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AVALIAÇÃO DE UMA PROTEÍNA HIPOTÉTICA ESPECÍFICA DE *Leishmania* NO SORODIAGNÓSTICO E DESENVOLVIMENTO DE UMA VACINA CONTRA AS LEISHMANIOSES

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, da Faculdade de Medicina da UFMG, como requisito parcial para a obtenção do Título de Mestre pelo referido Programa.

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LISTA DE ABREVIATURAS E SIGLAS

® ou ™ - Marca registrada

Abs - Absorbância

ANOVA - Análise de variância

BM - Medula óssea

CVL - Leishmaniose visceral canina

DAT - Teste de aglutinação direta

dLN - Linfonodo drenante

OD - Densidade ótica

elF-2 - Fator de alongamento 2

ELISA - Teste de imunoadsorção enzimática

FML - Ligante fucose-manose

FPLC - Cromatografia líquida rápida de proteínas

GM-CSF - Fator estimulador de colônias de macrófagos e granulócitos

gp63 - glicoproteína de superfície de 63 kDa

HASPB1 - Proteína acilada hidrofílica B1 de superfície

HbR - Receptor de hemoglobina

ICT - Teste de imunocromatografia rápida

IDRM - Intradermoreação de Montenegro

IFAT - Teste de imunofluorescência indireta

IFN-ɣ - Interferon-gama

IgG - Imunoglobulina G

IL – Interleucina

KMP-11 - Proteína de membrana de 11kDa dos cinetoplastídeos

iNOS - Óxido nítrico sintetase induzido

LB - Linfócito B

LC - Leishmaniose cutânea

LCD - Leishmaniose cutâneo-difusa

LiHyD - Proteína hipotética D de *Leishmania infantum*

LMC - Leishmaniose mucocutânea

LPG - Lipofosfoglicano

LT - Leishmaniose tegumentar

LT - Linfócito T

LV - Leishmaniose visceral

LVC - Leishmaniose visceral canina

Meio LB - Meio Luria Bertani

MHC - Complexo principal de histocompatibilidade

n - Número amostral

NCBI - *National Center for Biotechnology Information*

NO - Óxido nítrico

ORF - Fase de leitura aberta

pb - Pares de bases

PCR - Reação em cadeia da polimerase

PDI - Proteína dissulfeto isomerase

PPG - Proteofosfoglicano

PpSP15 - Proteína salivar 15 do *Phlebotomus papatasi*

Sap - saponina

SDS-PAGE - Gel de poliacrilamida desnaturante

SFB - Soro fetal bovino

SLA - Extrato proteico antigênico solúvel de *Leishmania spp.*

SLA*Li* - Extrato proteico antigênico solúvel de *Leishmania infantum*

SLA*Lm* Extrato proteico antigênico solúvel de *Leishmania major*

SLA*Lb* Extrato proteico antigênico solúvel de *Leishmania braziliesnis*

Syn. - Sinônimo

r – Recombinante

ROC - Característica de operação do receptor

RPMI - *Roswell Park Memorial Institute*

TGF-β - Fator de transformação de crescimento beta

Th - T auxiliar

TNF-α - Fator de necrose tumoral alfa

TPI - Triose fosfato isomerase

TPR - Tripanotiona redutase

UV - Ultra-violeta

RESUMO

A leishmaniose é um complexo de doenças incidente no Brasil e no mundo, apresentando elevada morbidade e mortalidade. Nosso país responde por aproximadamente 95% dos casos de leishmaniose visceral (LV) nas Américas, sendo o cão o principal reservatório doméstico da doença. O sorodiagnóstico da LV canina (LVC) apresenta problemas relacionados à sua sensibilidade e/ou especificidade. No presente estudo, uma proteína hipotética específica de *Leishmania,* LiHyD, sob sua forma recombinante (rLiHyD), foi avaliada em experimentos de ELISA para o sorodiagnóstico da LVC. Três epitopos de linfócitos B da proteína foram sintetizados (Peptídeo-1, Peptídeo-2 e Peptídeo-3) e também avaliados como marcadores diagnósticos. A proteína recombinante e o Peptídeo-3 mostraram os melhores resultados, tendo sido reconhecidos por anticorpos presentes em soros de cães com VL sintomática e assintomática, e não apresentaram reatividade cruzada com anticorpos presentes em soros de cães com doença de Chagas, ehrlichiose, babesiose ou de cães sem leishmaniose e/ou vacinados com a vacina Leish-Tec®. Na busca por se selecionar um antígeno candidato a compor uma vacina contra as leishmanioses, uma vacina baseada na combinação da proteína rLiHyD com saponina foi testada em camundongos BALB/c contra a infecção causada pelas espécies *Leishmania infantum*, *Leishmania major* e *Leishmania braziliensis*. A imunogenicidade da vacina foi avaliada e os resultados mostraram que os animais imunizados produziram níveis elevados de IFN-γ, IL-12 e GM-CSF após o estímulo *in vitro* de esplenócitos com a proteína ou usando os extratos proteicos de *L. infantum*, *L. major* ou *L. braziliensis*. Após o desafio, os animais vacinados mostraram reduções significativas na carga parasitária em todos os órgãos e tecidos avaliados, quando comparados com os animais que receberam salina ou que foram imunizados apenas com saponina ou com a proteína isolada. A proteção obtida com a vacina rLiHyD/saponina foi associada com a produção de IFN-γ específica contra o parasito e dependente de IL-12, que foi produzida principalmente por linfócitos T $CD4^+$. Nesses animais, uma redução na produção de IL-4 e IL-10, associada à presença de níveis elevados de anticorpos do isotipo IgG2a específicos à proteína e aos parasitos, foram também encontrados. O presente estudo mostrou que a proteína hipotética LiHyD, inicialmente identificada em *L. infantum*, pode ser utilizada como um antígeno para o sorodiagnóstico da LVC e, quando associada a um adjuvante Th1, pode também compor uma vacina para proteção contra as leishmanioses visceral e tegumentar.

Palavras-chave: Leishmaniose Visceral, Leishmaniose Tegumentar, proteína hipotética, epitopos, sorodiagnóstico, vacina.

ABSTRACT

Leishmaniasis is a disease complex with a large incidence in Brazil and in the world, presenting high morbidity and mortality. Our country accounts for approximately 95% of the VL cases in Americas, being the dog the main domestic reservoir of the parasites. The serodiagnosis of canine visceral leishmaniasis (CVL) presents problems related to its sensitivity and/or specificity. In the present study, a *Leishmania*-specific hypothetical protein, rLiHyD, in a recombinant form (rLiHyD), was evaluated in ELISA experiments for the CVL serodiagnosis. Three B cell epitopes of LiHyD were synthesized (Peptide-1, Peptide-2 and Peptide-3) and also evaluated as diagnostic markers. The recombinant protein and the Peptide-3 showed the best results, being recognized by antibodies in sera from dogs with asymptomatic and symptomatic VL, and did not show cross-reactivity with antibodies in dog sera of dogs with Chagas disease, ehrlichiosis, babesiosis or animals without leishmaniasis and/or vaccinated with the Leish-Tec® vaccine. In the search to also select a candidate antigen for composing a vaccine against leishmaniasis, a combination between rLiHyD and saponin was tested in BALB/c mice against infection by *Leishmania infantum*, *Leishmania major* and *Leishmania braziliensis*. The immunogenicity of rLiHyD/saponin vaccine was evaluated, and the results showed that immunized mice produced higher levels of IFN-γ, IL-12 and GM-CSF after splenocytes *in vitro* stimulation with rLiHyD or *L. infantum*, *L. major* or *L. braziliensis* protein extracts. After challenge, vaccinated animals showed significant reductions in the parasite burden in all evaluated organs and tissues, when compared to those that were inoculated with saline or immunized with saponin or the protein alone. The protection obtained with the rLiHyD/saponin was associated with a parasite-specific IL-12-dependent IFN-γ production, which was produced mainly by $CD4^+$T cells. In these animals, a decrease in the parasite-mediated IL-4 and IL-10 responses, associated with the presence of high levels of LiHyD and parasite-specific IgG2a isotype antibodies, were also found. The present study showed that the hypothetical protein LiHyD, which was firstly identified in *L. infantum*, can be used for the CVL serodiagnosis and, when combined with a Th1 adjuvant, can compose a vaccine and confer protection against visceral and tegumentary leishmaniasis.

Keywords: Visceral Leishmaniasis, Tegumentary Leishmaniasis, hypothetical protein, epitopes, serodiagnosis,vaccine.

1. INTRODUÇÃO E JUSTIFICATIVA

As leishmanioses são doenças causadas por parasitos protozoários da Ordem Kinetoplastida, Família Trypanosomatidae e gênero *Leishmania* (Grimaldi & Tesh, 1993), e que apresentam uma ampla distribuição geográfica no mundo. A gravidade da doença no hospedeiro mamífero pode alcançar desde uma lesão cutânea única e de cura espontânea, até a forma visceral, fatal, quando aguda e não tratada (WHO, 2010). O Brasil responde por grande parte dos casos das leishmanioses no Continente Americano, sendo responsável por aproximadamente 95% e 40% dos casos registrados das leishmanioses visceral (LV) e tegumentar (LT), respectivamente; fatos que tornam a doença um importante problema de Saúde Pública em nosso país (Alvar *et al.,* 2012).

A leishmaniose visceral canina (LVC) trata-se de uma doença zoonótica, sendo endêmica em diversos países da América Central, América do Sul, bacia do Mediterrâneo e Ásia (Gramiccia e Gradoni, 2005; Baneth *et al.*, 2008). Os cães são considerados os principais reservatórios domésticos dos parasitos. A LVC possui maior importância em relação à doença humana, uma vez que o número de casos registrados da doença em cães é significativamente maior quando comparado ao número de casos notificados no homem (Nicolato *et al.,* 2013). Atualmente, um diagnóstico precoce e preciso destes animais é de extrema importância como uma medida de controle efetivo contra a doença.

Os testes sorológicos utilizados no diagnóstico da LVC visam à detecção de anticorpos específicos aos antígenos dos parasitos presentes no soro dos animais (Da Silva *et al.,* 2006; Gomes *et al.,* 2008; Coelho *et al*., 2009). Atualmente, o Ministério da Saúde do Brasil determina que o diagnóstico imunológico da doença deva ser baseado nos testes sorológicos DPP-LVC® e ELISA (Coura-Vital *et al*., 2014; Laurenti *et al*., 2014). Tais técnicas, entretanto, podem apresentar sensibilidade e/ou especificidade variáveis e, nos estágios iniciais da doença, animais infectados podem apresentar-se soronegativos e outros, mesmo clinicamente curados, podem permanecer soropositivos por longos períodos (Ferrer *et al*., 1995; Silva *et al.,* 2011; Viol *et al.,* 2012). Ressalta-se também que a especificidade dos testes é comprometida quando os mesmos são aplicados em áreas endêmicas da doença; em animais sadios, mas vacinados; e em cães infectados com parasitoses relacionadas às leishmanioses como, por exemplo, babesiose e ehrlichiose (Tavares *et al*., 2003; Chávez-Fumagalli *et al*., 2013; Costa *et al.,* 2013). Dessa forma, a busca por antígenos a serem

aplicados em testes diagnósticos que apresentem elevada sensibilidade e especificidade torna-se desejável (Gomes *et al*., 2008).

As medidas de controle das leishmanioses visam, principalmente, à interrupção do ciclo biológico do parasito. Entretanto, o grande número de espécies de *Leishmania*, o caráter zoonótico da doença e a manutenção do parasito no ciclo silvestre dificultam a adoção de medidas efetivas de controle (Tesh, 1995). Dessa forma, devido à ineficiência de tais medidas, da dificuldade para o diagnóstico correto e dos problemas encontrados no tratamento da doença, como os efeitos tóxicos nos pacientes, a ocorrência de resistência dos parasitos aos fármacos convencionais e/ou seu elevado custo; o número de casos de leishmanioses tem aumentado em nosso país (Gontijo & Melo, 2004; Minodier & Parola, 2007, Mondal *et al*., 2010). Assim, o desenvolvimento de vacinas que sejam capazes de induzir imunidade protetora nos hospedeiros imunizados poderia ser considerado como uma medida alternativa e de menor custo para o controle da doença (Palatnik-de-Sousa, 2008). Uma vacina protetora contra as leishmanioses deve, preferencialmente, conter imunógenos conservados em diferentes espécies de *Leishmania spp.*, induzir uma resposta imune Th1, além de ser segura e apresentar um custo acessível à população (Grimaldi & Tesh, 1993; Coelho *et al*., 2003; WHO, 2010).

Proteínas hipotéticas são aquelas previamente descritas no genoma de organismos, mas que não apresentam aplicação biológica ainda definida (Fernandes et al., 2012). Recentemente, por meio de uma pesquisa imunoproteômica baseada na combinação das técnicas de eletroforese bidimensional, *immunoblotting* e espectrometria de massas, utilizando formas promastigotas em fase estacionária de crescimento e formas amastigotas axênicas de *L. infantum*; proteínas antigênicas dessa espécie foram reconhecidas por anticorpos presentes em soros de cães com LV sintomática e/ou assintomática (Coelho et al., 2012). Diversas proteínas foram identificadas, dentre elas proteínas já descritas na literatura como candidatas vacinais, alvos diagnósticos e/ou agentes terapêuticos contra as leishmanioses visceral e/ou tegumentar. Além disso, algumas proteínas tidas ainda como hipotéticas também foram identificadas e, devido ao fato de terem sido reconhecidas por anticorpos nos cães com LV, poderiam ser consideradas como possíveis alvos de potencial aplicação biológica. Dentre elas, uma proteína denominada LiHyD (LinJ.33.3150), foi reconhecida por anticorpos nos soros de cães com LV sintomática. Tal proteína, após estudos por bioinformática, mostrou-se ser específica de *Leishmania spp*., não sendo encontrada em outros parasitos ou em hospedeiros

mamíferos. Tal proteína poderia ser, portanto, considerada como um antígeno candidato para o sorodiagnóstico da LVC.

Atualmente, proteínas recombinantes têm sido muito estudadas com a finalidade de se desenvolver uma vacina segura, passível de padronização, pura e com custo acessível à população (Joshi *et al*., 2014). A proteína LiHyD, tendo sido reconhecida por anticorpos de animais com LV, e por ser conservada em diferentes espécies do parasito, poderia também apresentar-se como candidata a um antígeno vacinal contra a infecção por *Leishmania spp*.

Desta forma, o objetivo deste trabalho foi avaliar a eficácia da proteína recombinante LiHyD (rLiHyD) no sorodiagnóstico e como candidata a compor uma vacina contra as leishmanioses visceral e tegumentar. Três epitopos da proteína, dois lineares e um conformacional, sendo específicos de linfócitos B, foram identificados por programas de bioinformática e também avaliados no sorodiagnóstico da LVC por ELISA, utilizando-se um painel sorológico canino. A proteína rLiHyD foi avaliada como imunógeno vacinal na indução de proteção de camundongos BALB/c contra a infecção experimental com as espécies *L. infantum*, *L. braziliensis* e *L. major*.

2 REVISÃO DE LITERATURA

2.1 Epidemiologia das leishmanioses

As leishmanioses são um complexo de doenças infecto-parasitárias endêmicas em 98 países, afetando, principalmente, as regiões tropicais e subtropicais do mundo. Em relação à LV, aproximadamente 90% dos casos ocorrem na Índia, Bangladesh, Sudão, Sudão do Sul, Etiópia e Brasil. Já cerca de 70 a 75% dos casos da LT ocorrem no Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia, Sudão do Norte, Costa Rica e Peru (Alvar *et al.*, 2012).

É estimado que 310 milhões de pessoas encontram-se expostas ao risco de contrair a infecção pelo parasito *Leishmania*, cuja incidência global aproximada é de 0,2 a 0,4 milhões e de 0,7 a 1,2 milhões de novos casos de LV e LT, respectivamente, ao ano. O Brasil é responsável por aproximadamente 95% dos casos de LV e 40% dos casos de LT no continente Americano, fato que exalta a doença como um importante problema de Saúde Pública (Alvar *et al*., 2012; WHO, 2015).

O aumento da incidência de casos de leishmaniose na Europa, África, América e Ásia vêm ocorrendo nas últimas décadas devido a uma série de fatores, tais como a expansão do *habitat* dos flebotomíneos devido ao aquecimento global e o aumento do número de casos de cães de áreas não endêmicas que viajam com seus donos para locais onde o parasito é encontrado, podendo se infectar; além do aumento do desflorestamento devido à crescente urbanização (Baneth *et al*., 2008; Palatnik de Souza, 2012).

.2.2 A etiologia e o ciclo biológico do parasito *Leishmania spp.*

As leishmanioses são doenças causadas por parasitos protozoários pertencentes ao gênero *Leishmania*, Sub-Reino Protozoa, Filo Sarcomastigophora, Ordem Kinetoplastida e Família *Trypanosomatidae*. O vetor transmissor é um inseto da Ordem Diptera, Família Psychodidae, Sub-Família Phlebotominae, pertencente aos gêneros *Phlebotomus* em países do Velho Mundo e *Lutzomyia* nas Américas, sendo que apenas as fêmeas possuem hábitos hematófagos sendo assim, capazes de transmitir o parasito aos hospedeiros mamíferos (Grimaldi & Tesh, 1993).

Em seu ciclo biológico, o parasito *Leishmania spp.* pode apresentar-se sob duas formas morfológicas principais: amastigota e promastigota. As formas amastigotas são formas arredondadas, com flagelo rudimentar e cinetoplasto em forma de bastão, sendo parasitos intracelulares obrigatórios e se multiplicando no interior de vacúolos parasitóforos em células

do sistema fagocítico-mononuclear dos hospedeiros mamíferos. As formas promastigotas são formas alongadas, afiladas, com um único flagelo que lhes conferem motilidade, cinetoplasto localizado entre a porção anterior e o núcleo e são encontradas no interior do trato gastrointestinal do vetor transmissor. (Sacks & Kamhawi, 2001; Sacks & Sher, 2002).

A transmissão do parasito do hospedeiro mamífero para o vetor ocorre a partir da realização do repasto sanguíneo pela fêmea em um hospedeiro infectado, podendo assim ingerir, juntamente com o sangue, formas amastigotas livres ou células fagocíticas infectadas. Ao alcançarem o intestino do vetor, essas células se rompem e liberam os parasitos, que rapidamente irão se transformar em formas promastigotas procíclicas e migrar para o trato digestório médio e anterior do vetor. Os parasitos passam por um processo de multiplicação e assumem a forma paramastigota, e posteriormente sofrem a diferenciação em formas promastigotas metacíclicas, migrando para a parte anterior do aparelho bucal do inseto.

Quando o vetor já infectado realiza um novo repasto sanguíneo, o mesmo pode regurgitar formas promastigotas metacíclicas juntamente com a saliva, que por apresentarem elevada motilidade e capacidade migratória irão penetrar na derme do hospedeiro mamífero não infectado. Em seguida, as células do sistema fagocítico-mononuclear do hospedeiro, sendo principalmente macrófagos, migram para o local podendo fagocitar o parasito que, no interior de fagolisossomos nessas células, irão se diferenciar em formas amastigotas, que por sua vez irão se replicar. A replicação dos parasitos pode resultar no rompimento das células, liberando assim as formas amastigotas no organismo do hospedeiro. Os parasitos liberados podem ser novamente fagocitados, levando à continuidade da infecção e ao desenvolvimento da doença no hospedeiro mamífero. Dessa forma, um novo vetor não infectado pode ingerir as formas do parasito durante o repasto, completando assim o ciclo biológico do parasito (Pessoa & Martins, 1988; Medeiros *et al.*, 2005).

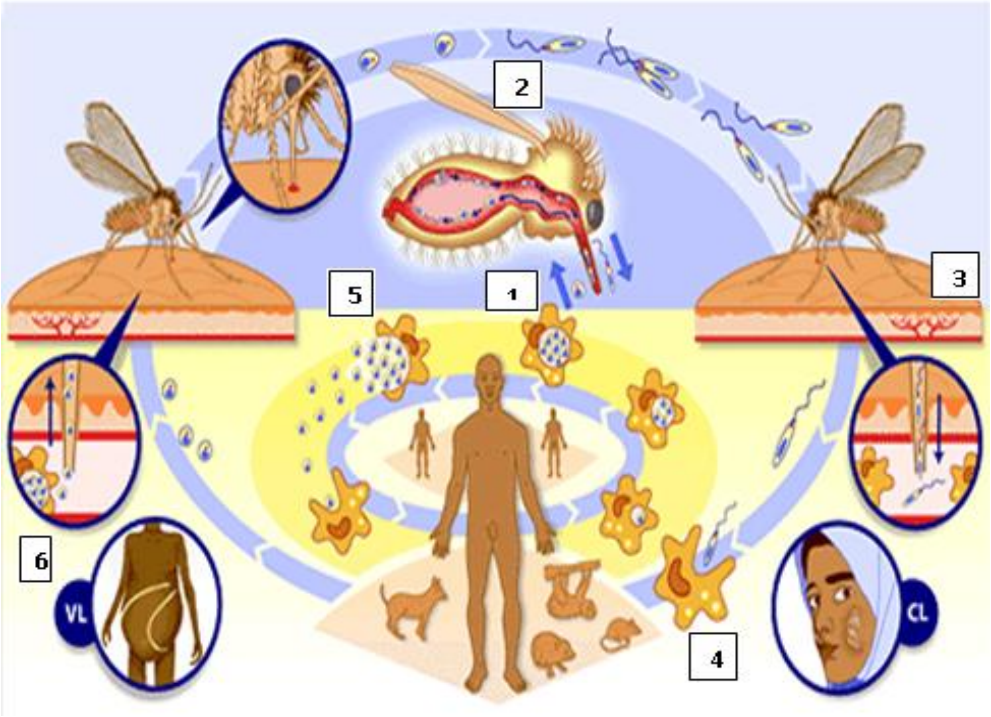

Figura 1: Ciclo biológico do parasito *Leishmania spp.* 1. Realização do repasto sanguíneo em um hospedeiro infectado e infecção do inseto vetor fêmea pela ingestão de formas amastigotas. 2. Diferenciação de formas amastigotas em promastigotas metacíclicas no interior do vetor. 3. Introdução de formas promastigotas metacíclicas na derme do hospedeiro através da picada do vetor. 4. Fagocitose dos parasitos por macrófagos do hospedeiro, onde ocorre a diferenciação em amastigotas e proliferação. 5. Rompimento da célula hospedeira com liberação das amastigotas podendo ser fagocitadas por novas células. 6. Disseminação dos parasitos para órgãos do hospedeiro mamífero, exemplificando a ocorrência da LV, e possibilidade de um novo repasto sanguíneo pelo inseto vetor. (Adaptado de WHO, 2003).

2.3 Manifestações clínicas da doença

As leishmanioses apresentam dois grandes espectros clínicos: a leishmaniose visceral (LV) e a leishmaniose tegumentar (LT) que, por sua vez, pode apresentar três tipos clínicos: a leishmaniose cutânea (LC), leishmaniose cutâneo-difusa (LCD) e a leishmaniose mucocutânea (LMC). A patogenia das doenças é determinada por fatores relacionados ao hospedeiro, como características genéticas e resposta imunológica, por fatores do parasito, como a virulência da espécie infectante e por fatores relacionados ao inseto vetor, como componentes salivares que portam os parasitos quando na infecção no homem. O resultado desta interação pode levar desde a resistência à infecção, passando pelo desenvolvimento da forma assintomática da doença, até a ocorrência da doença cutânea ou visceral aguda (Kane & Mosser, 2000).

A LC ocorre em cerca de 90% dos pacientes diagnosticados com a doença tegumentar sendo então a forma clínica mais encontrada da doença. A doença, geralmente, manifesta-se em partes mais expostas do corpo, como face, braços e pernas. A lesão apresenta-se como um

nódulo no local da picada do vetor que evolui formando uma úlcera granulosa contornada por uma borda elevada. Na maioria das vezes a lesão é única, mas nódulos satélites podem ocorrer nas proximidades da lesão inicial. Quando as úlceras são curadas, deixam cicatrizes permanentes, as quais podem levar a uma condição de morbidade. As principais espécies que podem causar a LC são: *L. braziliensis, L. amazonensis, L. mexicana, L. guyanensis* e *L. panamensis,* no Novo Mundo; e *L. major, L. tropica* e *L. infantum* em países do Velho Mundo (Stebut, 2014).

A LCD é uma forma rara e grave da doença, na qual as lesões apresentam-se como nódulos, de forma anérgica e que raramente ulceram. A ocorrência de lesões que se espalham por todo o corpo pode estar relacionada à uma deficiência na resposta imune celular do hospedeiro (Desjeux, 2004). A LCD, não apresenta cura espontânea, sendo de difícil tratamento. A ocorrência de recidivas frequentes tornou a doença um grave problema de Saúde Pública, pois os pacientes apresentam lesões desfigurantes e incapacitantes, excluindo-os do seu meio de vida social (Gontijo & Carvalho, 2003; Desjeux, 2004). A doença pode ser causada pelas espécies *L. mexicana, L. braziliensis, L. guyanensis* e *L. amazonensis* no Novo Mundo e por *L. aethiopica e L. major* no Velho Mundo (Stebut, 2014).

A LMC ocorre em diversos países no mundo, sendo causada pelas espécies *L. braziliensis, L. peruviana, L. panamensis* e *L. guyanensis* (Stebut, 2014). A doença, normalmente, manifesta-se com lesões de caráter infiltrante. A mucosa das cavidades nasal, da boca, faringe, laringe e traqueia são as mais afetadas e podem levar à lesões mutilantes no paciente. Esta manifestação da doença pode gerar uma elevada rejeição do paciente perante a sociedade, uma vez que gera uma grande deformidade física no local da lesão (WHO, 2015).

