015 - 2015 Curso de curta duração em Curso de proteção radiológica para área da pesquisa. (Carga horária: 40h).
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

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2015 - 2017 Vínculo: Professor Visitante , Enquadramento funcional: Estágio à Docência , Carga horária: 4, Regime: Parcial
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2014 - Atual Vínculo: Colaborador , Enquadramento funcional: Estudante de Doutorado , Carga horária: 40, Regime: Dedicação exclusiva

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Prêmios e títulos

2017 Trabalho premiado na categoria pôster na XXIV Semana de Biomedicina da UFPE., UFPE

2016 Trabalho Premiado na categoria apresentação oral "Avaliação da expressão gênica de grupos de genes parálogos de Gp63 de Leishmania braziliensis", FIOCRUZ - PERNAMBUCO. IV Semana de Biociências e biotecnologia em saúde.

2015 Trabalho premiado, SBBq - Brazilian Society for Biochemistry and Molecular Biology

2013 Trabalho premiado na categoria de apresentação oral, Programa de Pós-Graduação em Genética - UFPE

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Artigos completos publicados em periódicos

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Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis. PLoS One. , v.12, p.e0184867 - , 2017.

Trabalhos publicados em anais de eventos (resumo)

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2. DUTRA, A. K. O.; BRITO, A. N. A. L. M.; **CASTRO NETO, A. L.**; MELO NETO, O. P. Produção de páralogos recombinantes da proteína de virulência Gp63 de Leishmania braziliensis para avaliação de reconhecimento frente a anticorpos específicos In: XXIV Semana de Biomedicina da UFPE, 2017, Recife.
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Apresentação de trabalho e palestra

1. CASTRO NETO, A. L.; REZENDE, A.M.; MAGALHAES, F. B.; MELO NETO, O. P.
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2. CASTRO NETO, A. L.; REZENDE, A.M.; MAGALHAES, F. B.; MELO NETO, O. P.
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3. **CASTRO NETO, A. L. Tripanossomatídeos e seus mecanismos de evasão do sistema imune**, 2016. (Conferência ou palestra,Apresentação de Trabalho)

4. CASTRO NETO, A. L.; REZENDE, A.M.; MAGALHAES, F. B.; MELO NETO, O. P.
In Silico Characterization of Orthologs and Paralogs of the gp63 Virulence Protein from Leishmania sp., 2015. (Congresso,Apresentação de Trabalho)

5. CASTRO NETO, A. L.; NASCIMENTO, M. B.; MAGALHAES, F. B.; MELO NETO, O. P.
Assessment of subcellular localization and gene knockout of two promising Leishmania infantum antigens, 2014. (Congresso,Apresentação de Trabalho)

Educação e Popularização de C&T

Apresentação de trabalho e palestra

1. **CASTRO NETO, A. L.**
Tripanossomatídeos e seus mecanismos de evasão do sistema imune, 2016. (Conferência ou palestra,Apresentação de Trabalho)

Curso de curta duração ministrado

1. **CASTRO NETO, A. L.**
Clonagem em vetores bacterianos e extração de DNA plasmidial, 2016. (Aperfeiçoamento, Curso de curta duração ministrado)

2. **CASTRO NETO, A. L.**
Aspectos moleculares no desenvolvimento de novos métodos de diagnóstico e vacina, 2015. (Outro, Curso de curta duração ministrado)

3. **CASTRO NETO, A. L.**
Desenvolvimento de vacinas e imunoterápicos, 2015. (Especialização, Curso de curta duração ministrado)

Orientações e Supervisões

Orientações e supervisões concluídas

Trabalhos de conclusão de curso de graduação

1. Adriana Neuman Albuquerque Lins Moura de Brito. **Avaliação Da Variabilidade Gênica De Múltiplos Parálogos Da Proteína De Virulência Gp63 De Leishmania Braziliensis**. 2016. Curso (Biomedicina) - Universidade Federal de Pernambuco

Iniciação científica

1. Adriana Neuman Albuquerque Lins Moura de Brito. **Avaliação do perfil de expressão dos múltiplos genes de gp63 durante o ciclo de vida da L. braziliensis**. 2016. Iniciação científica (Biomedicina) - Universidade Federal de Pernambuco

Orientações e supervisões em andamento

Dissertações de mestrado : co-orientador

1. Adriana Neuman Albuquerque Lins Moura de Brito. **Avaliação Comparativa Do Perfil De Expressão De Genes Parálogos Codificantes Da Proteína De Virulência Gp63 De Leishmania Braziliensis**. 2017. Dissertação (Biociências de Biotecnologia em Saúde) - Centro de Pesquisa Aggeu Magalhães, FIOCRUZ-PE

Iniciação científica

1. Allana Kelly Oliveira Dutra. **Análise do perfil transcricional dos múltiplos parálogos de Gp63 de Leishmania braziliensis**. 2016. Iniciação científica (Biomedicina) - Universidade Federal de Pernambuco

Eventos

Participação em eventos

1. Apresentação de Poster / Painel no(a) **23rd Congress of the International Union of Biochemistry and Molecular Biology(IUBMB) and 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology(SBBq)**, 2015. (Congresso) IN SILICO CHARACTERIZATION OF ORTHOLOGS AND PARALOGS OF THE GP63 VIRULENCE PROTEIN FROM LEISHMANIA SP.

2. Apresentação de Poster / Painel no(a) **XXX Annual Meeting of the Brazilian Society of Protozoology**, 2014. (Congresso) Assessment of subcellular localization and gene knockout of two promising Leishmania infantum antigens.

Bancas

Bancas

Participação em banca de trabalhos de conclusão

Graduação

1. **CASTRO NETO, A. L.**; ASSIS, L. A.; FREIRE, E. R.; MELO NETO, O. P.
Participação em banca de Tallyta Tâmara da Silva Monteiro. **Avaliação das diferentes subpopulações de mRNAs associados aos fatores de tradução EIF4E3 e EIF4E4 em Trypanosoma brucei**, 2017
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2. CASTRO NETO, A. L.; FREIRE, E. R.; REIS, C. R. S.
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