A LV ocorre pela infecção com as espécies *L. donovani, L. infantum* e *L. tropica* em países do Velho Mundo e por *L. infantum* (*syn*. *L. chagasi*) no Novo Mundo (Stebut, 2014). A infecção atinge, principalmente, órgãos como baço, fígado, órgãos linfoides e medula óssea. Após um período de incubação, que geralmente varia entre dois a seis meses, indivíduos sintomáticos apresentam sinais de uma infecção sistêmica e persistente, como febre, fadiga, fraqueza, perda de peso, hepatoesplenomegalia e linfadenopatia, dentre outros sintomas clínicos (Chappuis *et al*., 2007). Devido ao fato da intensidade das manifestações clínicas da doença ser variável, muitos indivíduos podem permanecer assintomáticos por um longo tempo, o que dificulta seu diagnóstico clínico, embora cerca de 20% dos pacientes infectados

desenvolvam a forma aguda e sintomática da doença. Os sintomas são progressivos e as complicações decorrentes da evolução da infecção são responsáveis pela elevada letalidade da doença (Badaró *et al*., 1986; Gama *et al.,* 2004).

A LV canina (LVC) causa manifestações clínicas diversas, que dependem de vários fatores, tais como resposta imune do cão e a espécie do parasito. O período de incubação da doença pode variar de poucos meses a alguns anos, entretanto, cães infectados podem permanecer assintomáticos por longos períodos de tempo, ainda que permaneçam infectivos durante tal época (Lanotte *et al*., 1979; Keenan *et al*., 1984). As manifestações clínicas mais comuns da LVC são: linfoadenomegalia, enfraquecimento crônico, alopecia, úlceras, dermatite esfoliativa, onicogrifose, anemia, hepatoesplenomegalia, disfunção renal severa, hipergamaglobulinemia e colites (Abranches *et al*., 1991; Ferrer *et al*., 1991; Ciaramella *et al*., 1997; Tafuri *et al*., 2001).

2.4 Diagnóstico das leishmanioses

O diagnóstico das leishmanioses é realizado com base em evidências clínicas e epidemiológicas, em conjunto com exames laboratoriais (Tesh, 1995). O diagnóstico parasitológico, por meio de amostras de lesões, ou da punção de aspirados dos linfonodos ou da medula óssea, embora invasivos, mostram-se conclusivos na identificação dos parasitos através de análises microscópicas em lâminas e/ou culturas *in vitro* dos materiais coletados (Reithinger *et al*., 2007).

O diagnóstico imunológico pode ser realizado através do teste de intradermoreação de Montenegro (IDRM), tendo sido o primeiro método de diagnóstico laboratorial utilizado em diversas regiões no mundo, se baseia na resposta celular induzida pela memória imunológica de células T que são ativadas em indivíduos que tiveram contato prévio com o parasito. O IDRM, entretanto, pode apresentar especificidade reduzida, levando a detecção de resultados falso-positivos, com indivíduos não infectados de áreas endêmicas, com doença de Chagas, esporotricose, hanseníase ou tuberculose. Outra desvantagem do IDRM é que indivíduos imunosuprimidos, com a forma difusa da doença ou ainda com lesões recentes, podem ser diagnosticados como falso-negativos (Weigle *et al*., 1991; de Paiva-Cavalcanti *et al*., 2015).

Para o diagnóstico sorológico, testes baseados na detecção de anticorpos e/ou antígenos específicos em amostras de soro ou plasma de indivíduos como, por exemplo, o

ensaio imunoenzimático (ELISA), o ensaio de imunofluorescência indireta (IFAT), o teste de aglutinação direta (DAT), o Western-blot e o teste imunocromatográfico (ICT) podem também ser utilizados no diagnóstico das leishmanioses. Porém, esses testes também podem apresentar variações na sensibilidade e/ou especificidade (Marzochi & Marzochi, 1994; Tavares *et al*., 2003; de Paiva-Cavalcanti *et al.*, 2015).

Testes de diagnóstico molecular, como a Reação em Cadeia da Polimerase (PCR) e a PCR-*Real Time* também têm sido utilizados para a detecção de ácidos nucleicos do parasito. Entretanto, muitas vezes, há a ocorrência de problemas relacionados ao teste, como diferenças nos fragmentos do material coletado, que podem não conter parasitos prejudicando sua sensibilidade, bem como a necessidade de treinamento técnico especializado e a possibilidade de contaminação com agentes externos que podem ocasionar resultados falso-positivos. Além disso, tratam-se de testes que apresentam custo elevado (Sundar & Rai, 2002; Srivastava *et al*., 2011; de Paiva-Cavalcanti *et al.*, 2015).

Devido ao elevado número de cães com LV assintomática atuando como reservatórios do parasito e potenciais transmissores dos mesmos, a detecção sorológica dos mesmos é de extrema importância para o diagnóstico correto, para a realização de estudos epidemiológicos e para um controle mais efetivo da doença (Molina *et al.,* 1994; Costa-Val *et al.,* 2007; Michalsky *et al.,* 2007). A sensibilidade da técnica de ELISA utilizando extratos proteicos obtidos de formas promastigotas de *Leishmania spp.* não é satisfatória para a detecção dos casos de LVC assintomática (Porrozzi *et al.,* 2007; Miro *et al.,* 2008). A especificidade deste teste também é reduzida, confirmada pela ocorrência de resultados falso-positivos devido à reatividade cruzada com anticorpos dos soros de cães com doença de Chagas, ehrlichiose, babesiose ou toxoplasmose (Kar, 1995; Ferreira *et al.*, 2007; Porrozzi *et al.,* 2007). Outro problema relacionado à especificidade dos testes é que a mesma deva ser elevada para a diferenciação de amostras de soros de cães saudáveis vacinados de animais infectados. No entanto, muitas vezes, animais vacinados são detectados como falso-positivos. Diversas proteínas recombinantes dos parasitos vêm sendo testadas como antígenos para ELISA, visando minimizar esses problemas com o desenvolvimento de um teste de diagnóstico mais sensível e específico para LVC (Kubar & Fragaki, 2005).

No Brasil, o teste de ELISA foi utilizado como triagem, enquanto o RIFI foi usado como teste confirmatório para a LVC. Entretanto, por meio de uma Nota Técnica Conjunta, publicada em 01/2011, o Ministério da Saúde substituiu o protocolo de diagnóstico da

doença, passando a recomendar a utilização do teste rápido DPP® (Dual-Path Platform, Biomanguinhos, Fiocruz, Rio de Janeiro, Brasil) como método de triagem e o teste de ELISA (EIE-LVC kit, Biomanguinhos, Fiocruz, Rio de Janeiro, Brasil) como método confirmatório (Faria & Andrade, 2012; Coura-Vital *et al*., 2014; Laurenti *et al.*,2014). O DPP trata-se de um teste imunocromatográfico composto pelos antígenos recombinantes rK39 e rK26, e que apresenta elevada sensibilidade e especificidade na detecção de cães sintomáticos. Entretanto, em relação à detecção de animais assintomáticos, o teste apresenta uma variação em sua eficácia, com uma elevada redução na sensibilidade (Grimaldi *et al*., 2012). O teste confirmatório de ELISA, por sua vez, apresenta uma elevada sensibilidade em identificar cães doentes, entretanto, a especificidade do teste pode ser reduzida, uma vez que reações cruzadas podem ocorrer acusando resultados falso-positivos (Faria & Andrade, 2012). A ocorrência de variações de sensibilidade e especificidade nos testes diagnósticos pode prejudicar a detecção de cães com leishmaniose, gerando resultados falso-negativos, sendo estes, cães que permanecerão transmitindo a doença ou também ocasionar resultados falso-positivos em animais saudáveis, podendo acabar sacrificados em vão devido a tais erros nos testes (Werneck, 2014). Devido a esse fato, estudos buscando antígenos que apresentem melhores valores de sensibilidade e especificidade para a detecção desses animais, são de extrema relevância no cenário atual.

2.5 Medidas de prevenção e controle das leishmanioses

As medidas de prevenção e controle para as leishmanioses visam a interrupção do ciclo biológico do parasito, entretanto, o número elevado de espécies de *Leishmania spp.*, o caráter zoonótico da doença e a manutenção do seu ciclo silvestre dificultam a adoção de medidas eficazes de controle (Tesh, 1995).

O controle do inseto vetor pode ser realizado através da administração de inseticidas em ambientes domésticos, entretanto, a eficácia depende da classe de inseticida utilizada, da susceptibilidade do inseto vetor, do tipo de superfície ao qual o mesmo é aplicado e da dosagem e método de aplicação. Para que esta medida de controle seja eficiente, é necessário que se tenha procedimentos operacionais padrão, gerenciamento, logística, supervisão, monitoramento e avaliação da eficácia em determinados períodos de tempo. Algumas classes de inseticidas utilizadas são os organoclorados, organofosfatados, carbamatos e piretróides, e estes devem ser trocados em determinados períodos de tempo para que se evite o

aparecimento de insetos vetores resistentes (WHO, 2010). Entretanto, tal medida é pouco efetiva na redução do número de casos da doença.

No caso da doença humana, o controle consiste na detecção dos casos, no diagnóstico correto e no tratamento efetivo dos pacientes. Medidas de prevenção à reinfecção, como o uso de repelentes individuais e telas de proteção nas casas também devem ser empregadas. Tais medidas podem reduzir a transmissão da doença, mas não geram um impacto significativo (Grimaldi & Tesh, 1993; WHO, 2010).

Em relação à LVC, a principal forma de controle está relacionada ao cão, sendo ele o principal reservatório doméstico dos parasitos, e uma importante fonte de infecção para o vetor transmissor. Órgãos de Saúde Pública são responsáveis por realizar a triagem dos animais através da detecção de sinais clínicos e de testes sorológicos, bem como realizar a eutanásia de animais sintomáticos e assintomático visando a redução da transmissão do parasito. No entanto, tal medida apresenta falhas devido a recusa de novos a fazerem o teste em seus cães e à não autorização do procedimento de eutanásia em animais doentes. A ocorrência de uma rápida reposição de animais em áreas endêmicas, juntamente com a realização do tratamento de animais que ainda assim não eliminam totalmente o parasito, também contribuem para a ineficiência das medidas e dificultam a redução do número de casos da doença (Gontijo & Melo, 2004; WHO, 2010). A utilização de inseticidas tópicos ou impregnados em coleiras e a vacinação podem ser estratégias preventivas com algum impacto no controle da LVC (Werneck, 2014). Em relação aos hospedeiros selvagens, a eliminação não é uma medida executável ou ecologicamente correta. Além disso, existe a possibilidade de adaptação do parasito a outros reservatórios existentes em ambientes rurais (Grimaldi & Tesh, 1993; Gramiccia & Gradoni, 2005).

Segundo o Ministério da Saúde, além das medidas preventivas relacionadas diretamente ao controle do vetor e do hospedeiro reservatório, investimentos em programas de saneamento, educação social e conscientização ambiental também são de extrema importância em auxiliar o controle das leishmanioses.

2.6 Tratamento das leishmanioses

O tratamento das leishmanioses visa evitar a mortalidade causada pela LV e reduzir a morbidade provocada pelas lesões desfigurantes das formas mais graves de LT. Os fármacos mais utilizados para o tratamento atual das leishmanioses são os antimoniais pentavalentes, sendo eles o antimoniato de N-metil meglumina, produzido com o nome comercial de

Glucantime®pela empresa Rhône Poulenc Rorer (França) e o estibogluconato de sódio, produzido com o nome comercial de Pentostan® pela Wellcome Foundation (Inglaterra). Tais fármacos, entretanto, são tóxicos e causam efeitos colaterais aos pacientes (Figueiredo *et al*., 1999; Balasegaram *et al*., 2012). O tratamento é relativamente longo, durando cerca de 40 dias, além de ser de via de administração dolorosa podendo causar fadiga, artralgias, mialgias, além de toxicidade renal, hepática e cardíaca nos pacientes, que muitas vezes abandonam o tratamento, podendo levar a resistência dos parasitos aos fármacos utilizados (Grogl *et al.*, 1991; Carvalho *et al.*, 2000; Tavares *et al.*, 2003).

A anfotericina B constitui a droga de segunda escolha para o tratamento da doença, porém, seu uso também provoca efeitos colaterais e tóxicos aos pacientes como febre, cefaleias, náuseas, vômitos, diarreia, anorexia, mal-estar geral, mialgias, artralgias, flebite no local da infusão, anemia hemolítica e nefrotoxicidade. Sua formulação lipossomal (AmBisome™) , apresenta-se menos tóxica, porém seu custo é elevado e há necessidade de ser administrada em ambiente hospitalar (Balasegaram *et al*., 2012).

A miltefosina trata-se do primeiro medicamento oral utilizado para o tratamento da LV, entretanto, distúrbios gastrointestinais, nefro e hepatotoxicidade e teratogenicidade foram descritos, além de também apresentar custo elevado (Balasegaram *et al*., 2012).

Pacientes com lesões severas, infecções recorrentes, disseminadas ou com a forma visceral da doença, devem ser tratados sistemicamente. Entretanto, pacientes com lesões locais e menos complexas devem ser tratados apenas com métodos locais, a fim de se evitar os efeitos colaterais gerados pela maioria dos medicamentos utilizados. É recomendado que pacientes tratados façam um acompanhamento podendo durar de meses a anos, para acompanhar a ocorrência de uma possível recidiva da doença (WHO, 2010; Stebut, 2014).

Devido aos problemas relacionados à eficiência das medidas de controle e dos métodos de prevenção das leishmanioses, bem como por problemas de sensibilidade e especificidade dos testes diagnósticos e pelas dificuldades do tratamento da doença; o desenvolvimento de novas medidas profiláticas, tais como a busca por vacinas, torna-se uma alternativa atrativa e vem sendo muito pesquisada; entretanto, essa é uma tarefa complexa, embora seja considerada uma solução real e com melhor custo- benefício para o controle efetivo e prevenção da doença (Grimaldi & Tesh, 1993; Gonzalo *et al*., 2002; Sukumaran *et al*., 2003; Ramiro et al., 2003; Desjeux, 2004; Palatnik-de-Sousa *et al.*, 2012).

2.7 Resposta imune na leishmaniose murina

O modelo murino utilizando camundongos é o mais estudado para avaliação de antígenos vacinais e de novos medicamentos para o tratamento das leishmanioses. A escolha da espécie de camundongo para cada estudo varia com a espécie de *Leishmania* e o tipo de infecção a ser avaliada. Algumas linhagens de camundongos, como C57BL/6, C57BL/10, C3H e CBA apresentam um perfil de resistência à espécie *L. major*, mesmo quando um grande número de parasitos é inoculado pela via subcutânea nos animais; enquanto outras linhagens, como o BALB/c, apresentam um perfil de susceptibilidade à infecção por *L. major*, *L. amazonensis* ou *L. infantum*, entretanto, desenvolvem lesões limitadas quando infectados por *L. brasiliensis* (de Luca & Macedo, 2016). Camundongos BALB/c são utilizados como modelo de infecção para o estudo de candidatos vacinais contra várias *Leishmania spp.*, tais como *L. infantum* e *L. amazonensis* (Sacks & Noben-Trauth, 2002; Wilson *et al*., 2005). O perfil da resposta imune em camundongos BALB/c infectados experimentalmente pode variar de acordo com o inóculo de parasitos, a via e o tempo de infecção (Oliveira *et al*., 2012).

A resistência ou a susceptibilidade dos camundongos à infecção deve-se, principalmente, aos fatores genéticos dos animais. Camundongos geneticamente resistentes à LV, como o CBA, apresentam o gene *Slc1 1a1* funcional, o qual confere imunidade inata contra o parasito e possibilita o controle da infecção. Camundongos susceptíveis à LV, como BALB/c, apresentam uma mutação neste gene, o que permite a replicação de parasitos no fígado durante as primeiras semanas de infecção (Das & Ali, 2012; Kumar & Nylén, 2012).

As respostas imunes inata (macrófagos, células dendríticas e neutrófilos) e adaptativa (células T), são responsáveis pelo controle da infecção no hospedeiro mamífero. As células T $CD4^+$ assumem um papel crucial na resposta à infecção. Ao se diferenciarem em células Th1, produzem citocinas pró-inflamatórias que possuem um papel importante na resposta protetora e na resolução da infecção (Kedzierski e Evans, 2014). As células T $CD8^+$, por sua vez, contribuem na aquisição de uma resposta de memória na doença (Stager *et al*., 2000).

Estudos de infecção com modelos experimentais de camundongos para leishmaniose deram origem ao paradigma Th1/Th2 de resistência e susceptibilidade associado à infecção celular. Este perfil da resposta imune encontra-se mais relacionado à LT, causada pelo parasito *L. major*, não sendo bem definido para LV (Kedzierski e Evans, 2014). Na LV murina a proteção depende da produção de IL-12, induzindo uma resposta do tipo Th1, mediada pela produção de IL-2 e IFN- ɣ. O IFN-ɣ por sua vez, induz a ativação enzima iNOS levando à produção de NO pelos macrófagos do baço e fígado, permitindo o controle da

multiplicação dos parasitos nestes órgãos (Green *et al*., 1990; Blackwell, 1996). Citocinas como a IL-17 e IL-22 complementam o papel de proteção na LV, porém, ainda não se conhecem os mecanismos de atuação das mesmas (Faleiro *et al*., 2014).

Níveis elevados das citocinas anti-inflamatórias, como TGF-β, IL-4, IL-10 e IL-13, normalmente, ocasionam a supressão da resposta imune Th1, direcionando para o desenvolvimento de uma resposta Th2, responsável por um perfil de susceptibilidade à infecção (Wilson *et al*., 2005). Entretanto, baixas concentrações de IL-4 melhoram a proteção induzida por uma vacina, aumentando indiretamente a produção de IFN-ɣ por LT $CD8^+$ e impedindo uma exacerbação da infecção por *L. donovani* (Stager *et al.*, 2003).

Em camundongos, a depuração da carga parasitária hepática ocorre cerca de duas semanas após a infecção, devido à formação de granulomas resultantes da interação de células T com células dendríticas e macrófagos parasitados por *Leishmania spp*. A eficácia de uma vacina ou a progressão da doença pode ser predita, baseando-se no grau de maturação dos granulomas hepáticos juntamente com a imunidade mediada por células nos animais desafiados (Murray *et al*., 1992; Carrión *et al*., 2006). Em contraste com a resposta ocorrida no fígado, o baço e a medula óssea agem como locais de persistência dos parasitos, resultando na infecção crônica. A ausência de reação granulomatosa no baço e a produção reduzida de IL-12 pelos macrófagos locais direcionam para um aumento da carga parasitária neste órgão (Nieto *et al*., 2011).

Em relação à resposta humoral, observa-se que um aumento da produção de anticorpos do isotipo IgG2a ocorre em animais que apresentam um perfil de resistência; enquanto que o perfil de susceptibilidade e progressão da doença se caracteriza pelo predomínio de anticorpos do isotipo IgG1 específicos aos antígenos dos parasitos (Martins *et al*., 2013; Costa *et al*., 2014). Entretanto, alguns autores consideram que os anticorpos não desempenham papel de proteção contra a infecção por *Leishmania spp*., podendo inclusive contribuir para progressão da doença (Bohme *et al*., 1986; Coelho et al., 2003; Kedzierski e Evans, 2014).

2.8 Desenvolvimento de vacinas

A pesquisa por vacinas contra as leishmanioses provém do fato de que indivíduos curados de lesões cutâneas desenvolvem uma resposta imune frente aos parasitos e tornam-se protegidos contra a reinfecção pela espécie infectante (Grimaldi & Tesh, 1993; Handman, 2001). Diversas preparações já foram propostas e estudos têm sido conduzidos com a finalidade de se desenvolver uma vacina que apresente resultados satisfatórios na proteção

contra a infecção por *Leishmania spp*.

Uma das primeiras estratégias vacinais foi a leishmanização, usada a partir do ano de 1908 em combate às leishmanioses no Oriente Médio, União Soviética e Israel. Na leishmanização, uma baixa quantidade de parasitos vivos é inoculada em uma região não exposta do corpo, com o objetivo de que uma lesão cutânea local se desenvolva e que haja a cura espontânea, conferindo ao indivíduo uma imunidade protetora contra a reinfecção. Contudo, esta técnica foi abandonada por muitos países devido ao risco de se causar uma infecção crônica, levando o indivíduo a necessitar de tratamento; dando lugar à utilização de parasitos mortos como estratégia vacinal (Handman, 2001; Kumar & Engwerda, 2014).

A utilização de parasitos irradiados ou autoclavados para compor uma vacina foi descontinuada devido aos resultados conflitantes de efetividade obtidos na década de 1940 (Grimaldi & Tesh, 1993; Handman, 2001). Devido aos resultados obtidos em diversos experimentos realizados com parasitos mortos, foi observado que a persistência do patógeno no organismo do indivíduo tivesse certa importância para se obter uma resposta imune protetora e assim, foi sugerido que doses de vacinas de parasitos vivos atenuados virassem uma nova alternativa (Selvapandiyan *et al*., 2014).

Vacinas com parasitos vivos atenuados podem ser obtidas através de culturas *in vitro* com meios modificados, alterações de temperatura, irradiação gama ou mutações genéticas (Foroughi-Parvar & Hatam, 2014). Este tipo de vacina vem sendo testado em alguns modelos animais, demonstrando que os parasitos modificados são reconhecidos pelas células do hospedeiro da mesma forma que os parasitos virulentos e que conseguem permanecer no organismo por algum tempo, sem se replicarem. O sistema imune do hospedeiro consegue montar uma resposta específica contra os antígenos devido à presença desse parasito e dessa forma, células efetoras e de memória geradas podem conferir proteção. Uma das preocupações com a utilização desta vacina é a possível reversão da virulência dos parasitos, entretanto, uma alternativa para a resolução deste problema seria a eliminação de genes de virulência, permitindo que tais candidatos vacinais sejam estudados. A utilização de parasitos mutantes sensíveis a determinadas drogas, bem como a utilização de espécies não patogênicas, como *L. tarentolae*, seriam também possíveis alternativas, porém, ainda deve-se priorizar a segurança da vacina atentando-se para a possibilidade de que parasitos vivos estão susceptíveis a mutações espontâneas, além de se considerar a dificuldade de produção e análise de qualidade em larga escala (Kumar & Engwerda, 2014).

A utilização de frações proteicas de *Leishmania spp*. além de apresentar-se mais

segura, têm apresentado propriedades imunoprotetoras em modelos experimentais (Das & Ali, 2012). Vacinas utilizando extratos brutos dos parasitos, proteínas recombinantes purificadas, peptídeos sintéticos e frações de DNA inseridos em plasmídeos, vêm também sendo testadas com diferentes sistemas de entrega.

A formulação vacinal LBSap, baseada em extrato bruto de *L. brasiliensis* adicionada de saponina, foi administrada em cães e demonstrou ser capaz de induzir uma resposta humoral com anticorpos específicos anti-*Leishmania*, além de induzir um perfil celular ativando LT $CD4^+$ e LT $CD8^+$. Uma elevada produção de IFN-ɣ e IL-12 bem como uma redução na carga parasitária do baço demonstraram sua imunogenicidade e proteção contra a infecção experimental por *L. infantum* (Roatt *et al.*, 2012; Resende *et al*., 2013). Uma vacina similar foi também testada com extrato bruto de *L. amazonensis* e BCG, nomeada de Leishvaccine®. Esta formulação foi capaz de estimular uma ativação inicial de LT $CD4^+$ e uma ativação tardia de LT $CD8^+$ e LB, demonstrando um elevado potencial imunogênico contra as leishmanioses (Araújo *et al*., 2008).

Na Europa, a utilização de proteínas *L. infantum* secretadas e purificadas do sobrenadante da cultura também estão sendo aplicadas no desenvolvimento de vacinas. A formulação denominada LiESAP-MDP recebeu o nome comercial de CaniLeish® e apresentou eficácia de 92% nos animais vacinados (Lemesre *et al.*, 2007). Experimentos utilizando o composto ligante fucose-manose (FML) de formas promastigotas de *L. donovani* em conjunto com um adjuvante indutor de resposta Th1 apresentou resultados de imunogenicidade, imunoprofilaxia e imunoterapêutica em camundongos, hamsters e cães (Palatnik-de-Sousa *et al*., 1994; Santos *et al*., 2002, 2003, 2007; Borja-Cabrera *et al*., 2004, 2008, 2010), dando origem a vacina denominada Leishmune®, composta por FML-saponina, que foi capaz de induzir proteção na maioria dos animais vacinados (Palatnik-de-Sousa, 2012), tendo sido usada por muitos anos no Brasil, mas que agora foi retirada do mercado.

Vacinas com antígenos recombinantes podem ser consideradas em relação à eficiência, segurança e custo (Foroughi-Parvar & Hatam, 2014). Com o avanço na tecnologia do DNA recombinante, moléculas específicas de determinada espécie ou fase de vida do parasito estão sendo extensivamente estudadas, originando vários candidatos à vacina (Joshi *et al.*, 2014). O primeiro antígeno recombinante utilizado para imunização contra as leishmanioses foi a glicoproteína gp63, considerada uma proteína de virulência dos parasitos e encontrada conservada em todas as espécies de *Leishmania spp*. Tanto a

proteína nativa quanto a recombinante foram capazes de estimular uma resposta protetora em camundongos, demonstrando que esta imunogenicidade está relacionada ao processamento e apresentação de peptídeos e não apenas da estrutura conformacional nativa (Das & Ali, 2012).

Proteínas de *Leishmania spp*. como LCR1, A2, HASPB1 foram expressas em sua forma recombinante e testadas contra LV. A LCR1 é uma proteína específica da forma amastigota de *L. infantum* capaz de estimular a produção de IFN-ɣ e de proteger parcialmente camundongos BALB/c contra a infecção por esta espécie (Wilson *et al*., 1995). Stager e colaboradores (2000) confirmaram que a proteína rHASPB1 é altamente imunogênica e que gerou proteção significativa em camundongos desafiados com *L. donovani*. Fernandes e colaboradores (2008) investigaram a imunidade protetora da proteína recombinante A2 em associação com a saponina contra a LVC. Esta formulação tornou-se uma vacina comercial denominada Leish-Tec® e provou induzir um aumento significativo de IFN-ɣ nos cães vacinados, além de reduzida produção de IL-10 (Fernandes *et al*., 2012), sendo a única vacina para a LVC disponível no mercado atualmente no Brasil.

Proteínas específicas da forma promastigota dos parasitos têm demonstrado ser potentes estimuladoras de uma resposta imune do tipo Th1, como foi visto com a PDI, TPI, eIF-2, aldolase, enolase, P45 e TPR, as quais levaram à proteção variada de 60 a 90% em hamsters (Joshi *et al*., 2014). Outras proteínas recombinantes foram testadas e demonstram ser boas candidatas à vacina, como a F14 e proteínas ribossomais de *Leishmania spp.*, uma vez que ambas forneceram proteção parcial em hamsters e camundongos BALB/c, respectivamente (Bhardwaj *et al.*, 2009; Chávez-Fumagalli *et al*., 2010).

A estratégia de imunoproteômica vem sendo utilizada para identificar novas proteínas, consideradas ainda como hipotéticas, mas que podem se apresentar como potenciais candidatas à vacina (Coelho *et al*, 2012). Uma destas proteínas, LiHyp1, foi identificada na forma amastigota de *L. infantum* por anticorpos de cães com LV ativa e demonstrou ter um potencial vacinal em camundongos BALB/c, induzindo uma elevada produção das citocinas IFN-ɣ, IL-12 e GM-CSF, e uma produção reduzida de IL-4 e IL-10. A resposta imune frente à proteína foi capaz de induzir uma redução significativa da carga parasitária nos animais desafiados, tendo sido primada pela participação de LT $CD4^+$ (Martins *et al*, 2013).

O desenvolvimento de vacinas recombinantes ou mesmo de peptídeos sintéticos apresentam a vantagem de permitir a utilização de epitopos determinados e já

caracterizados, uma vez que é possível modificar tais moléculas, removendo epitopos desnecessários para o desenvolvimento de uma imunidade protetora (Handman, 2001; Skeiky *et al.,* 2002). Para o desenvolvimento de uma vacina peptídica, o estudo dos epitopos através de ferramentas de bioinformática e o conhecimento em relação à ativação de LT e LB são de grande importância.

As vacinas com peptídeos sintéticos podem apresentar algumas vantagens em relação aos outros compostos vacinais como, por exemplo, a ausência de material potencialmente infeccioso, possibilidade de inclusão de diversos e determinados epitopos, diminuição na quantidade de antígenos utilizados, dentre outras (Joshi *et al*., 2014). Entretanto, os peptídeos podem também apresentar desvantagens, como a incapacidade de determinados indivíduos a responderem ao estímulo ofertado. Vacinas com peptídeos isolados normalmente são incapazes de induzir uma resposta protetora, sendo necessária uma mistura a outros antígenos para que possam estimular esta resposta e/ou o uso de adjuvantes e diferentes sistemas de entrega (Handman, 2001). Peptídeos extraídos de proteínas muito estudadas, como a gp63, KMP-11, A2, LPG, cisteino-proteinases, dentre outras, vêm sendo utilizados para compor vacinas, uma vez que têm apresentado resultados satisfatórios em camundongos (Joshi *et al*., 2014). Mais recentemente, a utilização da técnica de phage diplay selecionou mimotopos de *Leishmania spp*. em clones de bacteriófagos, os quais foram capazes de induzir uma resposta imunogênica com elevada concentração de IFN-ɣ, produzido principalmente por LT $CD8^+$ e pela reduzida produção de IL-4, tendo sido capaz de proteger camundongos BALB/c contra a infecção experimental por *L. infantum* (Costa *et al*., 2014).

Vacinas de DNA também são atrativas em compor uma estratégia profilática contra as leishmanioses. Este método pode apresentar vantagens, como o custo reduzido, facilidade na produção e armazenamento, estabilidade do material, expressão de antígenos relevantes e eficiente indução de uma resposta imune efetora e de memória (Handman, 2001; Kumar & Engwerda, 2014). Questões relacionadas à segurança das vacinas de DNA são discutidas, como a possibilidade da integração do DNA ao genoma de mamíferos e a indução ao desenvolvimento de doenças autoimune, entretanto, nada foi comprovado até o momento. Vacinas de DNA contendo genes que expressam a proteína A2 (Zanin *et al.*, 2007), PPG (Samant *et al.*, 2009) ou HbR (Guha *et al.*, 2013) provaram estimular uma resposta do tipo Th1, com níveis elevados de IFN-ɣ e reduzida produção de IL-4; resultando na proteção de camundongos e hamsters imunizados contra infecções

experimentais com diferentes espécies de *Leishmania spp*.

Alguns indivíduos não infectados, mas expostos ao inseto vetor, já apresentarem uma resposta protetora contra a infecção pelo parasito e, devido a esse fato, vacinas utilizando proteínas salivares de *Phlebotomus spp*. ou *Lutzomyia spp.*, como PpSP15, LJM17 ou LJM143 vêm sendo estudadas como potentes imunógenos, sendo capazes de estimular infiltração de linfócitos, macrófagos e uma produção aumentada de IFN-ɣ (Morris *et al*., 2001; Joshi *et al*., 2014).

Outras estratégias vacinais contendo diferentes veículos de entrega, como lipossomas (Firouzmand *et al*., 2013), nanopartículas (Danesh-Bahreini *et al*., 2011), vetores virais (Guha *et al*., 2013), células dendríticas (Matos *et al*., 2013) e bactérias (Hugentobler *et al*., 2012) têm resultado em graus variados de eficiência, assim como uma variação na utilização dos diferentes adjuvantes vacinais (Vitoriano-Souza *et al*., 2012; De Jesus Pereira *et al*., 2015; Thakur *et al*., 2015).

Deste modo, a determinação dos antígenos, do veículo de entrega, do adjuvante e da via de administração da vacina são de extrema importância ao se definir a estratégia vacinal a ser utilizada para compor uma vacina contra as leishmanioses.

2.9 Utilização da saponina como adjuvante de resposta imune

Adjuvantes são substâncias capazes de iniciar uma resposta imune em relação a um dado antígeno ou auxiliar no desenvolvimento de uma resposta já existente (Claassen & Boersma, 1992). A associação de adjuvantes de resposta imune à vacinas de subunidades é de grande importância para que os mesmos auxiliem na ativação da resposta do sistema imune inato e adaptativo após o contato com o antígeno vacinal (Rueckert & Guzmán, 2012). Os adjuvantes proporcionam um aumento da imunogenicidade de antígenos mais fracos, sendo capazes também de reduzir a quantidade de antígeno necessária para a imunização, bem como o número de doses a serem administradas. Além disso, podem induzir um fenótipo de citocinas favorável para uma resposta imune benéfica ao hospedeiro, além de induzir uma resposta imune prolongada (Vitoriano-Souza *et al*., 2012).

As saponinas são obtidas a partir da casca da árvore *Quillaja saponária* e apresentam-se eficazes na utilização em experimentos vacinais, como adjuvantes de resposta imune (Liu *et al*., 2002; Ravindran & Ali, 2004). Tratam-se de glicosídeos tensoativos que contém um núcleo hidrofóbico de estrutura triterpenóide, com cadeias de carboidratos ligadas a ele (Rajput et al. 2007). A saponina apresenta uma formulação simples, custo reduzido e é

considerada segura para uso veterinário (Vitoriano-Souza *et al*., 2012). Devido ao fato de possuir efeitos citotóxicos, principalmente hemolíticos, o uso da saponina não é permitido em humanos (Palatnik-de-Sousa *et al.*, 2004).

A saponina é responsável por induzir um rápido recrutamento de células inflamatórias em seu local de administração e esta resposta pode ser mantida por dias após o estímulo (Taylor, 1995). A administração da saponina induz a migração diferencial de neutrófilos, monócitos e linfócitos para o sítio de aplicação, além de induzir a produção das citocinas IL-2, IFN-ɣ e IL-6, (Vitoriano-Souza *et al*., 2012), sendo um adjuvante de resposta do tipo Th1.

O presente trabalho objetivou a utilização da saponina em associação com a proteína recombinante LiHyD, a fim de avaliar a imunogenicidade e a eficácia protetora da mesma contra a infecção experimental de camundongos BALB/c com formas promastigotas estacionárias de *Leishmania spp*.

2.10 Antígeno utilizado no trabalho

A proteína LiHyD (proteína hipotética de *L. infantum* D) (XP_001468360.1), utilizada neste trabalho, foi recentemente identificada por Coelho et al. (2012), tendo sido reconhecida por anticorpos presentes em soros de cães com a doença ativa. A proteína foi identificada no genoma de *L. infantum* e possui uma sequência de 327 aminoácidos, os quais conferem à mesma um peso molecular de aproximadamente 36,7 kiloDaltons (kDa). O gene responsável por sua codificação foi identificado no cromossomo 33 e possui 984 pares de bases (pb). Trata-se de uma proteína específica de *Leishmania* e que é conservada em diferentes espécies do parasito, incluindo espécies causadoras de LT, com valores de identidade de 80% e 56% em relação à *L. major* (LmjF.33.2990) e *L. braziliensis* (LbrM.33.3270), respectivamente.

A partir de análises de bioinformática, confirmou-se a presença de epitopos específicos para linfócitos B e T na proteína e, portanto, a mesma foi selecionada como candidata a antígeno marcador para o diagnóstico da LVC e como candidata a compor uma vacina que ofereça proteção cruzada contra as leishmanioses.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar a proteína hipotética específica de *Leishmania spp.*, LiHyD (LinJ.33.3150), em sua forma recombinante bem como com seus três epitopos específicos de linfócitos B, dois lineares e um conformacional, no sorodiagnóstico da leishmaniose visceral canina. Avaliar a imunogenicidade e eficácia protetora da proteína em camundongos BALB/c contra a infecção experimental com *L. infantum* e *L. braziliensis* e *L. major.*

3.2 Objetivos específicos

- Clonar, expressar e purificar a proteína recombinante LiHyD (rLiHyD).

- Identificar epitopos específicos de linfócitos B da proteína utilizando, para tal, algoritmos e programas de bioinformática disponíveis na internet, para a seleção físico-química e predição das sequências de aminoácidos de interesse, a fim de realizar a construção dos peptídeos selecionados.

- Realizar a síntese química dos peptídeos selecionados, por meio da técnica de Fmoc-*synthesis*.

- Avaliar a reatividade dos anticorpos provenientes das amostras de soros de cães com LV sintomática e assintomática, em soros de cães não infectados e residentes em áreas endêmicas ou não endêmicas da doença; soros de animais não infectados, mas imunizados com a vacina Leish-Tec®; e soros de cães infectados com *Trypanosoma cruzi*, *Ehrlichia canis* e *Babesia canis*; frente à proteína rLiHyD e seus peptídeos sintéticos.

- Avaliar os parâmetros de sensibilidade e especificidade da proteína rLiHyD e de seus peptídeos sintéticos no sorodiagnóstico da LVC.

- Avaliar a proteção induzida pela proteína rLiHyD mais saponina em camundongos BALB/c contra a infecção com *L. infantum* e *L. braziliensis* e *L. major* por meio da avaliação do perfil da resposta imune celular e humoral gerada nos animais imunizados, além da carga parasitária em diferentes órgãos e tecidos dos animais.

4 MATERIAL E MÉTODOS

4.1 Animais de experimentação

Camundongos BALB/c fêmeas, de 8 semanas de idade, foram utilizados. Os animais foram adquiridos no Biotério do Instituto de Ciências Biológicas (ICB) da UFMG e mantidos em estante apropriada no Biotério do Departamento de Patologia Clínica do COLTEC, UFMG, sob as devidas condições de manejo. Este projeto foi aprovado junto ao Comitê de Ética em Experimentação Animal (CEUA) da UFMG, com o protocolo número 333/2015 (ANEXO 1).

4.2 Parasitos

Parasitos das cepas MHOM/BR/1970/BH46 de *Leishmania infantum*, MHOM/IL/1980/Friedlin de *L. major* e MHOM/BR/1975/M2903 de *L. braziliensis* foram cultivados em meio de cultura Schneider's (Schneider's Insect Medium, Sigma) completo, constituído por meio Schneider's acrescido com 20% de soro fetal bovino (SFB) inativado (Sigma), 20 mM de L-glutamina, 200 unidades por mililitro (U/mL) de penicilina e 100 microgramas por mililitro (µg/mL) de estreptomicina, pH 7,4. Os parasitos foram cultivados a 24°C e repiques das culturas foram efetuados de cinco em cinco dias, período que os parasitos apresentavam-se em fase logarítmica de crescimento. Estoques de parasitos foram mantidos em nitrogênio líquido a -196ºC.

4.3 Amostras de soros

Para a avaliação da antigenicidade da proteína rLiHyD, foram utilizadas 177 amostras de soro de cães (*Canis familiaris*), compostas por machos (n=100) e fêmeas (n=77) de diferentes raças e idades. Os soros positivos para LVC foram selecionados com base em dois testes sorológicos da Biomanguinhos (Fiocruz, Brasil): IFAT (IFAT-LVC Bio-Manguinhos kit) e ELISA (EIE- LVC Bio-Manguinhos kit) e pelo teste de PCR. Soros com titulação < 1/40 no IFAT e identificados abaixo do valor do ponto de corte indicado pelo fabricante foram considerados negativos. Animais com titulação de IFAT > 1/40 e valor de absorbância acima do ponto de corte foram considerados positivos. Os animais com LV sintomática (n=44) foram positivos nos testes IFAT, ELISA e PCR, e apresentaram três ou mais sinais clínicos da doença, como perda de peso, alopecia,

adenopatia, onicogrifose, hepatomegalia, conjuntivite e dermatite esfoliativa no focinho, cauda ou pontas das orelhas. Animais assintomáticos (n=9) também apresentaram resultados parasitológicos e sorológicos positivos, entretanto, não apresentavam sinais clínicos da doença. Soros de animais não infectados com *Leishmania spp.* foram selecionados de áreas endêmica (n=44) (Belo Horizonte, Minas Gerais, Brasil) e não endêmica (n=20) (Poços de Caldas, Minas Gerais, Brasil) para as leishmanioses e apresentaram resultados sorológicos, moleculares e clínicos negativos. Amostras de soros de animais não infectados, mantidos isolados em canil e imunizados com a vacina Leish-Tec® (n=30) e de animais experimentalmente infectados com *T. cruzi* (n=10), *E. canis* (n=10) e *B. canis* (n=10) também foram utilizadas nos ensaios.

4.4 Preparo do extrato antigênico de Leishmania infantum, Leishmania major e Leishmania braziliensis (SLALi/SLALm/SLALb)

Os extratos solúveis antigênicos (SLA) de *L. infantum*, *L. major* e *L. braziliensis* foram preparados como descrito previamente por Coelho *et al.* (2003). Uma estimativa da concentração de proteínas foi determinada conforme descrito por Bradford (1976).

4.5 Obtenção e análise das sequências da proteína LiHyD

As sequências de nucleotídeos e aminoácidos da proteína LiHyD (LinJ.33.3150), foram obtidas no banco de dados do TriTrypDB (http://tritrypdb.org). (Figura 2). Para analisar a conservação da proteína dentre o genoma de outros organismos foi realizado um alinhamento através da ferramenta de bioinformática BLAST.

Foi realizada uma análise *in silico* da sequência da proteína LiHyD que consistiu de três etapas: (i) busca por similaridade entre sequências depositadas em bancos de dados de proteínas, (ii) comparação com bancos de dados de outros Tripanosomatídeos, sendo esses *L. major, L. mexicana, L. braziliensis, T. cruzi, T. brucei e T. congolenses* (todos disponíveis em www.genedb.org), e (iii) avaliação da sequência da proteína para análise de suas propriedades físico-químicas usando a ferramenta ProtParam no servidor ExPASy (Gasteiger et al., 2005). Os parâmetros calculados pelo programa e apresentados neste trabalho incluem o peso molecular, o ponto isoelétrico teórico, a composição de aminoácidos, o número total de resíduos positivos e negativos, o coeficiente de extinção, o índice de instabilidade e o índice alifático.

Partindo da sequência gênica codificadora da proteína, um par de iniciadores foi

desenhado para permitir a amplificação do gene de interesse, por meio da técnica de PCR. Os primers *Forward* e *Reverse* (Eurofins mwg/operon), descritos abaixo, foram desenhados de acordo com a sequência da região ORF do DNA genômico para *L. infantum*.

F → (5'-GGATCCATGCSGATGCAAGGCAACATG-3')

R → (5'-AAGCTTATTGTTGCCGCATACTTGG -3')

Dois epitopos de células B lineares e um epitopo conformacional presentes na proteína LiHyD foram sintetizados. Os peptídeos contendo as sequências lineares: Peptídeo-1: PQPGYQPPPPMEHALP, posições 262-277 e Peptídeo-2: SSLRRQNSMRRNE, posições 296-307, foram preditos usando o software ABCpred Prediction Server (www.imtech.res.in/raghava/abcpred/), como descrito por Saha & Raghava (2006). O segundo epitopo foi também predito utilizando o algoritmo de escala de acessibilidade Emini, com base no programa IEDB (disponível em www.iedb.org), como descrito por Emini et al. (1985). O epitopo conformacional (Peptídeo-3) foi predito utilizando uma combinação de três algoritmos: ABCpred Prediction Server, Bepipred Linear Epitope Prediction (www.tools.immuneepitope.org/bcell) e escala de antigenicidade de Kolaskar eTongaonkar (www.tools.immuneepitope.org/bcell/), seguindo os protocolos técnicos descritos por Kolaskar & Tongaonkar (1990), Larsen et al (2006) e Saha & Raghava (2006). Este epitopo contém uma combinação de aminoácidos de duas regiões diferentes da proteína LiHyD: LYHPAPSSL (posições 221-229) e PQPGYQPP (posições 262-269). Todos os peptídeos foram sintetizados pela técnica de F-moc (Merrifield, 1963), seguindo modificações descritas por Machado-de-Avila et al. (2011) e, posteriormente, utilizados nos ensaios sorológicos.

LinJ.33.3150 hypothetical protein[Leishmania infantum]

MQMQGNMYPRQMEWAVHQQQPQSMQGNRQAVASRAYHLEPISTMQLRQQRGSMPGMMIGQ
QPGGGMMDGLSTYGPRPMIRDVQDGHMGPQQADAARAAGYGTQGMYGSHLMSYGAAGMGGV
NNLQNGNAALFAAGSAGQASEGNSINFNGIFNSAVNPQVQSSVAVQDDGKPLPFPPGNLLAQYPPEY
QQQLIFYYRLLRLQYPELYQQYVDYYVMYYEP**LYHPAPSSL**SKDDLNGGQQRKKEPLLQQTQRAHMQR
QQPAM**PQPGYQPPPPMEHALPS**EVMCRTTSNLSGGLKRQ**SSLRRQNSMRRNE**VNQLKNEGSLKRL
PSMRQQ

Figura 2: Sequência de aminoácidos da proteína recombinante LiHyD

4.6 Clonagem da proteína recombinante LiHyD

4.6.1 Extração do DNA genômico dos parasitos

A cepa MHOM/BR/1970/BH46 de *L. infantum* foi cultivada de acordo com protocolo descrito por Coelho *et al.* (2003). O DNA genômico foi extraído conforme descrito por Piarroux *et al.* (1993). Para verificar a qualidade e a quantidade do material obtido, foi realizada uma eletroforese em gel de agarose 1% peso por volume (p/v) (Sigma) em tampão TAE (constituído de Tris 40mM, ácido acético 20 mM e EDTA 1 mM). O gel foi submetido a uma corrente de 80 volts (V) e corado com brometo de etídio (10 μg/mL). A dosagem foi realizada em espectrofotômetro nos comprimentos de onda de 260 e 280 nm, e a amostra foi armazenada a -80°C.

4.6.2 Amplificação das sequências gênicas por PCR

A região codificadora da proteína foi amplificada por PCR, utilizando-se oligonucleotídeos iniciadores complementares aos fragmentos. Sítios de restrição para as enzimas *Bam*HI e *Hin*dIII foram inseridos na sequência da proteína. A reação de amplificação do genede interesse foi realizada utilizando o kit GoTaq® DNA Polymerase, de acordo com as instruções do fabricante (Promega). A termociclagem foi padronizada em um ciclador térmico (Tonegen Palm, Tonederm), utilizando-se o seguinte programa:

Passo 1 – Desnaturação a 95°C por 5 min.

Passo 2 – Desnaturação a 95°C por 45 seg;
Anelamento a 55°C por 45 seg;
Extensão a 72°C por 120 seg.
} 30 ciclos

Passo 3 – Extensão final a 72 °C por 5 min.

Após a reação, o produto da PCR foi aplicado em um gel de agarose 1% e corado com brometo de etídio. O produto da PCR foi quantificado utilizando o comprimento de onda 260/280 nm no aparelho NanoDrop® 2000 (Thermo scientific).

4.6.3 Ligação do gene em pGEM®-T *Easy Vector Systems*

O produto da amplificação do gene LiHyD foi ligado ao vetor de clonagem pGEM®-

T *Easy Vector Systems* de acordo com as instruções do fabricante (Promega). A ligação do inserto ao vetor ocorreu a 4°C, durante 16 h.

4.6.4 Preparo e transformação de bactérias *Escherichia coli* XL1*blue*

Células competentes *E. coli* XL1*blue* foram preparadas através da técnica de cloreto de cálcio, segundo Sambrook *et al*. (1989). A transformação das bactérias XL1 blue foi realizada através de choque térmico com os plasmídeos pGEM-LiHyD e plaqueadas como sugerido pelo fabricante (Agilent Technologies).

4.6.5 Extração do DNA plasmidial

Após a incubação das placas por 16 h a 37ºC, clones positivos foram selecionados a fim de se realizar a extração dos seus DNAs plasmidiais. As colônias selecionadas foram replicadas em meio LB e ampicilina (100 μg/mL) e seus DNAs plasmidiais extraídos através do kit *Wizard® Plus SV Minipreps DNA Purification System* (Promega). A digestão dos plasmídeos foi realizada com as endonucleases de restrição *Hin*dIII e *Bam*HI (Promega) de acordo com protocolo do fabricante (Promega). Os produtos foram confirmados em gel de agarose 1% corado com brometo de etídio e as respectivas bandas obtidas foram excisadas e purificadas com o *kit Invisorb® Fragment CleanUp* (Invitek).

4.6.6 Sequenciamento dos genes

Após a clonagem em vetor pGEM-T®, amostras do plasmídeo purificado foram separadas para a realização do sequenciamento automático de DNA de alta qualidade e para a confirmação da identidade do inserto (LiHyD) e do vetor (pGEM-T). O sequenciamento foi realizado em *MegaBACE 1000 DNA Sequencing System* (GE Healthcare) e analisado pelo Núcleo de Análise de Genoma (NAGE), do Departamento de Bioquímica e Imunologia do ICB, UFMG.

4.6.7 Clonagem do gene em vetor de expressão

O inserto e o plasmídeo de expressão [LiHyD e pQE30 (Qiagen)] foram digeridos com as enzimas de restrição descritas no item 4.6.5 de acordo com o protocolo sugerido pelo fabricante. A amostra foi confirmada em gel de agarose 1% e purificada com o *kit Invisorb® Fragment CleanUp* (Invitek). A ligação foi realizada com a enzima T4 ligase, como sugerido

pelo fabricante (Promega). Para a transformação por choque térmico, bactérias *E. coli* M15 foram utilizadas, seguindo o protocolo sugerido pelos fabricantes e plaqueadas em meio de cultura LB sólido acrescido de antibióticos específicos. As colônias crescidas na placa foram selecionadas e replicadas em meio de cultura LB, com os devidos antibióticos. Para confirmar a presença dos insertos ligados, os plasmídeos foram extraídos pelo método de lise alcalina e os DNAs das colônias recombinantes foram submetidos à digestão enzimática com as endonucleases específicas, sendo que um novo gel de agarose a 1% foi realizado para a confirmação dos insertos. Alíquotas das bactérias transformadas foram estocadas em glicerol 16%, a -80°C.

4.7 Expressão e purificação da proteína recombinante

Para verificar a cinética de expressão da proteína, foi realizado um experimento piloto utilizando 20 mL de cultura da bactéria transformada. Após a densidade ótica (DO) da cultura alcançar aproximadamente uma leitura de 0,5, em comprimento de onda de 600nm, uma alíquota de 1 mL foi removida, sendo correspondente ao tempo não induzido. A cultura foi induzida com 1 mM de IPTG (isopropyl-β-D-thiogalactopyranoside, Promega) e 1, 2, 3 e 4 h após a indução, alíquotas de 1 mL foram retiradas para confirmação da expressão em gel SDS-PAGE. A proteína foi expressa em maior concentração após um período de 3 h de indução, em temperatura de 37°C.

Para a purificação em larga escala, a expressão da proteína foi realizada em 2L de cultura, dentro das condições padronizadas. A lise das bactérias foi realizada em ultrassom (5 pulsos de 90MHz), a proteína foi purificada por cromatografia de afinidade em colunas de níquel e a amostra foi dialisada frente a PBS 1x. A proteína foi submetida à purificação por gel filtração (Superdex^{TM} 200) e em seguida, passadas em uma coluna de agarose-polimixina (Sigma), para remoção de qualquer endotoxina residual de bactéria. A proteína purificada foi dosada pelo método de Bradford, aplicada em gel SDS-PAGE a 10% para confirmação do peso molecular e estocada a -80°C, até o momento do uso.

4.8 Eletroforese em gel SDS-PAGE

As eletroforeses de proteínas em géis desnaturantes (SDS-PAGE) foram realizadas em sistema de Minigel da BioRad (Hercules, CA, USA) para géis de poliacrilamida descontínuos, segundo Laemmli (1970).

4.9 Western-blot

Os experimentos de Western-Blot foram realizados como descrito por Towbin *et al.* (1979), com modificações. Após o preparo do gel SDS-PAGE, 10 μg da proteína foram aplicados e a corrida eletroforética foi realizada a 200 V. A proteína foi transferida do gel para uma membrana de nitrocelulose (NC, poro de 0,2 μm, Sigma) em tampão de transferência (0,192 M glicina, 0,025 M tris e metanol 20%), a 100 V por 1 h e 30 min. As membranas foram bloqueadas com PBS-T 1x (PBS 1x, pH 7,4 e Tween 20 a 0,05% v/v), e albumina bovina a 5%, por 1 h, e posteriormente, lavadas com PBS-T 1x. As membranas foram incubadas individualmente frente a um *pool de* soros de camundongos BALB/c infectados com *L. infantum*, *L. major* e *L. braziliensis* ou não infectados (diluídos 1:100 em solução PBS-T 1x. Em seguida, as membranas foram lavadas e incubadas com anticorpo secundário específico (anti-IgG de camundongo) conjugado à enzima peroxidase (Sigma-Aldrich), diluído em PBS-T 1x (1:10.000). Após realização das lavagens com PBS-T 1x, seguida por PBS 1x, o substrato (cloronaftol 12,5 mg, metanol 4%, diaminobenzidina 25 mg, 20 μL de peróxido de hidrogênio (H_2O_2) 20 vol. e PBS 1x para um volume final de 50 mL) foi acrescentado para a revelação da reação. A reação foi interrompida com água destilada e as membranas foram secas em papel de filtro.

4.10 ELISA para o sorodiagnóstico da LVC

A avaliação da antigenicidade da proteína rLiHyD, foi realizada de acordo com Martins *et al*. (2013) com algumas modificações. Curvas de titulação foram realizadas a fim de determinar as concentrações mais apropriadas dos antígenos e a diluição dos soros para os experimentos. Placas de microtitulação de 96 poços (Falcon®) foram sensibilizadas com rLiHyD (1,0 μg), com os Peptídeos 1, 2 ou 3 (20,0 μg de cada), rA2 (1,0 μg) ou SLA *L. infantum* (2,0 μg), diluídos em 100 μL de tampão de sensibilização (carbonato de sódio 0,1M e bicarbonato de sódio 0,05 M, pH 9,6). As placas foram lavadas com PBS-T e bloqueadas por 1 h a 37°C. Após serem lavadas, as placas foram incubadas com 100μL por poço de soros de cães individuais, em duplicata, tendo sido diluídos a 1:100 em PBS-T . Após incubação por 1 h a 37°C, as placas foram lavadas novamente e 100 μL do anticorpo anti-IgG de cão (Sigma) conjugado à peroxidase foi utilizado em uma diluição de 1:5,000 em PBS-T 1x por 1 h a 37°C. As placas foram lavadas e incubadas com 100 μL da solução reveladora [10 mL de tampão citrato-fosfato (Na_2HPO_4 24 mM e ácido cítrico 17,5 mM para 1 L de água destilada, pH 5,0), 2 mg de orto-fenilenodiamina (OPD) e 2 μL de H_2O_2]

durante 30 min e ao abrigo da luz. A reação de revelação foi parada adicionando-se 25 μL de 2N H_2SO_4 e a leitura ocorreu ao comprimento de onda de 492 nm em espectrofotômetro (Molecular Devices, Spectra Max Plus, Canada). Controles positivos e negativos foram utilizados em todas as placas.

4.11 Imunização de camundongos BALB/c

Grupos de camundongos BALB/c (n=8, por grupo) fêmeas foram imunizados pela via subcutânea no coxim plantar esquerdo, com três doses em intervalos de 15 dias entre as mesmas. Os animais foram divididos em grupos dentro de quatro experimentos:

Primeiro experimento (*infecção L. infantum)*:

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rLiHyD + 25 μg de saponina;

Segundo experimento (infecção *L. major*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rLiHyD;
4) Grupo imunizado com 25 μg de rLiHyD+ 25 μg de saponina;

Terceiro experimento (*infecção L. braziliensis*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rLiHyD;
4) Grupo imunizado com 25 μg de rLiHyD+ 25 μg de saponina.

As concentrações dos imunógenos e do adjuvante foram determinadas com base em experimentos vacinais padronizados por nosso grupo de pesquisa.

4.12 Infecção desafio

A infecção desafio foi realizada 30 dias após a última dose da vacina, quando também ocorreu a eutanásia de metade do número dos animais de cada grupo para a realização de experimentos de imunogenicidade. Para tal, 1 x 10^7 promastigotas em fase

estacionária de crescimento de *L. infantum* (primeiro experimento), 1 x 10^6 promastigotas em fase estacionária de crescimento de *L. major (*segundo experimento) ou 1 x 10^7 promastigotas em fase estacionária de crescimento de *L. braziliensis* foram diluídas em 20 μL de PBS 1x e inoculadas no coxim plantar direito de cada animal. Os camundongos infectados foram monitorados durante 10 semanas após o desafio e então eutanasiados para realização dos experimentos de imunogenicidade e determinação da carga parasitária. Animais desafiados com as espécies *L. major* e *L. braziliensis* foram submetidos à medição da lesão causada na pata infectada semanalmente, durante um período de 10 semanas, com auxílio de um paquímetro (L.S. Starrett S.A., SP, Brasil).

4.13 Isolamento e cultura dos esplenócitos

Camundongos imunizados ou imunizados e infectados foram submetidos à eutanásia para a coleta do baço. O isolamento, cultura e estimulação de esplenócitos foram realizados de acordo com Martins *et al*. (2013), com modificações. Para tal, 5 x 10^6 células/mL foram incubadas em meio de cultura RPMI 1640 completo (acrescido com 20% de soro fetal bovino (SFB) inativado (Sigma), 20 mM de L-glutamina, 200 unidades por mililitro (U/mL) de penicilina e 100 microgramas por mililitro (μg/mL) de estreptomicina), na presença de estímulos específicos (proteína recombinante ou SLA) ou não (controle negativo). As placas foram incubadas em estufa com 5% de CO_2 durante 48 h a 37°C, e os sobrenadantes foram coletados para a dosagem das citocinas. A avaliação da participação de LTCD4$^+$ e LTCD8$^+$ na produção de IFN-ɣ, pelos esplenócitos dos animais imunizados e desafiados foi realizada com a utilização dos anticorpos monoclonais anti-CD4$^+$ (GK 1.5), anti-CD8$^+$ (53-6.7) ou anti-IL12 (C17.8) como recomendado pelo fabricante (todos da Pharmingen, San Diego, CA, USA).

4.14 Dosagem das citocinas

As citocinas IFN-ɣ, IL-4, IL-10, IL-12p70 e GM-CSF foram avaliadas. A dosagem das citocinas foi realizada através da técnica de ELISA de captura, utilizando-se os kits comerciais *BD OptEIA* ^{TM} *Set Mouse*, catálogo 555138, 555232 , 555167, 555256 e 555252, respectivamente (Pharmingen, San Diego, CA, USA), seguindo as instruções do fabricante.

4.15 Avaliação da produção de anticorpos IgG1 e IgG2a

O perfil da resposta humoral nos animais imunizados ou imunizados e desafiados foi avaliado através da produção de anticorpos IgG1 e IgG2a específicos ao SLA de *L. infantum, L. major* ou *L. braziliensis.* Amostras de sangue dos animais foram retiradas 30 dias após a última dose de imunização e na décima semana após a infecção, incubadas por 10 min a 37°C e centrifugadas a 5.000 x g por 10 min, para a obtenção de soro. Os ensaios de ELISA foram realizados em placas de 96 poços (Jet-Biofil®), sendo que 0,5 µg/poço de rLiHyD e 1,0 µg/poço de SLA*Li,* SLA*Lm* ou SLA*Lb* foram utilizados em 100 µL de tampão de sensibilização. As placas foram incubadas a 4°C por 18 h e após este período foram lavadas com PBS-T 1x e bloqueadas. Após uma nova lavagem, as amostras de soro foram aplicadas em duplicatas, diluídas 1:100 em PBS-T 1x e caseína 0,25%. Após incubação, as placas foram lavadas e os conjugados anti-IgG1 ou anti-IgG2a de camundongo, ligados à peroxidase (Sigma), foram acrescentados na diluição de 1:5.000 (no experimento com *L. infantum*) e 1:10000 (no experimento com *L. major* e *L. braziliensis*) em PBS-T 1x e caseína 0,25%. As amostras foram reveladas e a leitura das absorbâncias foi realizada em leitor de ELISA, com comprimento de onda de 492 nm. Em todos os ensaios, controles positivos e negativos foram utilizados.

4.16 Avaliação da carga parasitária

Para realizar a quantificação de parasitos, baço, fígado, linfonodo drenante, medula óssea e lesão da pata infectada (quando pertinente) foram removidos 10 semanas após a infecção. Os órgãos foram macerados em meio Schneider's incompleto na proporção de 1 mL de meio de cultura a cada 100 mg de tecido. Diluições seriadas foram realizadas utilizando meio de Schneider's completo, partindo de uma diluição de 10^{-1} até uma diluição máxima de 10^{-12}. As placas de cultura celular de 96 poços (Nunc) foram incubadas em estufa a 24ºC durante 7 dias e os resultados foram representados pela maior diluição na qual os parasitos puderam ser visualizados utilizando-se um microscópio trinocular invertido (Axiovert 25, Zeiss), de acordo com Coelho *et al.* (2003).

4.17 Análise estatística

As análises estatísticas foram realizadas no *software* Graph Pad Prism (versão 6.0

para Windows). Curvas ROC (*Receiver Operating Characteristic*) foram preparadas para análise dos dados de antigenicidade da proteína LiHyD e seus peptídeos. Para determinar se a variável foi distribuída normalmente, utilizou-se o teste de normalidade D'Agostino & Pearson. O teste t de Student não pareado também foi utilizado e diferenças significativas foram consideradas quando $P < 0,05$. A capacidade de diagnóstico de cada antígeno foi medida avaliando a sensibilidade (IC 95%), especificidade (IC 95%), área sob a curva (AUC) e acurácia (AC). Para a estatística da imunogenicidade, os valores encontrados para os diferentes grupos experimentais foram analisados pelo teste de análise de variância (ANOVA), seguido pelo teste de Bonferroni's. Diferenças foram consideradas significativas quando o valor de $P < 0.05$.

5. RESULTADOS E DISCUSSÃO

Conforme as normas que regem a redação das dissertações de mestrado e teses de doutorado do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, as seções de Resultados e Discussão serão apresentadas sob a forma de três artigos científicos já publicados.

5.1 Artigo 1 – Breve introdução e objetivo

O primeiro artigo a ser apresentado é intitulado *"A new Leishmania-specific hypothetical protein and its non-described specific B cell conformational epitope applied in the serodiagnosis of canine visceral leishmaniasis"* e foi publicado na revista internacional *Parasitology Research.*

O presente estudo avaliou as propriedades antigênicas da proteína LiHyD em experimentos de ELISA como marcador para o sorodiagnóstico da leishmaniose visceral canina. Com o objetivo de comparar a eficácia entre a proteína e seus peptídeos sintéticos, três epitopos de linfócitos B de LiHyD foram preditos, sendo dois lineares e um conformacional, e também avaliados como marcadores de diagnóstico.

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ORIGINAL PAPER

A new *Leishmania*-specific hypothetical protein and its non-described specific B cell conformational epitope applied in the serodiagnosis of canine visceral leishmaniasis

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Abstract The serodiagnosis of canine visceral leishmaniasis (CVL) presents problems related to its sensitivity and/or specificity. In the present study, a new *Leishmania*-specific hypothetical protein, LiHyD, was produced as a recombinant protein (rLiHyD) and evaluated in ELISA experiments for the CVL serodiagnosis. LiHyD was characterized as antigenic in a recent immunoproteomic search performed with *Leishmania infantum* proteins and the sera of dogs developing visceral leishmaniasis (VL). Aiming to compare the efficacy between whole proteins and synthetic peptides, two linear and one conformational B cell epitopes of LiHyD were synthesized and also evaluated as diagnostic markers. The four antigens were recognized by the sera of dogs suffering VL. On the contrary, low reactivity was observed when they were assayed with sera from non-infected healthy dogs living in endemic or non-endemic areas of leishmaniasis. In addition, no reactivity was found against them using sera from dogs experimentally infected by *Trypanosoma cruzi*, *Babesia canis*, or *Ehrlichia canis*, or sera from animals vaccinated with the Leish-Tec® vaccine, a prophylactic preparation commercially available for CVL prevention in Brazil. As comparative diagnostic tools, a recombinant version of the amastigote-specific A2 protein and a soluble crude *Leishmania* extract were studied. Both antigens presented lower sensitivity and/or specificity values than the LiHyD-based products. The rLiHyD presented better results for the CVL serodiagnosis than its linear epitopes, although the peptide recreating the conformational epitope resulted also appropriate as a diagnostic marker of CVL. To the best of our knowledge, this is the first study showing the use of a conformational epitope derived from a *Leishmania* protein for serodiagnosis of CVL.

Keywords Hypothetical proteins · Conformational epitopes · *Leishmania* · Serodiagnosis · Canine visceral leishmaniasis · ELISA

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Introduction

Leishmaniasis is a disease complex that presents a high morbidity and mortality in the world, where about 380 million people are at risk in 98 countries, with approximately 1.5 to 2.0 million new cases being registered annually (Alvar et al.

2012). Zoonotic visceral leishmaniasis (VL) is a disease caused by *Leishmania infantum* in the Mediterranean area, Middle East, Africa, Asian countries, and Latin America (WHO 2010), and dogs are considered important domestic reservoirs of parasites (Petersen 2009). The disease is also emerging in the USA, Canada, Northern Italy, and Germany and in the Americas, with about 95 % of the cases registered in Brazil (Ready 2010; Alvar et al. 2012).

Upon infection, dogs can develop asymptomatic or symptomatic forms of disease (Solano-Gallego et al. 2011). In symptomatic canine VL (CVL), cutaneous and organic alterations are observed correlating with the presence of high parasite burdens, and the disease usually results in the death of the infected animals (Ciaramella et al. 1997). For diagnosis, parasitological methods based on direct demonstration of amastigote forms by direct staining or amplification of the parasite DNA by the polymerase chain reaction (PCR) in collected samples from infected organs or tissues can be employed. PCR-based tests applied for *Leishmania* detection are more reliable than direct observation of parasites (Antinori et al. 2007). However, these methods require invasive procedures for sample collection and false negative results can be obtained when diagnosing individuals with low parasite burdens, like in asymptomatic patients (Coura-Vital et al. 2011). In addition, for PCR-based diagnosis, a careful standardization of protocols is needed in terms of design of primers and DNA extraction procedures (Alvar et al. 2004; Baneth & Aroch 2008; Deborggraeve et al. 2008; Maia & Campino 2008).

Serological tests have been recommended for the CVL diagnosis due to the fact that they use less invasive methods of sample collection. Infected dogs can present a moderate to strong humoral response, which generally accompanies the development of disease (Porrozzi et al. 2007; Maia & Campino 2008). However, antigens used present cross-reactivity with antibodies generated against proteins of other pathogens, leading to the occurrence of false positive results (Coura-Vital et al. 2011; Almeida-Leal et al. 2014). Moreover, in areas in which CVL is endemic, non-infected animals can also develop an antileishmanial serology, and they can be confused with infected dogs (Courtenay et al. 2002; Moshfe et al. 2009).

The Brazilian Ministry of Health has recommended that, for a CVL serological diagnosis, the "Dual Path Platform" (DPP®; Bio-Manguinhos, Fiocruz, Rio de Janeiro, Brazil) combined with the "Canine Leishmaniasis ELISA Kit" (EIE-LVC kit; BioManguinhos, Fiocruz, Rio de Janeiro, Brazil) should be employed (Coura-Vital et al. 2014; Laurenti et al. 2014). However, their efficacy has been hampered by factors affecting their sensitivity and/or specificity, mainly related to the antigens employed. Thus, it is necessary to find new antigenic proteins that serve to design serodiagnostic systems with higher degree of sensitivity and specificity than current kits. Different recombinant proteins have been evaluated as diagnostic markers of disease (Soto et al. 1998; Candido et al. 2008; Martins et al. 2013), although a precise antigen does not exist. In parallel to the use of recombinant proteins, synthetic peptides could be also considered, since these antigens are simpler, stable, and cheaper to produce (Noya et al. 2003; Chávez-Fumagalli et al. 2013).

In a recent immunoproteomic search performed with *L. infantum* proteins, a *Leishmania*-specific hypothetical protein was recognized by CVL sera (Coelho et al. 2012). This protein, namely, LiHyD (LinJ.33.3150), was obtained as a recombinant molecule in the present study (rLiHyD). It was chosen because it is only present in the *Leishmania* genus, it is highly conserved among *Leishmania* species, and it is predicted to have B cell epitopes. Aiming to evaluate new candidates for the CVL serodiagnosis, this study employed the rLiHyD protein and three of its specific B cell epitopes (two linear and one conformational) contained in three different synthetic peptides. Also, to the best of our knowledge, this study evaluates for the first time the diagnostic properties of a conformational epitope derived from a *Leishmania* hypothetical protein.

Materials and methods

Ethics statement

This study was approved by Committee on the Ethical Handling of Research Animals from Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil, under the protocol number 043/2011.

Canine sera

The sample size used was composed of 177 domestic animals (*Canis familiaris*) and consisted of males ($n=100$) and females ($n=77$), of different breeds and ages. CVL-positive animals presented positive parasitological results for *L. infantum* DNA assayed by a PCR technique (Reis et al. 2013). All of them presented positive serological results in two commercial tests (IFAT-LVC Bio-Manguinhos kit and EIE-LVC Bio-Manguinhos kit). Symptomatic VL dogs (CVLS, $n=44$) were those with positive parasitological and serological results, as well as showing three or more clinical signals and/or symptoms. Asymptomatic VL dogs (CVLA, $n=9$) were those presenting positive parasitological and serological results, but without any clinical signals of leishmaniasis. Non-infected dogs were selected from endemic (HEA, $n=44$; Belo Horizonte, Minas Gerais, Brazil) or non-endemic (HNEA, $n=20$; Poços de Caldas, Minas Gerais, Brazil) areas of leishmaniasis. All of them presented negative serological results, as well as were free of any signal of

disease. In this study were also included sera samples of healthy animals vaccinated with Leish-Tec® (HV, $n=30$), and sera from dogs infected with *Ehrlichia canis* (EC, $n=10$), *Babesia canis* (BC, $n=10$), or *Trypanosoma cruzi* (TC, $n=10$). These last animals were maintained in kennels to prevent their contact with transmitting vectors of leishmaniasis.

Parasite

The *L. infantum* (MHOM/BR/1970/BH46) strain was used. Parasites were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA) supplemented with 10 % inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was prepared from 1×10^9 stationary-phase promastigote cultures (5–7 days old), as described (Coelho et al. 2003).

Sequence analysis of the LiHyD protein

The process of in silico analysis of the *L. infantum* LiHyD sequence consisted of (i) the search for similarity among sequences deposited in non-redundant protein databases, (ii) comparison with the databases of other trypanosomatids whose genomes have been sequenced completely or are in the phase of annotation, i.e., *Leishmaniamajor*, *Leishmania mexicana*, *Leishmania braziliensis*, *T. cruzi*, *Trypanosoma brucei*, and *Trypanosoma congolenses* (all available at www.genedb.org), and (*iii*) evaluation of the sequence for analysis of its physicochemical properties using the ProtParam tool in the ExPASy server (Gasteiger et al. 2005). The parameters computed by the program and reported here include the molecular weight, theoretical isoelectric point, amino acid composition, total number of positive and negative residues, extinction coefficient, instability index, aliphatic index, and grand average of hydropathicity (GRAVY).

Cloning, expression, and purification of recombinant LiHyD protein

The cloning, expression, and purification of the LiHyD protein were performed as described by Lage et al. (2015). The *L. donovani* A2 recombinant protein used as an antigen control was produced as described by Zhang et al. (1996). After purification, the recombinant proteins were passed through a polymyxin-agarose column (Sigma), in order to remove residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, MD, USA).

Mapping of specific B cell epitopes of the LiHyD protein

Two linear and one conformational B cell epitope of LiHyD were synthesized. Two peptides containing the linear sequences (Peptide-1, PQPGYQPPPPMEHALP, 262-277 positions; and Peptide-2, SSLRRQNSMRRNE, 296-307 positions) were predicted using the ABCpred Prediction Server software (www.imtech.res.in/raghava/abcpred/), as described by Saha and Raghava (2006). The second epitope was also predicted using the Emini Surface Accessibility Scale algorithm, based on the program IEDB (Immune Epitope DataBase and Analysis Resource; available at www.iedb.org), as described by Emini et al. (1985). The conformational epitope (Peptide-3) was predicted using a combination of three algorithms, ABCpred Prediction Server, Bepipred Linear Epitope Prediction (www.tools.immuneepitope.org/bcell), and Kolaskar and Tongaonkar antigenicity scale (www.tools.immuneepitope.org/bcell/), following technical protocols described by Kolaskar and Tongaonkar (1990), Larsen et al. (2006), and Saha and Raghava (2006). This epitope contains a combination of amino acids from two different protein regions of LiHyD: LYHPAPSSL (221-229 positions) and PQPGYQPP (262-269 positions). All peptides were synthesized by the F-moc technique of Merrifield (1963), with modifications following Machado-de-Ávila et al. (2011). Briefly, peptides were released from the amine resin by trifluoracetic acid treatment in the presence of the appropriate scavengers. Then, they were diluted in Milli-Q water and purified by high-performance liquid chromatography (HPLC) on a C18 reverse phase column (flow rate 1.0 mL/min; Vydac). Finally, they were submitted to a MALDI-TOF-TOF analysis.

ELISA for CVL serodiagnosis

Previous titration curves were performed to determine the most appropriate concentration of antigens and sera sample dilutions to be used in the ELISA experiments. Microtiter immunoassay plates (Falcon) were coated with rLiHyD, rA2, Peptide-1, Peptide-2, Peptide-3, or *L. infantum* SLA (1.0, 1.0, 20.0, 20.0, 20.0, and 2.0 μg per well, respectively), dissolved in 100 μL coating buffer (50 mM carbonate buffer, pH 9.6), for 18 h at 4 °C. Next, free binding sites were blocked using 200 μL of PBS-T (phosphate-buffered saline plus Tween 20 0.05 %), containing 5 % albumin, for 1 h at 37 °C. After washing the plates three times with PBS-T, they were incubated with 100 μL of canine sera (1:100, diluted in PBS-T), for 1 h at 37 °C. Plates were subsequently washed four times in PBS-T and incubated with anti-dog IgG horseradish-peroxidase-conjugated antibody (1:5000, diluted in PBS-T; catalog A6792, Sigma Aldrich, USA), for 1 h at 37 °C. After washing the plates five times with PBS-T, the reactions were developed by incubation with 100 μL per well of a solution consisting of 2 μL H_2O_2, 2 mg orto-

phenylenediamine, and 10 mL citrate-phosphate buffer at pH 5.0, for 30 min and in the dark. Reaction was stopped by adding 25 μL 2 N H_2SO_4. The optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The mean optical density (OD) value was calculated by subtracting the mean blank OD from the mean OD for each individual sample. The lower limits of positivity (cutoff) for the diagnostic antigens were established for optimal sensitivity and specificity using the receiver operating characteristic (ROC) analysis. The curves were plotted with the values from symptomatic and asymptomatic CVL groups versus the control groups, following a sick/non-sick rating method. The result of the division between the mean OD obtained for the sample and its respective cutoff was called "optical density index" (ODI). The D'Agostino & Pearson normality test was used to determine whether a variable was normally distributed. An unpaired Students *t* test was also used, and significant differences were considered with $P<0.05$. The diagnostic capacity of each antigen was measured by assessing its sensitivity (95 % confidence interval, CI 95%), specificity (CI 95%), area under the curve (AUC), and accuracy (AC). The degree of agreement between the assays was determined by kappa (κ) index (with CI 95%) and classified according to the Fleiss scale: 0.00–0.20 (poor), 0.21–0.40 (fair), 0.41–0.60 (moderate), 0.61–0.80 (good), 0.81–0.99 (very good), and 1.00 (perfect).

Results

Sequence database and physicochemical evaluation of LiHyD protein

In the present study, the hypothetical LiHyD protein was defined as a *Leishmania* spp. specific protein, and its identity degree found was 60, 79, and 80 % for the *L. braziliensis*, *L. major*, and *L. mexicana* species, respectively. No orthologue sequence was found in the *T. cruzi*, *T. brucei*, *Trypanosoma vivax*, and *T. congolenses* species. Based on the fact that LiHyD is a hypothetical protein, a physicochemical evaluation was in silico performed (Table 1). The results showed that it presents 327 amino acids in its primary sequence, having a molecular weight of 36 kDa, and an isoelectric point of 9.49.

Table 1 Physicochemical evaluation of the LiHyD protein

Parameters	Results
Number of amino acids	327
Estimated molecular weight (kDa)	36.0
Theoretical isoelectric point	9.49
Total number of negatively charged residues	20
Total number of positively charged residue	28
Ext. coefficient (M^{-1} cm^{-1})	33810
Instability index (classification)	56.4
Aliphatic index	56.15
Grand average of hydropathicity (GRAVY)	-0.806

Evaluation of the rLiHyD protein for CVL serodiagnosis

The rLiHyD protein was evaluated for CVL serodiagnosis (Fig. 1). For comparison, a recombinant version of the A2 protein and the *L. infantum* SLA were also employed in the ELISA assays. The results showed that 100 % of the CVL sera presented OD values over the cutoff, when rLiHyD was employed as an antigenic source (Fig. 1a). The ODI value of reactivity of rLiHyD was calculated and is also shown (Fig. 1b). The cutoff values for accessing the sensitivity and specificity of antigens were determined using receiver operating characteristic (ROC) analysis, and the area under the curve (AUC) was calculated to assess the accuracy of the tests (Fig. 1c). When the rA2 protein was used as a comparative diagnostic marker, its sensitivity and specificity values were 100 and 72.6 %, respectively (Fig. 2). The *L. infantum* SLA was not able to identify the asymptomatic animals. In addition, a poor specificity was observed, since it was recognized by 30 % of the sera from *T. cruzi*-infected dogs (Fig. 2a). The ODI values (Fig. 2b) and the result of the ROC analysis for rA2 and SLA (Fig. 2c) are also shown. The individual evaluation of each antigen for the CVL serodiagnosis was performed (Table 2). The AUC was used to compare the efficacy between the different evaluated diagnostic antigens. The rLiHyD protein presented the highest AUC value (1.000), followed by rA2 (0.992) and *L. infantum* SLA (0.964). The maximum sensitivity and specificity values (100.0 % in both cases), the maximum accuracy value (100.0%), and a total agreement were obtained using the rLiHyD protein. On the other hand, when the rA2 and *L. infantum* SLA were used, they presented sensitivity values of 98.1 and 83.0 %, respectively, and accuracy values of 96.0 and 93.0 %, respectively.

Comparative efficacy between the rLiHyD protein and its linear and conformational B cell epitopes for CVL serodiagnosis

Next, the antigenicity of the linear and conformational epitopes was investigated by an ELISA assay. Results are shown

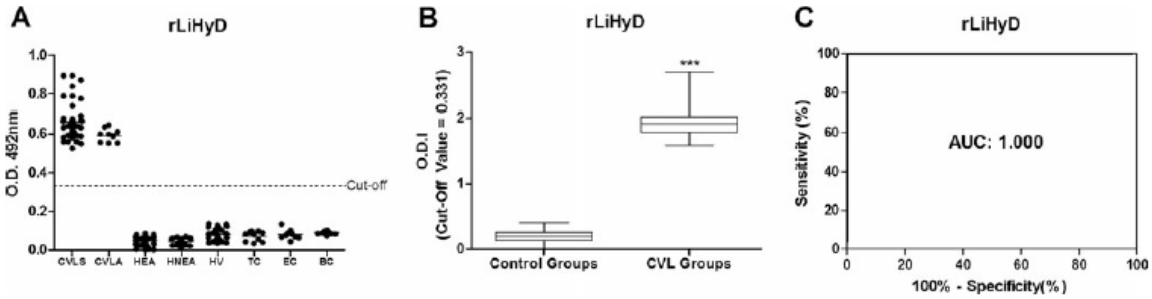

Fig. 1 Evaluation of ELISA reactivity with the rLiHyD protein using a canine serological panel. The recombinant LiHyD protein was employed as an antigen for ELISA assays performed with canine sera samples obtained by the next animal groups: symptomatic (CVLS, $n=44$) or asymptomatic (CVLA, $n=9$) visceral leishmaniasis (VL) dogs, healthy dogs living in endemic (HEA, $n=44$) or non-endemic (HNEA, $n=20$) areas of leishmaniasis, dogs immunized with Leish-Tec® vaccine (HV, $n=30$), and animals infected with *Trypanosoma cruzi* (TC, $n=10$), *Ehrlichia canis* (EC, $n=10$), or *Babesia canis* (BC, $n=10$). The individual OD values are shown (**a**). The *dotted line* represents the cutoff value calculated by a ROC analysis. The box and whisker plots of the ODI values from sera grouped in non-infected (control) or CVL (*Leishmania*-infected) groups are also shown (**b**), as well as the ROC curves obtained for LiHyD protein (**x**). Statistically significant differences (***$P<0.001$) were observed between the CVL group and the control groups

in Fig. 3. The three peptides were recognized by CVL sera. However, an unexpected reactivity against Peptide-1 and Peptide-2 was observed when sera from non-infected dogs living in endemic area of leishmaniasis were employed. Of note, Peptide-3 was able to react specifically with CVL samples, irrespective of the presence of clinical signs (Fig. 3a). In fact, this antigen presented the best results to distinguish the CVL sera from the other samples, since all sera reactivities were higher than the cutoff value calculated by ROC analysis. The ODI values from *L. infantum*-infected dogs and those from non-infected animals were also determined (Fig. 3b). The AUC was used to compare the efficacy between the

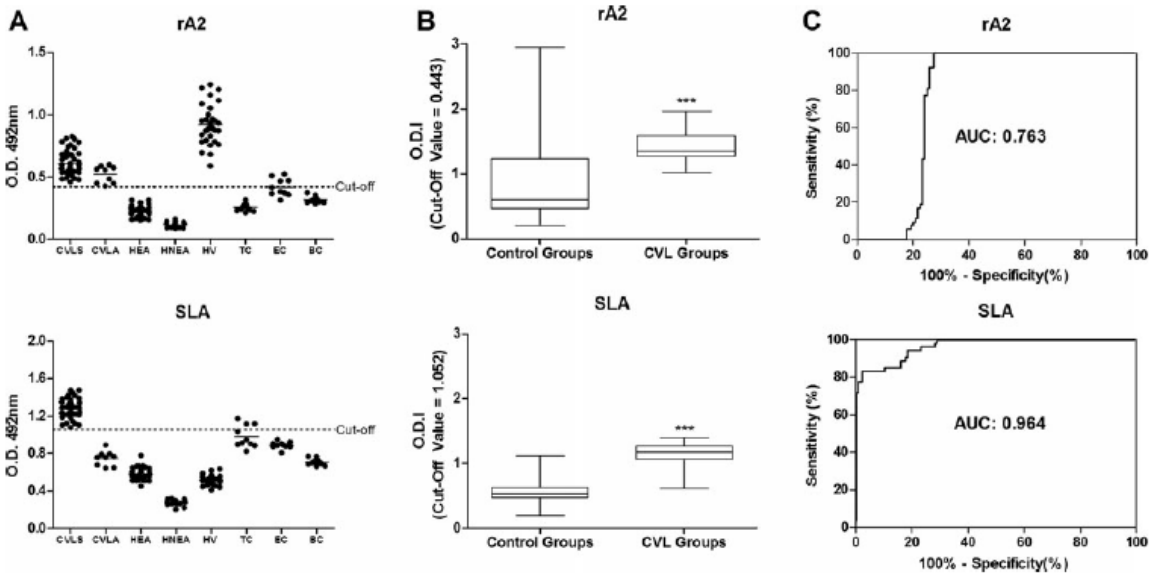

Fig. 2 Evaluation of ELISA reactivity using the rA2 and *L. infantum* SLA against a canine serological panel. Two antigenic preparations were studied: the recombinant A2 protein (*upper panels*) and a soluble *L. infantum* antigenic (SLA) preparation (*bottom panels*). ELISA assays were performed using sera samples obtained by the next animal groups: symptomatic (CVLS, $n=44$) or asymptomatic (CVLA, $n=9$) visceral leishmaniasis (VL) dogs, healthy dogs living in endemic (HEA, $n=44$) or non-endemic (HNEA, $n=20$) areas of leishmaniasis, dogs immunized with Leish-Tec® vaccine (HV, $n=30$), and animals infected with *Trypanosoma cruzi* (TC, $n=10$), *Ehrlichia canis* (EC, $n=10$), or *Babesia canis* (BC, $n=10$). The individual OD values are shown (**a**). The *dotted line* represents the cutoff value calculated by a ROC analysis. The box and whisker plots of the ODI values from sera grouped in non-infected (control) or CVL (*Leishmania*-infected) groups are also shown (**b**), as well as the ROC curves obtained for LiHyD protein (**c**). Statistically significant differences (***$P<0.001$) were observed between the CVL group and the control groups

Table 2 Diagnostic efficacy of rLiHyD protein for CVL serodiagnosis

Antigen	Parameters[a]					
	Se (%) (CI 95%)	Sp (%) (CI 95%)	AUC (CI 95%)	κ (CI 95%)	Agreement[b]	AC (%)
rLiHyD	100.0 (93.3-100.0)	100.0 (97.1-100.0)	1.000 (1.000-1.000)	1.0 (1.0-1.0)	Perfect	100.0
rA2	100.0 (93.3-100.0)	72.6 (63.9-80.2)	0.760 (0.690-0.840)	0.6 (0.5-0.7)	Good	80.9
SLA	83.0 (70.0-91.9)	97.6 (93.1-99.5)	0.960 (0.940-0.990)	0.9 (0.8-1.0)	Very Good	93.2

ROC curves were used to determine ELISA sensitivity (Se), specificity (Sp), confidence interval (CI 95%), and area under curve (AUC) of each diagnostic antigen

[a] Parameters were calculated using all 177 samples presented in this study

[b] Agreement was calculated using parasitological assay (PCR technique) as a gold standard

Se, sensitivity, *Sp* specificity, *CI* confidence interval, *AUC* area under curve, κ kappa index, *AC* accuracy

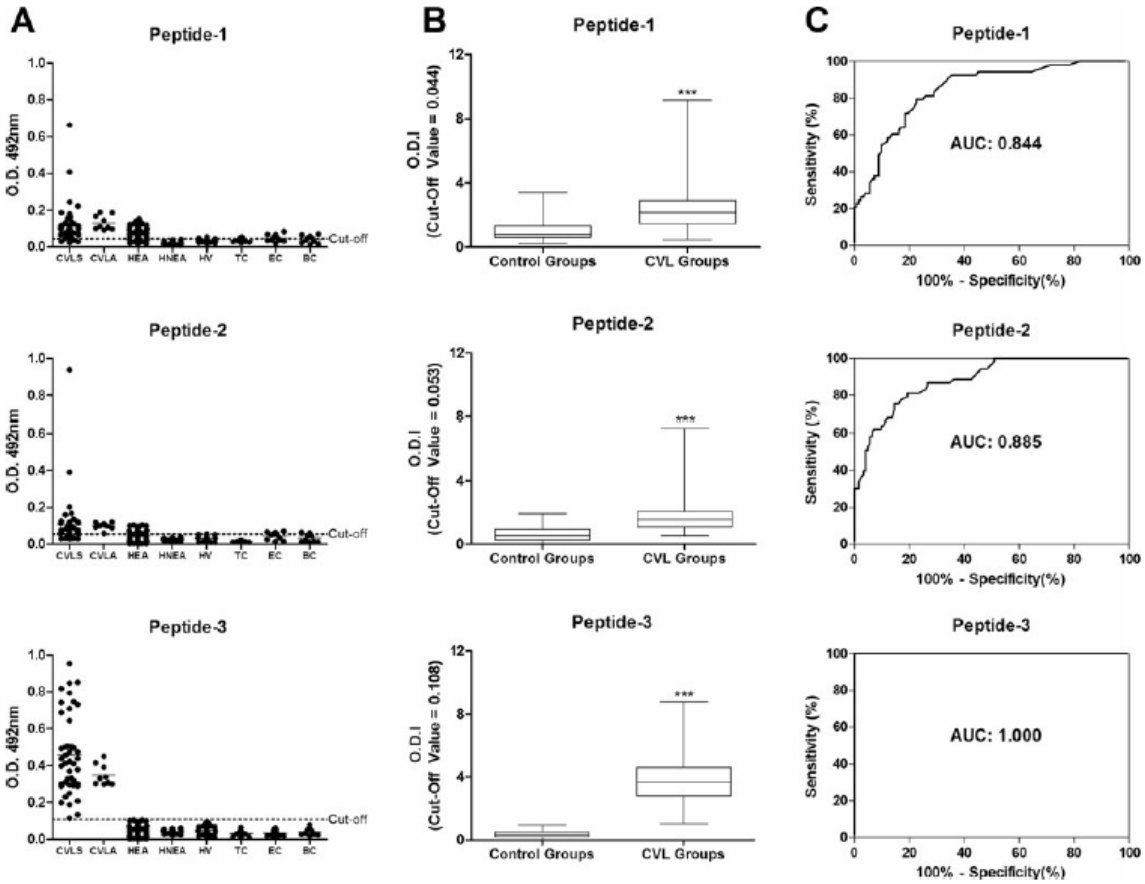

Fig. 3 Analysis of the specific B cell epitopes derived from LiHyD protein for CVL serodiagnosis. ELISA assays were performed using three synthetic epitopes designed on the basis of a computation analysis looking for linear (Peptide-1 [*upper panels*] and Peptide-2 [*middle panels*]) and the conformational epitopes (Peptide-3). Sera samples were obtained from symptomatic (CVLS; $n=44$) or asymptomatic (CVLA; $n=9$) visceral leishmaniasis (VL) dogs, from healthy dogs living in endemic (HEA; $n=44$) or non-endemic (HNEA; $n=20$) areas of leishmaniasis, and from dogs immunized with Leish-Tec® vaccine (HV; $n=30$) or from animals infected with *Trypanosoma cruzi* (TC; $n=10$), *Ehrlichia canis* (EC; $n=10$), or *Babesia canis* (BC; $n=10$). The individual OD values are shown (**a**). The *dotted line* represents the cut-off value calculated by a ROC analysis. The box and whisker plots of the ODI values from sera grouped in non-infected (control) or CVL (*Leishmania*-infected) groups are also shown (**b**), as well as the ROC curves obtained from the same groups (**c**). Statistically significant differences (***$P<0.001$) were observed between the CVL group and the control groups

different peptides (Fig. 3c). Peptide-3 presented the highest AUC value (1.000), followed by Peptide-2 (0.885) and Peptide-1 (0.844). Parameters related to the sensitivity and specificity of these antigens applied in the CVL serodiagnosis were determined and are also shown (Table 3). Peptide-3 presented the maximum sensitivity and accuracy values (100.0 % in both cases), similar to the values obtained using the rLiHyD protein. Peptide-1 and Peptide-2 showed sensitivity values of 92.5 and 81.1 %, respectively, and an accuracy of 72.8 and 80.8 %, respectively (Table 3). Evaluating the specificity of the synthetic epitopes, Peptide-3 showed the highest value (100.0 %), followed by Peptide-2 (80.7 %) and Peptide-1 (64.5 %).

Discussion

The development of a cost-effective and affordable diagnostic tool for CVL is still needed. It will allow the establishment of field assays within the national control strategic programs of the endemic countries for detecting canine infections (Desjeux 2004). Similarly, some problems have been reported for an accurate CVL serodiagnosis. The first one is the variable sensitivity of the tests, especially in determining asymptomatic but infected animals that can present low titers of antileishmanial antibodies. An additional difficulty is the low specificity of the tests when they are employed in areas endemic for other pathogens related or not to *Leishmania spp*. This lack of specificity usually produces false positive results (Coelho et al. 2009; Laurenti et al. 2014; Wolf et al. 2014; Peixoto et al. 2015).

In recent years, studies have been developed aiming to improve the quality of the CVL serodiagnosis. Some of these were directed to produce and characterize recombinant versions of parasite individual antigens to be employed for CVL diagnosis (Celeste et al. 2004; Fonseca et al. 2014; Menezes-Souza et al. 2015). Derived from this line of investigation, detection of the major antigenic determinants within these proteins and construction of synthetic peptides able to diagnose the disease are also the focus of current research (Costa et al. 2011; Martins et al. 2015). It should be noted that peptides are usually more stable, easier to produce, and cheaper than recombinant proteins (Chávez-Fumagalli et al. 2013). In this light, the present study evaluated the antigenic properties of a *Leishmania* hypothetical protein that was recently identified by an immunoproteomic study performed with *L. infantum* total extracts (Coelho et al. 2012). Aiming to compare the efficacy between different antigenic compositions based on the same protein, studies were completed using three putative B cell epitopes derived from LiHyD, two being linear and the other a conformational epitope.

SLA-based ELISA has been evaluated in the CVL serodiagnosis (Coelho et al. 2009; Chávez-Fumagalli et al. 2013). The main inconvenience is that total *Leishmania* preparations share common epitopes with other microbial antigens, resulting in the cross-reaction with serum samples from dogs infected with related diseases (De Arruda et al. 2013; Kubar & Fragaki 2005). Another limitation has been related to the standardization of the production of these extracts, affecting the reproducibility of the tests. In fact, the commercial EIE-LVC® kit can present false positive results. Marcondes et al. (2011) reported a high degree of cross-reactivity between *Leishmania* spp. and *T. cruzi* (57%) species, as well as between *Leishmania* spp. and *E. canis* (57%) species. Zanette et al. (2014), using three serological methods for the CVL serodiagnosis, showed cross-reactivity among the sera from dogs infected with *E. canis*, *B. canis*, *Toxoplasma gondii*, *Neospora caninum*, and *T. cruzi*.

In the present study, it was observed that all CVL sera recognized the rLiHyD protein. In addition, a null cross-reactivity was observed when sera of *T. cruzi*-, *B. canis*-, or *E. canis*-infected dogs were evaluated. When the putative B cell epitopes were studied, different results were obtained. Although the three peptides were clearly antigenic, only Peptide-3, designed to contain a conformational epitope, presented the same sensitivity and specificity values as the

Table 3 Diagnostic evaluation of specific linear and conformational B cell epitopes for CVL serodiagnosis

Antigen	Parameters[a]					
	Se (CI 95%)	Sp (CI 95%)	AUC (CI 95%)	κ (CI 95%)	Agreement[b]	AC (%)
Peptide-1	92.5 (81.8–97.9)	64.5 (55.4–72.9)	0.844 (0.783–0.905)	0.5 (0.4–0.6)	Moderate	72.8
Peptide-2	81.1 (68.0–90.6)	80.7 (72.6–87.2)	0.885 (0.834–0.925)	0.6 (0.5–0.7)	Moderate	80.8
Peptide-3	100.0 (93.3–100.0)	100.0 (97.1–100.0)	1.000 (1.000–1.000)	1.0 (1.0–1.0)	Perfect	100.0

ROC curves were used to determine ELISA sensitivity (Se), specificity (Sp), confidence interval (CI 95%), and are under curve (AUC) of each diagnostic peptide

[a] Parameters were calculated using all 177 samples presented in this study

[b] Agreement was calculated using parasitological assay (PCR technique) as a gold standard

Se sensitivity, *Sp* specificity, *CI* confidence interval, *AUC* area under curve, κ kappa index, *AC* accuracy

recombinant protein. The development and use of a new generation of biotechnological products has been based on identification of linear or conformational epitopes. Peptides recognized by the antibodies present in sera of patients developing different diseases can be employed for their diagnosis (Chávez-Fumagalli et al. 2013; Menezes-Souza et al. 2014). Also, peptides have emerged as vaccine candidates against rotavirus infection (Jafarpour et al. 2015) or dengue (Amat-ur-Rasool et al. 2015). However, to the best of our knowledge, the present study is the first to employ a peptide containing a conformational *Leishmania* epitope for CVL serodiagnosis. The better results observed for the conformational peptide relative to the linear ones offers an alternative approach to find new antigenic molecules that can be easily constructed and reproduced for diagnostic purposes.

Aiming to compare the diagnostic efficacy of the rLiHyD with other known diagnostic antigenic markers, the rA2 protein (Porrozzi et al. 2007; Akhoundi et al. 2013) was included in the analysis. This antigen is expressed in the amastigote stage of some *Leishmania* species and belongs to a protein family that displays a variable number of repeated sequences of 10 amino acid residues (Zhang et al. 1996). In 2007, the Brazilian Ministry of Agriculture licensed the use of Leish-Tec® vaccine (based the recombinant A2 protein) to prevent CVL. One problem associated with this vaccination is that about 30.9 % of the vaccinated dogs tested seropositive when an SLA-based ELISA is employed (Fernandes et al. 2014). Data obtained here showed that the rA2-based ELISA failed not only to distinguish between *Leishmania*-infected dogs from those vaccinated with Leish-Tec® but also between *Leishmania* and *E. canis*-infected dogs. The possibility to distinguish Leish-Tec® vaccinated dogs from the *Leishmania*-infected ones is another advantage of using the rLiHyD protein or Peptide-3. Both molecules have improved the diagnostic values found with other antigens, such as other recombinant single proteins (Fonseca et al. 2014; Rodríguez-Cortés et al. 2013) or chimeric proteins (Boarino et al. 2005; Faria et al. 2015), synthetic linear peptides (Chávez-Fumagalli et al. 2013), and phage-derived mimotopes (Costa et al. 2013).

One drawback of this work is that we have not demonstrated the presence of the LiHyD protein in *Leishmania*. However, the presence of antibodies recognizing this protein in the sera of infected dogs may be taken as an indication that it is expressed by the parasites during the active disease. Database searches performed in this study demonstrated the presence of LiHyD encoding genes in different *Leishmania* species. The protein is highly conserved in *Leishmania* spp. and no orthologue form was found in other Trypanosomatidae. This specificity together with its high antigenicity allows its use as a diagnostic tool for VL. However, further studies should be performed to understand its expression pattern, as well as the biological function that the protein plays in the parasite.

Although nearly 200 serum samples had been used in the present work, other studies are also necessary to evaluate a larger canine serological panel, in order to further corroborate the efficacy of these diagnostic markers for the CVL serodiagnosis. For instance, our panel did not contain samples from *L. braziliensis*-infected dogs, although in Brazil there are endemic areas for both tegumentary and visceral leishmaniasis (Courtenay et al. 2002; Coura-Vital et al. 2011). In this context, the present study should be taken as a proof-of-concept of the capacity of the proposed antigens for the CVL serodiagnosis and may well serve as a reference for further assays. However, due to scarcity of antigens to diagnose this important neglected disease, this study irradiates new possibilities to use both the rLiHyD protein and its conformational epitope as possible diagnostic markers for CVL.

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Compliance with ethical standards

Conflict of interest The authors confirm that they have no conflicts of interest in relation to this work.

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5.2 Artigo 2 – Breve introdução e objetivo

O segundo artigo foi intitulado *"Prophylactic properties of a Leishmania-specific hypothetical protein in a murine model of visceral leishmaniasis"* e publicado na revista internacional *Parasite Immunology.*

O presente estudo avaliou a proteína LiHyD como potencial candidata a compor uma vacina contra a leishmaniose visceral. Os efeitos da proteína obtida na sua forma recombinante (rLiHyD) administrada em camundongos BALB/c (associada à saponina) e sua capacidade protetora frente à infecção por *L. infantum* foram estudados. A progressão da infecção e a resposta imune relacionada à proteção são discutidas neste trabalho.

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Prophylactic properties of a *Leishmania*-specific hypothetical protein in a murine model of visceral leishmaniasis

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SUMMARY

In this work, the effect of vaccination of a newly described Leishmania infantum *antigenic protein has been studied in BALB/c mice infected with this parasite species. The LiHyD protein was characterized after a proteomic screening performed with the sera from dogs suffering visceral leishmaniasis (VL). Its recombinant version was expressed, purified and administered to BALB/c mice in combination with saponin. As a result of vaccination and 10 weeks after challenge using an infective dose of* L. infantum *stationary promastigotes, vaccinated mice showed lower parasite burdens in different organs (liver, spleen, bone marrow and footpads' draining lymph nodes) than mice inoculated with the adjuvant alone or the vaccine diluent. Protected mice showed anti-*Leishmania *IgG2a antibodies and a predominant IL-12-driven IFN-γ production (mainly produced by CD4$^+$ T cells) against parasite proteins, whereas unprotected controls showed anti-*Leishmania *IgG1 antibodies and parasite-mediated IL-4 and IL-10 responses. Vaccinated mice showed an anti-LiHyD IgG2a humoral response, and their spleen cells were able to secrete LiHyD-specific IFN-γ, IL-12 and GM-CSF cytokines before and after infection. The protection was correlated with the* Leishmania*-specific production on nitric oxide. Altogether, the results indicate that the new LiHyD protein could be considered in vaccine formulations against VL.*

Keywords *BALB/c mice, experimental vaccine, hypothetical protein,* Leishmania infantum, *saponin*

INTRODUCTION

Visceral leishmaniasis (VL) caused by *Leishmania donovani* and *L. infantum*/*L. chagasi* is an important neglected disease in the world (1). About 350 million people are at risk of contracting the infection in 98 countries, and 0·2–0·5 million of new cases of VL are registered annually (1, 2). The treatment of this disease is yet mostly based on the parenteral administration of pentavalent antimonials. However, increased parasite resistance and side effects registered in the patients have been the important problems (3, 4). Alternative drugs, such as amphotericin B and its liposomal formulations, pentamidine and miltefosine, have showed encouraging results; however, their toxicity, the increase of parasite resistance and/or high cost had limited their use (5–7). A VL elimination campaign has been initiated around the world, which will focus on vector control, early diagnosis and drug treatment strategies (8, 9). However, it is presumed that a vaccine will be required for successful elimination of the disease.

The development of a type 1 T helper (Th)-mediated immunity to prevent leishmaniasis has inspired the development of prophylactic vaccination against the disease, but few have progressed beyond the experimental stage using mice models for cutaneous and VL (10–14). The induction of specific CD4$^+$ Th1 cells against parasite proteins is crucial in controlling the infection caused by *Leishmania* spp. Both T CD4$^+$ and CD8$^+$ cells are the major weapons for an anti-*Leishmania* immune response, and

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they play a crucial role through the IL-2 and IFN-γ production. These cytokines activate the effector functions of macrophages, inducing the production of nitric oxide (NO), a molecule able to destroy the intracellular amastigotes (15, 16). Progression of the disease is associated with the induction of parasite-specific TGF-β-, IL-10- and IL-13-mediated responses (17–19). In addition, control of the IL-4-mediated Th2 humoral response against *Leishmania* commonly enhances vaccine-induced protection by indirectly increasing IFN-γ production by T cells (20–23).

The search for new targets to compose a vaccine is an important strategy for leishmaniasis control, mainly when employing proteins that are recognized by patients' sera, indicating that these antigens are present in the intracellular stages of the parasites and that they are able to interact with the host immune system. In recent decades, most of the studies evaluating vaccines for leishmaniasis have focused on *Leishmania* spp. proteins possessing orthologue forms in other protozoan or mammalian species (16, 24–28). As a result of the application of proteomics for the search of new antigens, some proteins considered in the database as hypothetical (because of their lack of sequence similarity to previous described proteins) were characterized. As they were recognized by sera of mammalian hosts infected by cutaneous or viscerotropic *Leishmania* species, they have emerged as putative new vaccine candidates. Besides its antigenicity, these proteins do not exhibit a high degree of sequence conservation with any host counterparts, decreasing the possibility to induce cross-reactive responses against the host.

We have designed a study to evaluate a new vaccine candidate, a *Leishmania* spp.-specific hypothetical protein namely LiHyD. The effects of the administration of the protein obtained as a recombinant protein (rLiHyD) in BALB/c mice (combined with saponin) in VL evolution due to *L. infantum* infection have been studied. This protein was selected for the analysis, because of its reactivity with sera from dogs with active VL (29). In addition, it is conserved among different *Leishmania* species, but it is not present in other trypanosomatids or in mammalian species. The decrease in *L. infantum* infection progression and the immune correlate with protection in vaccinated mice is discussed, presenting the LiHyD hypothetical protein associated with Th1-type adjuvants as a formulation able to protect against VL.

MATERIALS AND METHODS

Ethics statement

This study was approved by Committee on the Ethical Handling of Research Animals from Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, under the protocol number 043/2011.

Mice and parasite

Female BALB/c mice (8 weeks of age) obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, were maintained under specific pathogen-free conditions. Experiments were carried out using the *L. infantum* (MHOM/BR/1970/BH46) strain. Parasites were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin, at pH 7·4. The soluble *L. infantum* antigenic extract (SLA) was prepared from 1×10^{10} stationary-phase promastigote cultures (5–7 days old), as described (30).

Obtaining the *Leishmania*-specific hypothetical protein, LiHyD

The LiHyD (LinJ.33.3150) nucleotide and amino acid sequences used in this study were obtained from TriTrypDB (http://tritrypdb.org). The local alignment of the LiHyD sequence against the available complete genomes of other organisms was performed by BLAST. For obtaining the recombinant protein (rLiHyD), its coding region was amplified by PCR using genomic *L. infantum* DNA (as *Leishmania* protein genes lacks introns) and the next primers: *forward* (5'-GGATCCATGCAGATGCAAGG CAACATG-3') and *reverse* (5'-AAGCTTATTGTTGCCG CATACTTGG-3'). The coding region was cloned into the *p*GEM®-T Vector Systems (Promega, Madison, WI, USA) and double stranded sequenced. After the DNA insert was obtained by a *BamHI* and *HindIII* double digestion (taking advance of the corresponding cut sites included in the primers [underlined]) and transferred to the same cut sites of the pQE30 plasmid (Qiagen, Hilden, Germany). Recombinant plasmid was transformed into *Escherichia coli* M15 strain where the overexpression of the recombinant protein was performed by adding 1·0 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Promega, Montreal, Canada), for 3 h at 37°C. For protein purification, cells were lysed by a homogenizer after five passages through the apparatus. The product was centrifuged at 13 000 × *g* for 20 min at 4°C. The rLiHyD protein, containing a tag of 6× residues of histidine fused at its N-terminal region, was purified under nondenaturing conditions, using a 5 mL His-Trap column (GE Healthcare Bio-Sciences, Pittsburgh, USA), attached to an FPLC (GE Healthcare Life Science) system. The recombinant protein was dial-

ysed using saline as a buffer. After dialysis, rLiHyD was passed through a polymyxin–agarose column (Sigma), to remove residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000; BioWhittaker, Walkersville, USA). In addition, the purity of the recombinant protein was checked by a one-dimensional 10% SDS-PAGE as described below.

SDS-PAGE and immunoblotting analysis

To analyse the purity of the recombinant LiHyD protein, bacterial total extracts containing the expressed protein and the protein obtained at the end of the purification process (10 μg each sample) were submitted to a 10% SDS-PAGE. Gels were stained with Coomassie blue. Similar gels containing the purified were blotted onto a nitrocellulose membrane (0·2 μm pore size, Sigma). Membranes were blocked with PBS-T (phosphate-buffered saline plus Tween-20 0·05%) plus 5% BSA for 1 h and were independently incubated for 2 h with two pools of sera samples obtained from naive mice ($n = 8$) or *L. infantum*-chronically infected BALB/c mice (1 : 100 dilution in PBS-T solution). VL animals ($n = 8$) were subcutaneously infected by 1×10^7 stationary promastigotes of *L. infantum,* and sera were collected at week 10 after infection. As secondary antibody, blots were incubated with peroxidase-conjugated anti-mouse IgG (1 : 10 000; Sigma) for 2 h. Reactions were revealed by adding chloronaphthol, diaminobenzidine and H_2O_2, and stopped by adding distilled water.

Immunization and challenge infection

For immunization, three groups ($n = 8$ animals per group) were made. Vaccinated group were inoculated subcutaneously in their left hind footpad with 25 μg of rLiHyD associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Control mice were similarly inoculated with the adjuvant (25 μg of saponin) or the vaccine diluent (PBS). Three doses were administered at 2-week intervals. Four weeks after the final immunization, animals ($n = 4$ per group) were euthanized to analyse the immune response elicited by vaccination. At the same time, the remaining animals ($n = 4$, per group) were subcutaneously infected in the right hind footpad with 1×10^7 stationary-phase promastigotes of *L. infantum*. To determine parasite burden and to evaluate the immune response post-challenge, mice were euthanized at week 10 after infection. Vaccination experiments were repeated and presented similar results. Data shown in this study represent the mean ± standard deviation of the individual data pooled from two independent experiments, which presented similar results.

Estimation of parasite load

Single-cell suspensions of the spleen, liver, draining lymph nodes (dLN) and bone marrow (BM) from mice were independently prepared for parasite quantification, following a limiting-dilution protocol (30). Briefly, the organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by centrifugation at $150 \times g$, and cells were concentrated by centrifugation at $2000 \times g$. Sediment cells were suspended in 1 mL of supplemented Schneider's insect medium (prepared as depicted above, but using 20% FBS). Ten-fold serial dilutions (from 10^{-1} to 10^{-12}) were made in the same medium using 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark). Each individual sample was plated in triplicate, and parasites' presence was analysed by microscopy after 7 days of culturing at 24°C. Pipette tips were discarded after each dilution step to avoid carrying adhered parasites from one well to another. Results are expressed as the log of the titre (*i.e.* the dilution corresponding to the last positive well) adjusted per milligram of tissue.

Cytokine and nitrite production

Splenocyte cultures were established from individual mice ($n = 4$, per group) at the time of the two described sacrifices. For this, single-cell preparations were plated in duplicate in 24-well plates (Nunc) at 5×10^6 cells per mL. Cells were incubated in complete DMEM medium (DMEM supplemented with 10% heat-inactivated foetal bovine serum, 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin) in the absence (negative control) or in the presence of rLiHyD protein (20 μg/mL) or *L. infantum* SLA (25 μg/mL), at 37°C in 5% CO_2 for 48 h. IFN-γ, IL-4, IL-10, IL-12 and GM-CSF levels were assessed in the supernatants by a sandwich ELISA kit (BD OptEIA TM set mouse IFN-γ (AN-18), IL-4, IL-10, IL-12 and GM-CSF; Pharmingen®, San Diego, CA, USA) following manufacturer's instructions. When indicated, and in order to block IL-12-, $CD4^+$- and $CD8^+$-mediated T-cell cytokine release, spleen cells of mice vaccinated with rLiHyD plus saponin and challenged with *L. infantum* were *in vitro* stimulated with SLA (25 μg/mL), in the absence or in the presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12 (C17·8), CD4 (GK 1·5) or CD8 (53-6·7). Appropriate isotype-matched controls (rat IgG2a (R35-95) and rat IgG2b (95-1)) were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®). The nitrite production in the cultures supernatant was assessed by the Griess reaction (31) in the

supernatants of spleen cells cultures established from individual infected mice ($n = 4$, per group), and stimulated and cultured as above. Data were expressed as μM per 5×10^6 cells.

Evaluation of the antibody production

The humoral response was evaluated by collecting sera samples of the animals ($n = 4$, per group) after the last immunization and before infection (4 weeks after last doses), as well as at the 10th week after challenge. For this, The LiHyD- and *L. infantum* SLA-specific IgG1 and IgG2a isotypes levels were measured by an ELISA technique, as described (12). Recombinant protein was employed at 5 μg/mL, and SLA was assayed at 10 μg/mL (100 μL per well). The sera samples were diluted at 1 : 100, and the anti-mouse IgG1 and IgG2a horseradish peroxidase-conjugated antibodies (Sigma-Aldrich) were used in a 1 : 5000 dilution.

Statistical analysis

The results were processed using MICROSOFT EXCEL (version 10.0) or GRAPHPAD PRISM™ (version 6.0 for Windows). The one-way analysis of variance (ANOVA), followed by Bonferroni's post-test, was used for multiple comparisons between the groups. Differences were considered significant when $P < 0.05$. As indicated above, individual data from two different experiments were employed for the statistical analysis.

RESULTS

Expression, purification and antigenicity of the rLiHyD

To analyse the immunological properties of the rLiHyD protein, the recombinant version of the protein was overexpressed *E. coli* (Figure 1a, lane 2) and purified by affinity chromatography (Lane 1A, lane 3, black arrow). A band of 36·0 kDa, molecular weight expected from its primary structure, was observed in a Coomassie-stained 10% SDS-PAGE gel (Figure 1a). Next, a Western blot of the purified protein was incubated with sera samples obtained from naïve BALB/c mice or sera from the same animals but chronically infected with *L. infantum*. Results shown in Figure 1(b) demonstrate that the purified protein was recognized only by the sera from the infected mice (lane 3, black arrow).

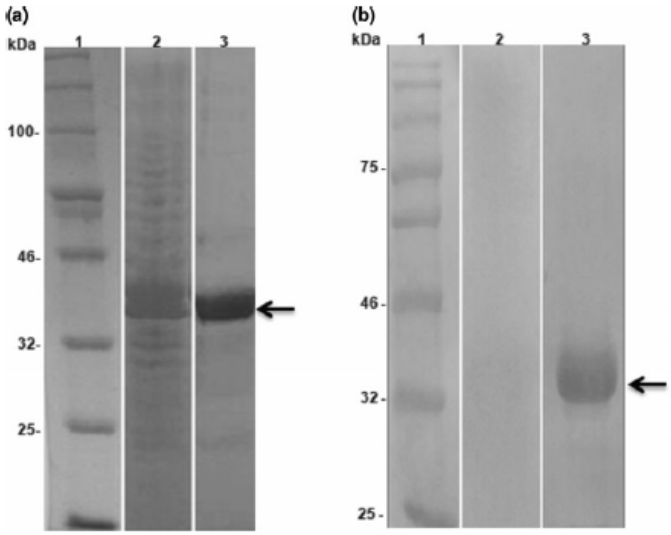

Figure 1 Antigenicity of the purified recombinant LiHyD protein. Analysis of the purification process is shown in (a). Molecular weight markers (lane 1), 10 μg of total bacterial cultures expressing the rLiHyD protein (lane 2) and protein purified by affinity chromatography (lane 3) were electrophoresed on a SDS-PAGE 10% gel. Coomassie staining of the gel is shown. In (b), similar gels loaded with molecular weight markers (lane 1) and 10 μg of the LiHyD purified protein (lanes 2 and 3) were electrophoresed and blotted onto a nitrocellulose membrane. Blots were incubated with pools of sera samples ($n = 8$ each group) from noninfected (lane 2) or chronically *Leishmania infantum*-infected mice (lane 3), and revealed with chloronaphthol. A scan from the blots is shown. Black arrows label the position of the rLiHyD protein in gels and blots.

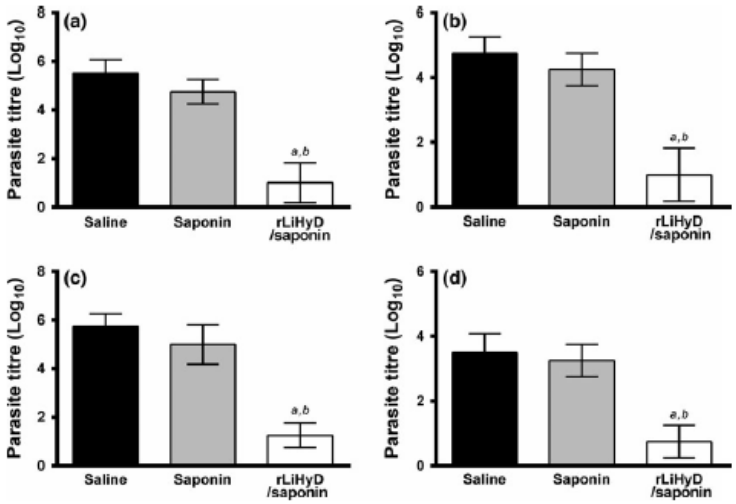

Figure 2 Protection of BALB/c mice vaccinated with rLiHyD plus saponin against *Leishmania infantum* infection. Mice inoculated with saline, with saponin or with rLiHyD plus saponin ($n = 4$, per group) were subcutaneously challenged with 1×10^7 stationary-phase promastigotes of *L. infantum*. The number of parasites in the liver (a), spleen (b), bone marrow (c) and paws' draining lymph nodes (d) was measured, 10 weeks after challenge, by a limiting-dilution technique. Mean ± standard deviation (SD) in each group is shown. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$). Data shown in this figure represent the mean ± standard deviation of two independent experiments.

Protective efficacy of a vaccine composed by rLiHyD plus saponin against *L. infantum*

To study the prophylactic properties of the rLiHyD protein, a vaccination–infection experiment was performed. For that purpose, a group of mice was immunized with the recombinant protein administered in combination with saponin. Two control groups were performed: mice inoculated with the same dose of saponin and mice inoculated with the vaccine diluent. A comparative analysis of the parasite burdens found in the spleen, liver, dLN and BM of the animals from the three groups was performed 10 weeks after a *L. infantum* challenge, performed subcutaneously in the footpads. It was observed that vaccinated mice presented significant reductions (between 4-log and 5-log) in the parasite load in the spleen (Figure 2a), liver (Figure 2b), dLN (Figure 2c) and BM (Figure 2d) when compared to both, saline and saponin groups.

The immune response generated in the animals against the parasite total proteins was analysed to compare the post-infection immune parameters found in the three groups of mice. Ten weeks after infection, vaccinated mice had a Th1 profile against a preparation of soluble leishmanial antigen (SLA). Thus, following *in vitro* stimulation with *L. infantum* SLA, spleen cells from vaccinated and infected mice produced higher levels of IFN-γ, IL-12 and GM-CSF, than those secreted by spleen cells from control mice groups (Figure 3a). As a correlate of disease progression, spleen cells from saline and saponin inoculated mice produced predominant IL-4 and IL-10 cytokine responses (Figure 3a). To evaluate the involvement of $CD4^+$ and $CD8^+$ T cells and the dependence of IL-12 in the parasite-specific IFN-γ production found in protected mice, their spleen cells were stimulated with SLA in the absence or presence of anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies (Figure 3b). IFN-γ production was significantly inhibited when the three antibodies were employed. The highest inhibition was obtained when the IL-12 blocking antibody was used, implicating to this cytokine in the parasite-specific IFN-γ response found in the protected mice. Higher decrease was found after anti-CD4 treatment than inhibiting the stimulation of $CD8^+$ T cells with monoclonal antibodies. These data were indicating that a predominant Th1 response was found against the parasite after infection in the protected mice. This finding correlates with the presence of a predominant IgG2 parasite-specific humoral response after infection in rLiHyD plus saponin vaccinated mice, in opposition to control groups, having a predominant IgG1 humoral response against SLA (Figure 4).

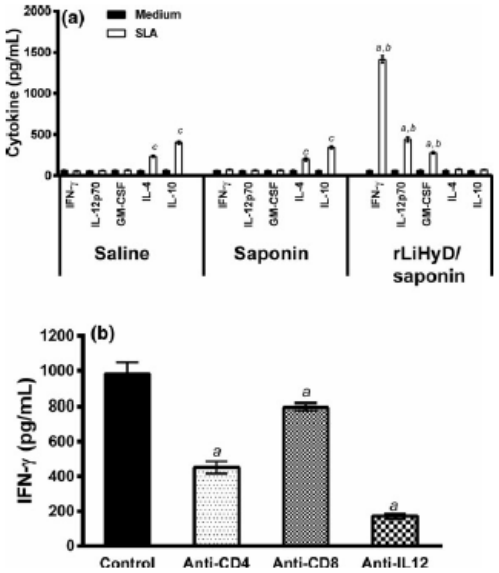

Figure 3 Cellular response against parasite proteins after *Leishmania infantum* challenge. Single spleen cells from mice that received saline or were inoculated with saponin or rLiHyD plus saponin ($n = 4$, per group) and were infected with *L. infantum* were collected, and independently cultured without stimulus (medium; negative control) or *in vitro* stimulated with SLA (25 μg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by ELISA in the culture supernatants (panel a). (*a*) indicates statistically significant increase in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant increase in relation to the saponin group ($P < 0{\cdot}001$). (*c*) indicates statistically significant increase in relation to the rLiHyD/saponin group ($P < 0{\cdot}001$). Also, spleen cells from mice vaccinated with rLiHyD plus saponin ($n = 4$) were *in vitro* stimulated with SLA (25 μg/mL; control) and incubated in the absence (positive control) or presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$ (panel b). The levels of IFN-γ in supernatants are shown. (*a*) indicates statistically significant differences from antibody treated samples and the untreated control sample ($P < 0{\cdot}001$). Bars represent the mean ± standard deviation (SD) of the groups.

rLiHyD-specific cellular and humoral response elicited by immunization using rLiHyD plus saponin before and after challenge infection

Next, the immune response elicited against the parasite antigen contained in the vaccine was studied. For that, the production of cytokines in the supernatants of spleen cells cultures stimulated with rLiHyD was analysed before and 10 weeks after challenge infection (Figure 5). Before challenge, it was observed that spleen cells derived from mice vaccinated with rLiHyD plus saponin produced higher levels of rLiHyD-specific IFN-γ, IL-12 and GM-CSF cytokines, than those secreted by spleen cells from control

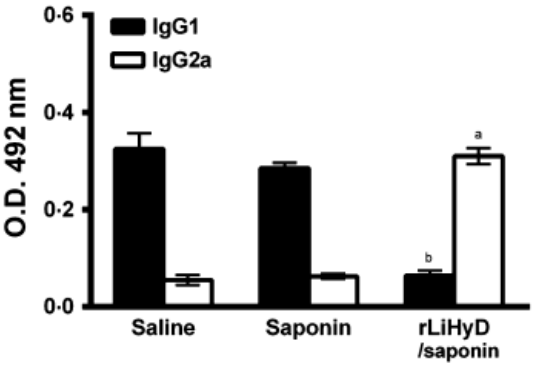

Figure 4 Anti-*Leishmania infantum* SLA humoral response after challenge. Sera samples were obtained from control and vaccinated mice ($n = 4$, per group), 10 weeks after infection with *L. infantum*. The levels of IgG1 and IgG2a isotypes against *L. infantum* SLA are shown. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant increase in IgG2a levels in vaccinated group regarding the saline and saponin groups ($P < 0{\cdot}001$). (*b*) indicates statistically significant decrease in IgG1 levels between vaccinated and both control groups ($P < 0{\cdot}001$).

groups (Figure 5a). In contrast, no production LiHyD-derived IL-4 and IL-10 could be observed in any experimental group. Mice that were immunized and lately challenged with *L. infantum* maintained the Th1 profile after infection, as their spleen cells cultures produced similar level of rLiHyD-specific cytokines than those secreted by spleen cells from vaccinated but uninfected mice (Figure 5b).

Evaluating the rLiHyD-specific humoral response in the vaccinated and/or infected animals (Figure 6), it was observed that before (Figure 6a) and after (Figure 6b) challenge, mice vaccinated with rLiHyD plus saponin presented higher levels of anti-rLiHyD-specific IgG1 and IgG2a isotype antibodies than the saline and saponin groups. The higher reactivity of the IgG2a antibodies found in the sera from vaccinated animals against the rLiHyD protein correlated with the Th1 cellular response determined by cytokine analysis. Although similar profiles were found for the humoral response before and after infection, the higher reactivity found post-challenge (comparing data from panel A and data shown in panel B; Figure 6) was indicating that the infection was able to boost the immune response elicited by vaccination, without changing its quality.

Analysis of the macrophage effector capacities after infection

It is well documented that macrophages stimulated by IFN-γ are able to activate NO synthesis to destroy the

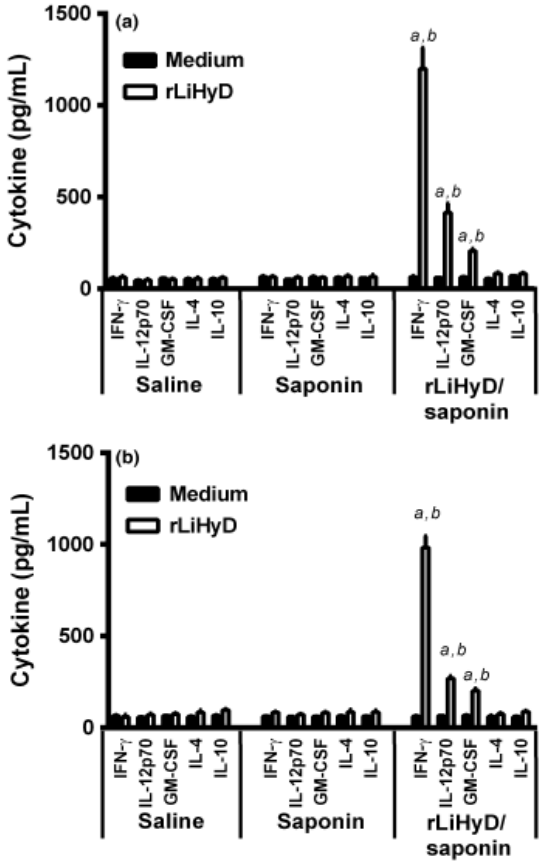

Figure 5 Vaccine-induced rLiHyD-specific cellular response. Single spleen cells of mice that received saline or were immunized with saponin or rLiHyD plus saponin ($n = 4$, per group) were collected before and after *Leishmania infantum* infection, and were nonstimulated (medium, negative control) or *in vitro* stimulated with rLiHyD protein (20 μg/mL), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured by ELISA in the culture supernatants before (a) and after (b) parasite challenge. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

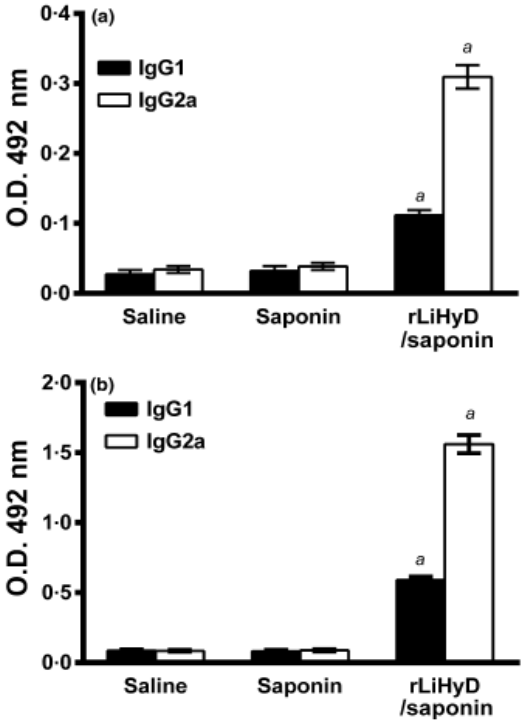

Figure 6 Vaccine-induced anti-rLiHyD humoral response. Sera samples were obtained from mice that received saline or were immunized with saponin or rLiHyD plus saponin, before ($n = 4$) (a) or after *Leishmania infantum* infection ($n = 4$) (b). Bars represent the mean ± standard deviation (SD) of IgG1 and IgG2a anti-LiHyD reactivity values from mice sera analysed in each experimental group. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

intracellular amastigotes. In an attempt to evaluate the parasite antigen-specific activation of macrophages in the three groups of mice, the presence of nitrite in culture supernatants was assayed as an indicator of NO production. For that purpose, spleen cells from infected mice (control and vaccinated mice groups) were cultured in the absence or in the presence of *L. infantum* SLA or the rLiHyD protein. Antigen-specific nitrite production was only detected in supernatants of the vaccinated and protected mice for both tested antigenic preparations (Figure 7).

DISCUSSION

In a recent immunoproteomic approach performed in stationary promastigote and axenic amastigote total extracts of *L. infantum* (29), different parasite proteins recognized by antibodies in sera of dogs suffering from active VL were characterized. Among them, some proteins annotated as hypothetical proteins were identified. These proteins are usually considered to have not defined functions, as they are predicted by genomics but they have not known domains or enough sequence conservation with other proteins (32). However, their antigenicity may be indicating

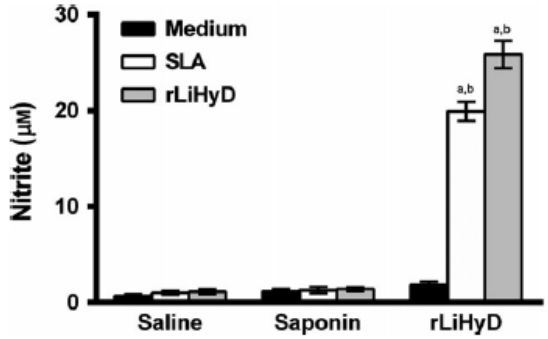

Figure 7 *Leishmania*-specific nitrite production after infection. Spleen cells from mice that received saline, saponin or rLiHyD plus saponin ($n = 4$, per group) and were challenged with *L. infantum* were collected 10 weeks after challenge. Spleen cells cultures were *in vitro* established and independently stimulated with SLA (25 μg/mL) or rLiHyD (20 μg/mL) for 48 h at 37°C in 5% CO_2. The presence of nitrite in the supernatants was analysed by the Griess method. Bars represent the mean ± standard deviation (SD) of the level of nitrites expressed in μM. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

that they are expressed in the promastigote or the amastigotes forms of the parasite during the active disease, and importantly that they are interacting with the host immune system. The LiHyD protein can be considered within this family of *Leishmania* antigens. It is recognized by sera from *L. infantum*-infected dogs (29), but also by antibodies present in the sera of *L. infantum*-chronically infected BALB/c mice. Interestingly, searches in the nucleotide or protein database have revealed that this protein is only found in the *Leishmania* genus. One gene copy exists in different *Leishmania* genomes, with identity ranging from 60% to 80%, in *L. major, L. braziliensis* and *L. mexicana*. No orthologue genes were found in the *Trypanosoma cruzi, T. brucei, T. vivax* and *T. congolense* annotated genomes. In this context, we have obtained the antigen as a purified recombinant protein and its immunoprophylactic properties have been analysed in a murine model of VL: BALB/c infected with *L. infantum*.

Although the use of murine models to test VL vaccines presents a limitation related to the organ-specific responses (parasites are cleared in the liver but infect chronically the spleen) (33), BALB/c mice infected with *L. infantum* or *L. donovani* have been widely employed with this purpose. In this sense, we have employed the subcutaneous challenge instead the intravenous infection, as similar evolution of the disease is produced (34, 35), and intravenous infection may undervalue the potential of some vaccine candidates (36). In addition, the rLiHyD protein has been combined with saponin, as it is an adjuvant able to induce cellular responses that have been employed for some canine vaccines (32, 37). Combination of parasite antigens with cellular adjuvants has resulted in the characterization of effective prophylactic tools against tegumentary and VL (16, 25, 30, 38–40).

Different previous studies evaluating vaccine candidates against leishmaniasis in murine models have showed different degree of success employing proteins evolutionary conserved (16, 27, 28, 41, 42). On the other hand, hypothetical proteins have emerged as interesting alternatives for vaccine development. As an example, Martins *et al.* (2013) evaluated the immunogenicity and protective efficacy of an amastigote-specific *L. infantum* hypothetical protein, LiHyp1, which was administered in association with saponin in BALB/c mice challenged subcutaneously with stationary promastigotes of *L. infantum* (16). The rLiHyp1/saponin vaccine induced a specific production of IFN-γ, IL-12 and GM-CSF. Interestingly, vaccinated animals showed significant reductions in the parasitism in organs such as liver, spleen, BM and dLNs, when compared to the control groups. Similarly, rLiHyD plus saponin-based vaccine was also able to induce a Th1 response against the hypothetical protein. This response did not change after challenge in quality, but it was boosted as a result of infection, as demonstrated by the higher anti-LiHyD immunoglobulin reactivity found in mice after challenge. The immune response elicited by the vaccine was able to diminish parasite numbers in all the evaluated organs (liver, spleen, BM and dLN). This decrease was correlated with high changes in the immune response elicited against the infective agent. Protected mice showed a pro-inflammatory profile against parasite antigens, showing an IL-12-dependent production of IFN-γ, whereas unvaccinated controls present parasite-specific IL10- and IL-4-driven responses. It was concluded that the induction of cellular inflammatory responses against the LiHyD was able to change the immune response against the parasite.

In the present study, the involvement of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated using monoclonal antibodies in the *in vitro* cultures, a technical protocol previously described by us (12, 16, 24, 39) and others (43, 44). This strategy has permitted to evaluate the contribution of both $CD4^+$ and $CD8^+$ T cells in the production of this cytokine in experimental vaccine models, based on the block antigen presentation. Data indicated here shown that $CD4^+$ T cells were the main source of IFN-γ in the vaccinated and infected mice, as the *in vitro* deactivation of these cells using the anti-CD4 monoclonal antibody significantly abrogated the parasite-dependent production of this cytokine. In agreement, $CD8^+$ T cells

also contribute, although in a less extension, to the production of parasite-specific IFN-γ-mediated response.

Our results also showed that the spleen cells from protected mice produced higher levels of parasite-specific GM-CSF than controls, a cytokine related with macrophage activation and related to protection against different *Leishmania* species in murine models (12, 38, 45, 46). Macrophage activation was also demonstrated by the *in vitro* production of nitrites after stimulation with the recombinant protein and total parasite extracts. According to these data, it could be speculated a possible activation of anti-*Leishmania* effector mechanisms mediated by IFN-γ production, as previously described by others (39, 47, 48), mainly the induction of the synthesis of the NO effector molecule.

Visceralizing and noncuring forms of leishmaniasis have been evaluated on extensive works in BALB/c mice, and disease progression has been generally thought to be associated with a Th2-type response and, in particular, with an early and sustained production of IL-4. Indeed, elevated levels of IL-4, IL-10 and/or IL-13 have been associated with VL progression (49, 50). Many authors have also related the induction of IL-4-dependent production of IgG1 antibodies, to be associated with the disease progression due to different *Leishmania* species, such as *L. amazonensis* (38, 51) and *L. infantum* (12, 16, 25, 39). In this context, a high production of IL-4 could induce to high levels of parasite-specific IgG1 isotype antibodies in the infected animals, this being an indicator of the polarization of Th2 response. On the other hand, IFN-γ has been implicated with the switch of antibody isotype on BALB/c mice to the IgG2a production. In consequence, high levels of this cytokine can induce a higher production of parasite-specific IgG2a isotype antibodies, being this humoral response an indicator of the development of a Th1 immune response in the animals (12, 25, 30).

Recombinant protein-based vaccines, although offering advantages in terms of safety and production costs, must be supplemented with immune adjuvants, to improve their immunogenicity (Cerpa-Cruz *et al.*, 2013). This situation has been observed in several studies that evaluate recombinant proteins, also considered effective vaccine candidates to protect against tegumentary and VL, but whose the association of an immune adjuvant is also considered critical to vaccine efficacy (16, 25, 29, 30, 38, 40, 52). In this context, saponins are natural glycosides derived from steroid or triterpene, which exhibit distinct biological and pharmacological activities. Notably, they can activate the mammalian immune system, which has led to their use as immune adjuvants in vaccines. Their unique capacity to stimulate both the Th1 immune response and the production of cytotoxic T cells makes the saponins ideal for use in vaccine compositions to protect against intracellular pathogens, as is the case of the *Leishmania* parasite (53, 54). Although they are considered toxic for use in humans, due to the possibility to cause local and granulomatous reactions, haemolysis and local pain (55); they are one of the few products authorized to use in dogs, being the adjuvant employed in a commercial Brazilian vaccine to protect against canine VL, namely Leish-Tec® (56).

In the present study, vaccinated mice were able to down-regulate the *Leishmania*-specific IL-10-mediated responses generated in the control nonprotected mice. The immuno-modulatory effects of IL-10, making macrophages unresponsive to IFN-γ, is another determinant factor for disease progression caused by viscerotropic *Leishmania* species (57–59). This would imply that the changes observed in the present work regarding the balance between IFN-γ and IL-10 during infection in protected vs. nonprotected mice are particularly important for the VL control as previously reported (60). Overall, our results validate the possibility to employ LiHyD, a *Leishmania*-specific protein, as a vaccine candidate against VL.

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DECLARATION OF INTEREST

The authors hereby declare that there is no conflict of interest.

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5.3 Artigo 3 – Breve introdução e objetivo

O terceiro artigo foi intitulado *"Cross protective efficacy of Leishmania infantum LiHyD protein against tegumentary leishmaniasis caused by Leishmania major and Leishmania braziliensis species",* publicado na revista internacional *Acta Tropica.*

No presente estudo, a combinação entre rLiHyD e saponina foi testada em camundongos BALB/c para verificação de proteção contra a infecção causada pelas espécies *Leishmania major* e *Leishmania braziliensis*, no sentido de se desenvolver uma vacina que seja capaz de induzir proteção heteróloga contra espécies de Leishmania que podem causar as leishmanioses tegumentar e visceral.

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Cross-protective efficacy of *Leishmania infantum* LiHyD protein against tegumentary leishmaniasis caused by *Leishmania major* and *Leishmania braziliensis* species

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ABSTRACT

Vaccination can be considered the most cost-effective strategy to control neglected diseases, but nowadays there is not an effective vaccine available against leishmaniasis. In the present study, a vaccine based on the combination of the *Leishmania*-specific hypothetical protein (LiHyD) with saponin was tested in BALB/c mice against infection caused by *Leishmania major* and *Leishmania braziliensis* species. This antigen was firstly identified in *Leishmania infantum* and showed to be protective against infection of BALB/c mice using this parasite species. The immunogenicity of rLiHyD/saponin vaccine was evaluated, and the results showed that immunized mice produced high levels of IFN-γ, IL-12 and GM-CSF after *in vitro* stimulation with rLiHyD, as well as by using *L. major* or *L. braziliensis* protein extracts. After challenge, vaccinated animals showed significant reductions in the infected footpad swellings, as well as in the parasite burden in the infection site, liver, spleen, and infected paws draining lymph nodes, when compared to those that were inoculated with the vaccine diluent (saline) or immunized with saponin. The immunization of rLiHyD without adjuvant was not protective against both challenges. The partial protection obtained by the rLiHyD/saponin vaccine was associated with a parasite-specific IL-12-dependent IFN-γ secretion, which was produced mainly by $CD4^+$ T cells. In these animals, a decrease in the parasite-mediated IL-4 and IL-10 responses, associated with the presence of high levels of LiHyD- and parasite-specific IgG2a isotype antibodies, were also observed. The present study showed that a hypothetical protein that was firstly identified in *L. infantum*, when combined to a Th1 adjuvant, was able to confer a cross-protection against highly infective stationary-phase promastigotes of two *Leishmania* species causing tegumentary leishmaniasis.

1. Introduction

Leishmaniasis comprises a group of diseases ranging from cutaneous to visceral forms. Although tegumentary leishmaniasis (TL) is not a fatal disease, it is endemic in more than 70 countries and 90% of the cases are registered in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria (Desjeux, 2004; Alvar et al., 2012). Different species of the parasites can act as causative agents of TL (Grimaldi and Tesh, 1993; Reithinger et al., 2007). In Brazil, it is most commonly caused by *Leishmania braziliensis* species, whereas *Leishmania major* and *Leishmania tropica* have been associated with disease in the Old World (Desjeux, 2004). The disease control has been difficult due to the natural features of reservoir and vectors, making the elimination of both components hard to obtain. Also, therapeutic measures are ineffective and the development of a vaccine would be the most effective measure to eliminate or, at least,

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control the disease (Marzochi and Marzochi, 1994; Silveira et al., 2004).

Most strains of mice infected by *L. major* develop small cutaneous lesions that spontaneously resolve over several weeks (Howard et al., 1980; DeTolla et al., 1981). On the other hand, certain mice strains, such as the BALB/c, develop severe and non-healing infections that visceralize to internal organs and can be fatal (Djoko-Tamnou et al., 1981; Li et al., 1997). The development of a healing versus non-healing infection is determined by immune responseí type that usually develops after parasite inoculation. Healing infections are characterized by development of a Th1 type response mediated mainly by $CD4^+$ cells producing the macrophage-activating cytokine, IFN-γ. On the other hand, IL-4 and IL-10 produced by Th2 cells predominate in susceptible animals (Scott et al., 1988; Heinzel et al., 1991).

An additional factor which might potentially influence the course of disease and/or resistance of the animals is related to the parasitesí infectivity. In this context, *L. braziliensis* species is the causative agent of cutaneous and mucosal leishmaniasis, and the latter is a severe and disfiguring form of TL in humans (David and Craft, 2009). There are studies showing that BALB/c mice are partially resistant to infection with this *Leishmania* species: they do not develop severe lesions and usually cure the infection developing a Th1/Th2 mixed response (Childs et al., 1984; Dekrey et al., 1998; Oliveira et al., 2004). However, others have shown that the outcome of the infection of BALB/c mice by *L. braziliensis* can be completely changed by treating them with previous injections of *Leishmania amazonensis* amastigote extracts, when the animals thus become susceptible to the disease (Araújo et al., 2014; Silva et al., 2015). In addition, it was also observed that pre-isolated parasites from lesions of chronically-infected BALB/c mice were more infective than those that were *in vitro* cultured for long periods of time, as was observed in the *L. amazonensis* species (Magalhães et al., 2014).

It is postulated that to be an effective vaccine candidate against leishmaniasis an antigen should be shared by different parasite species, immunogenic in all or in the most of them, present few polymorphisms, and not present in other Trypanosomatidae (Coelho et al., 2012; Fernandes et al., 2012). However, the most of studies have worked with *Leishmania* proteins also expressed in other *Leishmania*-related parasites (Modabber, 2010; Zanin et al., 2007; Todolí et al., 2012). In this context, *Leishmania*-specific proteins could be considered as interesting targets for constructing effective vaccines against disease.

We have designed this study to evaluate a new vaccine candidate, a *Leishmania*-specific hypothetical protein, namely LiHyD (LinJ.33.3150). This protein is conserved between different *Leishmania* species including those causing TL, with identity values of 80% and 56% with respect *L. major* (LmjF.33.2990) and *L. braziliensis* (LbrM.33.3270) homologues, respectively. Besides of this, LiHyD putative T cell epitopes evaluated by bioinformatics tools are located in regions conserved among the different parasite species. In this context, the effects of the administration of this protein obtained as a recombinant product (rLiHyD) in BALB/c mice (combined with saponin), a formulation previously showed to be protective against *Leishmania infantum* (Lage et al., 2015), was evaluated as a protective vaccine against *L. major* or *L. braziliensis* challenge infection. The vaccination using rLiHyD/saponin was able to mount a Th1 response before and after infection, which was based on rLiHyD- and parasite-specific IFN-γ production mainly by $CD4^+$ T cells, and was able to reduce the parasite burden in the infected and vaccinated animals. In this context, the present study showed a new candidate to compose a pan-*Leishmania* vaccine to protect against parasite species causing TL.

2. Materials and methods

2.1. Ethics statement and animals

The Committee on the Ethical Handling of Research Animals from Federal University of Minas Gerais (UFMG) approved this study with the protocol number 043/2011. Female BALB/c mice (8 weeks old) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG; and were maintained under specific pathogen-free conditions.

2.2. Parasites

L. major (MHOM/IL/1980/Friedlin) and *L. braziliensis* (MHOM/BR/1975/M2903) were used. Parasites were grown at 24 °C in complete Schneider's medium (Sigma-Aldrich, USA), which was composed by Schneiderís medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extracts (SLA) were prepared from 2×10^8 stationary-phase promastigotes of *L. major* (SLAm) or *L. braziliensis* (SLAb), as described (Coelho et al., 2003). Highly infective stationary-phase promastigotes of the parasites were used to challenge infections, since they were obtained after their passing in BALB/c mice or hamster (*L. major* and *L. braziliensis*, respectively), and *in vitro* new cultures derived from two passages were used to perform the infections.

2.3. Cloning of the LiHyD protein and immunoblotting assay

The cloning, expression and purification of rLiHyD protein was performed as described (Lage et al., 2015). To verify the antigenicity of the protein in BALB/c mice infected with *L. major* or *L. braziliensis* species, rLiHyD (10 μg) was submitted to a 10% SDS-PAGE and blotted onto a nitrocellulose membrane (0.2 μm pore size, Sigma, St. Louis, USA). Then, sera obtained from *L. major*- or *L. braziliensis*-chronically infected BALB/c mice (n = 8 per group), as well as sera of non-infected animals (n = 8) were individually incubated with the blots. The technical protocol was performed as described (Martins et al., 2015).

2.4. Vaccination, challenge infection and cutaneous lesion development

BALB/c mice (n = 16 per group) were subcutaneously immunized in their left hind footpad with 25 μg of rLiHyD protein, with or without the association of 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). In addition, other animals were immunized with 25 μg of saponin or received saline (vaccine diluent). Each group was boosted two and four weeks later using the same dose. Thirty days after the third and last vaccine dose, immunized mice were subcutaneously challenged into the right hind footpad using 1×10^6 or 1×10^7 stationary-phase promastigotes of *L. major* or *L. braziliensis* (n = 8 in each group). Then, the footpad swellings were measured weekly using a metric caliper (the thickness of the left footpad minus thickness of the right footpad is shown) until 10 weeks after challenge, when animals were euthanized to the evaluation of the parasitological and immunological parameters associated with the vaccination.

2.5. Parasite burden and cytokine production

The parasite load was evaluated by a limiting-dilution technique, as described (Martins et al., 2013). For this, the infected footpads, spleen, liver and infected paws draining lymph nodes

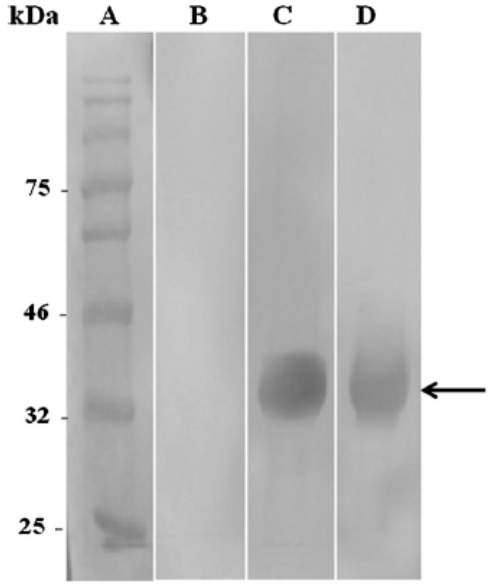

Fig. 1. Immunoblotting assays using the recombinant LiHyD protein. A low range protein ladder standard (Invitrogen™, Life Technologies, USA) was used (lane A). The individual reactions of the recombinant LiHyD protein (36 kDa) with the pools of sera from non-infected mice (n = 8) (lane B), or those chronically infected with *L. major* (n = 8) (lane **C**) or *L. braziliensis* (n = 8) (lane D) are shown (reaction with the rLiHyD is indicated by a black arrow). Immunoblottings were derived from three independent experiments, and one representative preparation is shown.

(dLNs) of the animals were collected, 10 weeks after infection and were independently processed. Results were expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well), which were adjusted per milligram of tissue or organ. To evaluate the cytokine production, spleen cells culture and cytokine assays were performed 30 days after the last vaccine dose (before infection), as well as at the 10th week after challenge. For this, splenocytes obtained were seeded and independently cultured in duplicate in 24-well plates (Nunc), at 5×10^6 cells per mL. Cells were incubated in complete DMEM medium (background, negative control), which was composed by DMEM medium plus 20% FBS, 20 mM L-glutamine, 200 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7.4; or separately stimulated with rLiHyD (20 $\mu g\ mL^{-1}$), concanavalin A (ConA, 5 $\mu g\ mL^{-1}$) and *L. major* or *L. braziliensis* SLA (25 $\mu g\ mL^{-1}$ each one), at 37 °C in 5% CO_2 for 48 h. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were assessed in the supernatants by a sandwich ELISA provided in commercial kits (BD OptEIA TM set mouse, Pharmingen®, USA); following the manufacturer's instructions. Aiming to block the IL-12, $CD4^+$ and $CD8^+$ mediated T cell cytokine release, spleen cells of mice vaccinated with rLiHyD/saponin and lately challenged with *L. major* or *L. braziliensis* were *in vitro* stimulated with rLiHyD (20 $\mu g\ mL^{-1}$), *L. major* or *L. braziliensis* SLA (25 $\mu g\ mL^{-1}$, each one) and incubated in the presence of 5 $\mu g\ mL^{-1}$ of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5) or CD8 (53-6.7). Appropriate isotype-matched controls—rat IgG2a (R35-95) and rat IgG2b (95-1)—were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®).

2.6. Humoral response

The antibody production was evaluated 30 days after the third and last vaccine dose (before infection), as well as at 10th week after challenge. The LiHyD- and parasite-specific IgG1 and IgG2a isotypes antibodies were measured by ELISA, as described (Martins et al., 2015). The concentrations used of rLiHyD, and *L. major* or *L. braziliensis* SLA were of 0.5, 1.0 and 1.0 μg per well, respectively. Sera samples were diluted at 1:100 in PBS-T, and the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were used 1:10,000 diluted in PBS-T. Reactions were developed through incubation with 2 mg orto-phenylenediamine, 2 μL H_2O_2 30 vol., and 10 mL citrate-phosphate buffer pH 5.0, for 30 min and in the dark; when they were stopped by adding 25 μL H_2SO_4 2 N. Then, the optical density was read in an

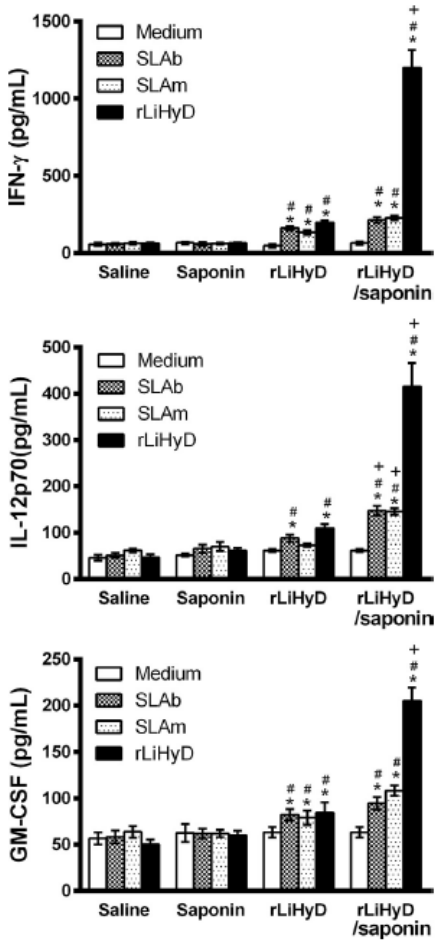

Fig. 2. Cellular response induced by immunization of BALB/c mice before challenge. Single cell suspensions were obtained from the spleens of mice (n = 8 for each group), 30 days after the third and last immunization and before challenge infection. Single cell suspensions were incubated in complete DMEM medium (negative control), which was composed by DMEM medium plus 20% FBS, 20 mM L-glutamine, 200 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7.4; or separately stimulated with rLiHyD (20 $\mu g\ mL^{-1}$), *L. braziliensis* or *L. major* SLA (25 $\mu g\ mL^{-1}$, each one), for 48 h at 37 °C in 5% CO_2. IFN-γ (A), IL-12 (B), and GM-CSF (**C**) levels were measured by a capture ELISA in the culture supernatants. Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups.

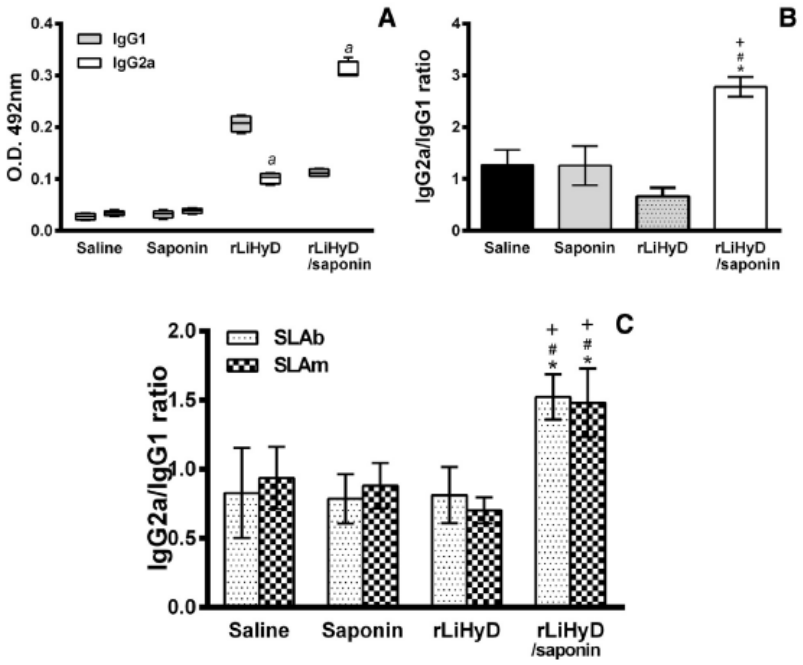

Fig. 3. Humoral response induced after immunization of BALB/c mice.
Sera samples were obtained from vaccinated mice (n = 8 for each group), 30 days after the third and last vaccine dose and before challenge infection. The reactivity against the rLiHyD protein, based on the levels of protein–specific IgG1 and IgG2a isotypes, was determined and is shown as whisker (min to max) plots (A). In addition, the ratios between the anti-LiHyD IgG2a and IgG1 isotypes levels were calculated and are also shown (B). With the evaluation of the parasite–specific IgG1 and IgG2a isotypes levels, the ratios between the IgG2a and IgG1 values were calculated and are shown (**C**). Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups. (a) indicates the existence of statistically significant difference in relation to the IgG1 levels in the same group.

ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada), at 492 nm.

2.7. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The one-way analyses of variance (ANOVA), following by Bonferroni's post-test, were used for multiple comparisons between the groups. Differences were considered significant when $P < 0.05$. Data shown represent the mean ± standard deviation of two independent experiments.

3. Results

3.1. rLiHyD is recognized by antibodies in sera of L. major or L. braziliensis-chronically infected mice

In this study, a *Leishmania* hypothetical protein, which was firstly identified as protective against *L. infantum*, was evaluated in immunoblotting assays by its reaction with antibodies in sera of *L. major* or *L. braziliensis*-infected BALB/c mice (Fig. 1). In the results, using a Ladder standard as a marker (lane A), it was shown that whereas the recombinant protein was not reactive with antibodies in sera of non-infected mice (lane B), it was specifically recognized by antibodies in sera of *L. major* or *L. braziliensis*-chronically infected mice (lanes C and D, respectively). These data demonstrated that the *L. infantum* LiHyD protein is also expressed in these species causing TL.

3.2. Immunization with rLiHyD/saponin elicits high levels of IFN-γ, IL-12 and GM-CSF after SLAm or SLAb stimulation, which is associated with a high production of IgG2a isotype antibodies

Aiming to evaluate the cellular effects of immunization using rLiHyD/saponin, we have characterized the levels of some cytokines in the culture supernatants after stimulation using rLiHyD or the *Leishmania* antigenic preparations (Fig. 2). Following the *in vitro* stimulation with rLiHyD, SLAm or SLAb, it was observed that spleen cells from vaccinated mice produced higher levels of IFN-γ (Fig. 2A), IL-12 (Fig. 2B) and GM-CSF (Fig. 2C) than those secreted by cultures established from control (saline and saponin) groups. When animals were immunized with rLiHyD without a Th1 adjuvant, no significant production of IFN-γ, IL-12 and GM-CSF was observed. In addition, no difference in IL-4 and IL-10 levels was observed in any experimental group, using all stimuli (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.actatropica.2016.03.011). When ConA was used as a positive control, all cytokines were produced in high levels by the *in vitro* stimulated spleen cells in all experimental groups, demonstrating the cellular viability in the different performed experiments (data not shown).

To characterize the humoral response triggered by immunization, we have analyzed the profile of IgG1 and IgG2a isotypes antibodies specific to LiHyD, SLAm or SLAb (Fig. 3). Data showed that mice vaccinated with rLiHyD/saponin produced higher lev-

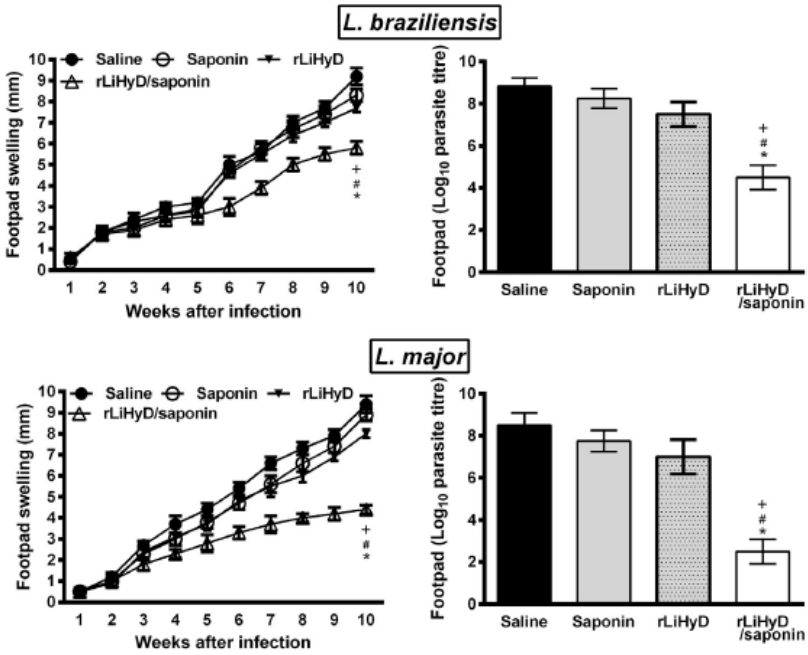

Fig. 4. Effect of the vaccination using rLiHyD against the murine tegumentary leishmaniasis.
BALB/c mice (n = 16 per group) were subcutaneously immunized in their left hind footpad with 25 μg of the rLiHyD protein, with or without the association of 25 μg of saponin. In addition, other animals were immunized with 25 μg of saponin or received saline. Each group was boosted two and four weeks later using the same dose. Thirty days after the third and last vaccine dose, immunized mice were subcutaneously infected into their right hind footpad with 1×10^6 or 1×10^7 highly infective stationary-phase promastigotes of *L. major* or *L. braziliensis* (n = 8 in each group), respectively. The lesion development and parasite load in the infection site were monitored during 10 weeks in mice infected with *L. braziliensis* (A and B, respectively) or *L. major* (C and D, respectively). Lines and bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups.

els of LiHyD-specific IgG2a isotype, when compared to the IgG1 levels (Fig. 3A). On the other hand, animals immunized with rLiHyD without adjuvant showed higher IgG1 levels in comparison to the IgG2a isotype. In addition, the ratio between the IgG2a and IgG1 levels was calculated, and it was showed that mice vaccinated with rLiHyD/saponin presented a higher IgG2a/IgG1 ratio, when compared to the other groups (Fig. 3B). Evaluating the antibody production against the parasite extracts, animals immunized with rLiHyD/saponin showed a higher production of anti-*Leishmania* antibodies than those produced by control mice, which was associated with a predominance of parasite-specific IgG2 isotype in relation to the obtained IgG1 levels (Fig. 3C).

3.3. *The rLiHyD/saponin vaccine induces a cross-protective response against* L. major *and* L. braziliensis *challenge infection*

To evaluate the protection induced by *L. infantum* LiHyD protein against the challenge caused two *Leishmania* species causing TL, rLiHyD/saponin immunized mice were separately challenged by subcutaneous injection using stationary-phase promastigotes of *L. braziliensis* or *L. major*. For the *L. braziliensis* model, mice were infected with 1×10^7 parasites and the development of dermal lesion and parasite load were evaluated 10 weeks after infection (Fig. 4). In the results, it was observed that immunized mice with rLiHyD/saponin displayed significant reductions in the footpad swellings, when compared to saline and saponin mice (37% and 30% respectively) (Fig. 4A). In addition, these animals showed a 2.0-log and 1.8-log reduction in the number of parasites in the infected footpads, when compared to saline and saponin groups, respectively (Fig. 4B). Using rLiHyD to immunize the animals, no significant difference was observed in relation to the control groups. For the *L. major* model, 1×10^6 stationary-phase promastigotes of the parasites were used for challenge. Vaccinated mice showed significant reductions in the footpad swelling when compared to saline and saponin groups (53% and 51%, respectively) (Fig. 4C). Also, rLiHyD did not shown any significant difference in relation to the control groups. Animals vaccinated with rLiHyD/saponin showed 3.4-log and 3.1-log reductions in the number of parasites in the infected footpads, when compared to the saline and saponin groups, respectively (Fig. 4D). Besides of the parasite burden estimated in the infected footpads, the presence of parasites was also evaluated in the popliteal lymph nodes (draining the site of infection). Also, the dispersion of parasites to internal organs such as the spleen and liver was evaluated (Fig. 5). For the *L. braziliensis* model, significant reductions in the number of parasites were observed in all evaluated organs of the rLiHyD/saponin vaccinated mice, when compared to the control mice. Vaccinated mice presented reductions in the parasite load in spleen (2.5- and 2.2-log reductions, Fig. 5A), liver (2.4- and 2.3-log reductions, Fig. 5C) and dLNs (3.0- and 2.7-log reductions, Fig. 5E), when compared to the saline and saponin groups, respectively. For the *L. major* model, significant reductions were observed in the vaccinated mice, when the spleen (5.0- and 4.3-log reductions, Fig. 5B), liver (5.5- and 5.0-log reductions, Fig. 5D) and dLNs (4.2- and 3.8-log reductions, Fig. 5F) were evaluated, in comparison to the saline and saponin groups, respectively. When the immunization was performed using rLi-

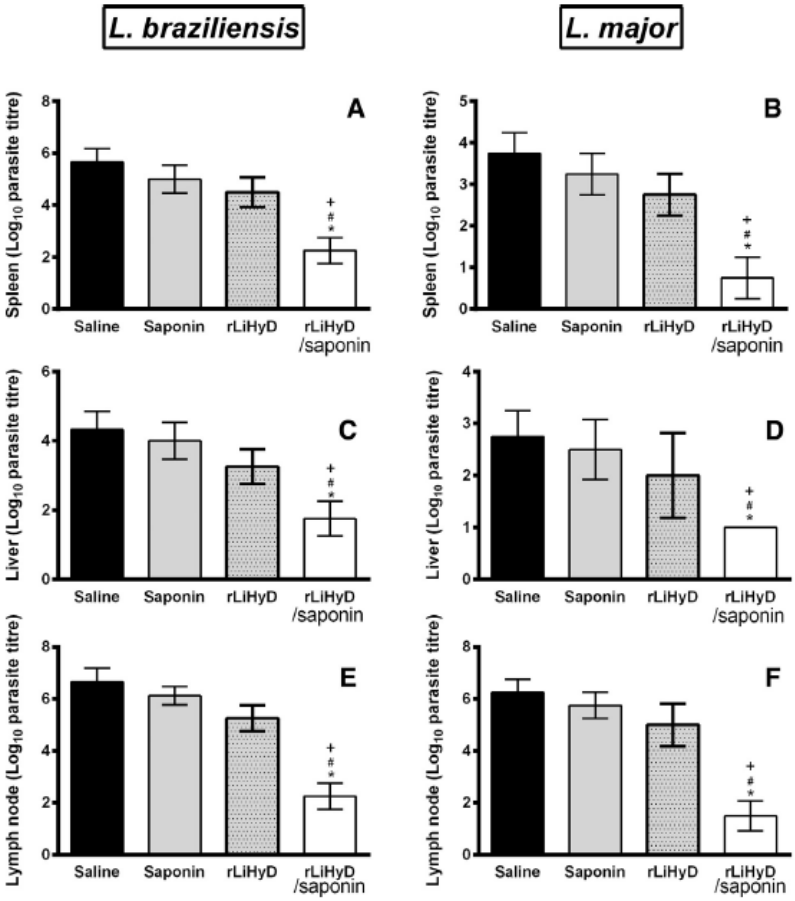

Fig. 5. Parasite load in different organs of the infected and vaccinated animals.
Mice inoculated with saline or vaccinated with the rLiHyD protein, saponin or rLiHyD plus saponin were challenged subcutaneously in their left footpad with 1×10^7 or 1×10^6 highly infective stationary-phase promastigotes of *L. braziliensis* and *L. major* (n = 8 for each group), respectively. The results of the parasite load obtained after *L. braziliensis* (A, C and E) or L. major (B, D and F) infection are shown. The number of parasites in the spleen (A and B), liver (C and D) and infected paws draining lymph nodes (E and F) were determined, 10 weeks after challenge. Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups.

HyD, although a reduction in the parasite load had been observed, no significant difference was obtained in relation to the control groups.

3.4. Immunization using rLiHyD/saponin induces higher production of th1-related cytokines, 10 weeks after challenge

The cytokine profile in the infected and immunized mice was evaluated in the supernatants of spleen cell cultures using rLiHyD, SLAm or SLAb as stimuli, 10 weeks after challenge (Fig. 6). Our results showed that, after *L. major* infection, spleen cells from mice vaccinated with rLiHyD/saponin produced higher levels of rLiHyD- and parasite-specific IFN-γ, IL-12 and GM-CSF cytokines, when compared to those secreted by spleen cells from the control groups (Fig. 6A, C and E, respectively). The similar immune profile was observed after *L. braziliensis* infection (Fig. 6B, D and F, respectively). In contrast, the *Leishmania*-driven production of IL-4 showed that vaccination using rLiHyD/saponin induced any production of this cytokine after either *L. major* (Fig. 6G) or *L. braziliensis* (Fig. 6H) infection, whereas spleen cells of the control mice produced higher levels of IL-4. Similar results were obtained when the IL-10 levels were evaluated, using either *L. major* (Fig. 6I) or *L. braziliensis* (Fig. 6J) infection model. Similarly to the observed for the saline and saponin groups, animals immunized with rLiHyD showed a higher production of IL-4 and IL-10 and lower levels of IFN-γ, IL-12 and GM-CSF after stimuli using rLiHyD and SLA, when compared to the results obtained in the rLiHyD/saponin group. In addition, the involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production after stimuli using rLiHyD and SLA in the rLiHyD/saponin group was also evaluated (Fig. 7). Results in both *L. major* (Fig. 7A) and *L. braziliensis* (Fig. 7B) infection models showed that the IFN-γ production was significantly inhibited when the anti-CD4 monoclonal antibody was added to the *in vitro* cultures. On the other hand, although the addition of the anti-CD8 monoclonal antibody has decreased the IFN-γ production in the cultures, when compared to the cell cultures without treatment; the IFN-γ levels were higher in relation to those treated with the anti-CD4 monoclonal antibody.

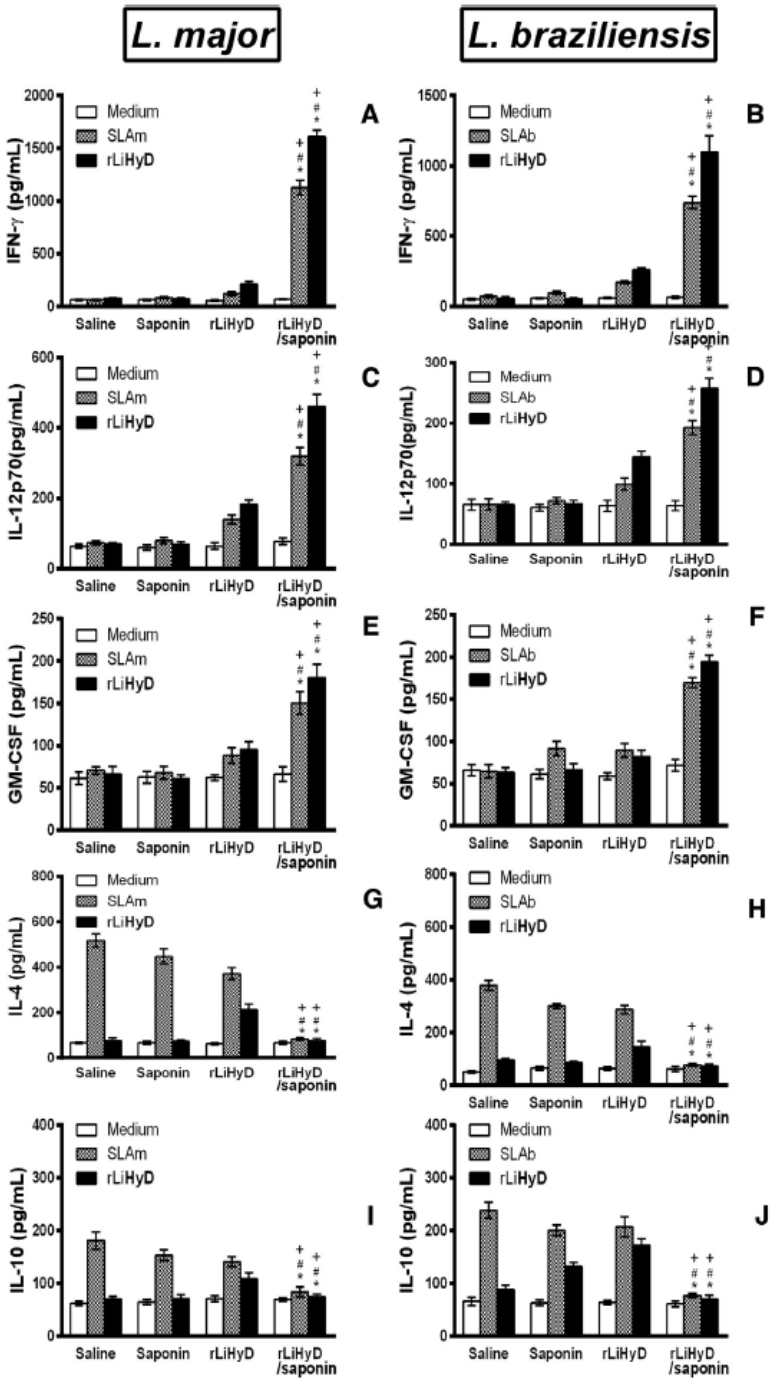

Fig. 6. Cytokine profile in the vaccinated BALB/c mice, which were challenged with *L. major* or *L. braziliensis*.
Single cell suspensions were obtained from the spleens of mice (n = 8 for each group), 10 weeks after the challenge infection. The cytokine levels after *L. major* (A, C, E, G and I) or *L. braziliensis* (B, D, F, G and J) infection are shown. IFN-γ (A and B), IL-12 (C and D), GM-CSF (E and F), IL-4 (G and H), and IL-10 (I and J) were measured by ELISA in the culture supernatants. Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups.

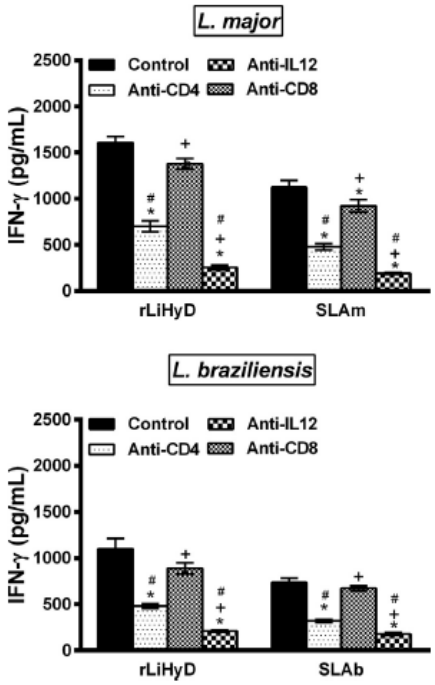

Fig. 7. Involvement of $CD4^+$ and $CD8^+$ T cells in IFN-g production in the infected and vaccinated animals.
Spleen cells of mice that were vaccinated with rLiHyD/saponin and challenged with *L. braziliensis* or *L. major* (n = 8 for each group) were collected, 10 weeks after challenge, when they were *in vitro* stimulated with the rLiHyD protein (20 μg mL^{-1}), *L. braziliensis* or *L. major* SLA (25 μg mL^{-1} each one) in the absence (positive control) or presence of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$. The results obtained after *L. major* or *L. braziliensis* infections are indicated in the graph. Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference in relation to the control group. (#) indicates the existence of statistically significant difference in relation to the anti-CD8 monoclonal antibody group. (+) indicates the existence of statistically significant difference in relation to the anti-CD4 monoclonal antibody group.

3.5. Humoral response elicited after challenge

We analyzed the humoral response profile elicited in the infected and immunized mice, aiming to evaluate the global anti-*Leishmania* humoral response induced by infection using both *Leishmania* species (Fig. 8). In the results, low levels of anti-rLiHyD antibodies were observed in the sera from saline, saponin and rLiHyD groups, after either *L. major* (Fig. 8A) or *L. braziliensis* SLA (Fig. 8B) infection. However, when the rLiHyD/saponin group was evaluated, animals produced significantly higher levels of anti-LiHyD IgG2a antibodies in comparison to the IgG1 levels. The ratio between IgG2a/IgG1 levels was also calculated and, in both models, the IgG2a isotype production was predominant in relation to the IgG1 levels (Fig. 8C and D, respectively). Evaluating the anti-parasite response, it was observed that the rLiHyD/saponin vaccinated animals presented higher levels of parasite-specific IgG2a antibodies, when compared to the levels found in the saline, saponin and rLiHyD groups; in both infection models (Fig. 8E and F, respectively). On the other hand, control mice showed higher levels of anti-*Leishmania* IgG1 antibodies in relation to the IgG2a levels. Also, as observed before infection, the ratio between the IgG2a and IgG1 levels showed that mice vaccinated with rLiHyD/saponin, and lately infected, showed a higher production of anti-parasite IgG2a isotype in comparison to the IgG1 levels, when both parasite extracts were used in the plates (Fig. 8G and H, respectively).

4. Discussion

The measures currently available to control the spread of tropical parasitic diseases are mainly based on therapeutic interventions such as drug-treatment programs and in the vector control, which have shown unsatisfactory results and reinforced the need for prophylactic vaccination (Sacks, 2014). Studies have shown that protection against leishmaniasis is mainly based on the development of a parasite-specific Th1 immune response, mainly linked to IFN-γ secretion (Scharton and Scott, 1993; Barbiéri, 2006). In this way, the present study analyzed whether a hypothetical protein, which was firstly identified in *L. infantum* and was shown to be protective against this *Leishmania* species (Lage et al., 2015), when administered in combination with saponin, could be cross-protective against infection caused by *L. major* or *L. braziliensis* species. The induction of partial protection in BALB/c mice by the rLiHyD/saponin vaccine was based on the IL-12-driven IFN-γ secretion, which was produced mainly by $CD4^+$ T cells. In the infected and vaccinated animals, high levels of parasite-specific IFN-γ, IL-12 and GM-CSF, associated with the presence of anti-*Leishmania* IgG2a antibodies, as well as by low levels of IL-4 and IL-10, were found.

Protein-based vaccines, although offering considerable advantages over extracts-based vaccines in terms of safety, standardization and costs of production, can present limited immunogenicity and require the addition of adjuvants, in order to induce a more protective immune response. In the present study, the absence of a Th1 adjuvant associated to rLiHyD was detrimental to the protective efficacy of this antigen, since when animals were immunized with rLiHyD and challenged, no significant protection was achieved. In this context, and as described in other studies (Coelho et al., 2003; Martins et al., 2013; Ramirez et al., 2014; Jesus-Pereira et al., 2015; Lage et al., 2015; Martins et al., 2015); rLiHyD should be inoculated in combination to a Th1 adjuvant aiming to induce a protective response against challenge infection.

The protection and/or susceptibility to *L. major* have been well established in murine models, where the development of a Th1 response, based on the NO production, as well as of IL-12, IFN-γ and TNF-α, usually result in parasitesí killing (Bogdan et al., 1996; McSorley et al., 1996; Soto et al., 2015). In contrast, the development of a Th2 response, based on the production of cytokines such as IL-4, IL-10 and IL-13, usually result in their susceptibility to infection (Roberts et al., 2005; Schwarz et al., 2013). In the *L. braziliensis* model, a Th1 response has been also showed to be necessary for protection in murine models (Rocha et al., 2007). However, it has been difficult to evaluate experimental models for studying susceptibility factors in this parasite species, since the most of the evaluated models develop a Th1 response after infection, being able to control the infection (Childs et al., 1984). In addition, in a previous study performed, Costa et al. (2011) showed that the immune modulation induced by IL-4 contributed to the development of disease in *L. braziliensis*-infected BALB/c mice, when the inoculation using more infective parasites was performed in the animals. Immunological assays performed in their spleen cells showed high levels of IL-4, which were associated with higher lesion size and parasite load in the infected animals, when compared to those that were inoculated with less infective parasite strains. In concordance, in our study, since highly infective parasites were used to the challenge infections, animals that received saline or were immunized with saponin showed higher levels of IL-4 and an increased parasite burden, which did not permit to them revert the infection.

Animals immunized with rLiHyD plus saponin displayed significant reductions in their infected footpad swellings, as well as in

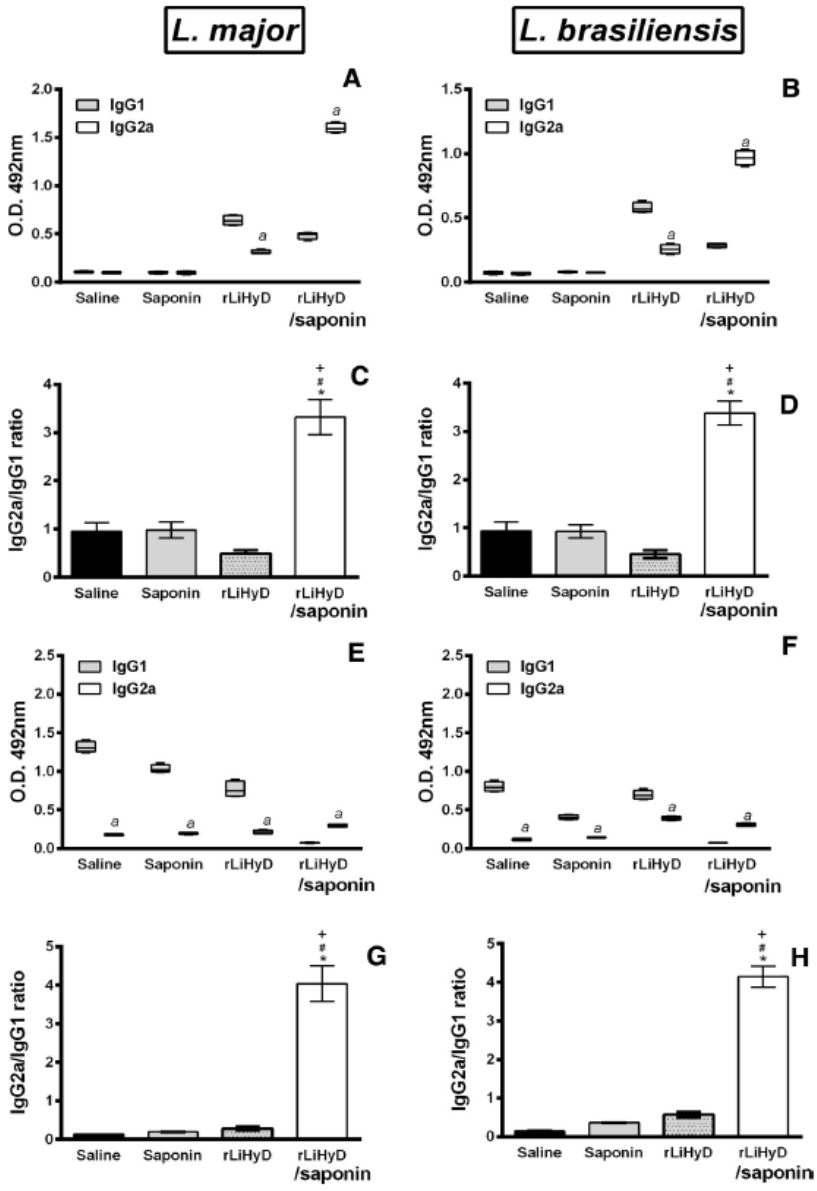

Fig. 8. Humoral response generated in vaccinated BALB/c mice after *L. major* or *L. braziliensis* challenge infection.
Sera samples were obtained from infected and vaccinated mice (n = 8 for each group), 10 weeks after infection. The results obtained after *L. braziliensis* (A, C, E, and G) or L. major (B, D, F, G and J) infections are shown. Also, the reactivity against rLiHyD (A, B, C and D) or parasite extracts (E, F, G and H) are shown. The levels of IgG1 and IgG2a isotypes antibodies are shown as whisker (min to max) plots (A, B, E and F) or ratios between the IgG2a and IgG1 values (C, D, G and H). Bars represent the mean ± standard deviation of the groups. (*) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saline group. (#) indicates the existence of statistically significant difference between rLiHyD or rLiHyD plus saponin vaccinated mice and saponin group. (+) indicates the existence of statistically significant difference between rLiHyD and rLiHyD plus saponin groups. (a) indicates the existence of statistically significant difference in relation to the IgG1 levels in the same group.

the parasite burden in the infection site (infected footpad), liver, spleen and dLNs. The decrease in the number of parasites in the infected footpads was accompanied by a reduction of the dermal inflammatory lesions in the infected and vaccinated animals, in both *Leishmania* infection models. The most of studies evaluating these species in murine models have used an infection based on the inoculation of 1×10^5 promastigotes of the parasites (Salay et al., 2007; Tonui and Titus, 2007; Carneiro et al., 2012; Santos et al., 2012). In our study, 10–100 times more parasites were used to infect the animals, aiming to guarantee an effective infection and to

test the vaccine candidate in stringent conditions reflected by high parasitism found in the infected animals (controls) in a lineage that is highly susceptible to *L. major*, although not to *L. braziliensis*. The decrease in parasite burden found in the infected and vaccinated mice reinforces the prophylactic properties of this tested vaccine, mainly due to its capacity to control parasite replication inside the infected mammalsí hosts by both *Leishmania* species.

Evaluating the involvement of both $CD4^+$ and $CD8^+$ T cells in the IFN-γ production in the rLiHyD/saponin vaccinated mice, it was observed that $CD4^+$ T cells proved to have a higher participation in the production of this cytokine. IFN-γ has showed to play with a central role in determine the resistance to *Leishmania* infection, since it has been related to induce a protective immunity in murine models against distinct *Leishmania* species, such as *L. infantum* (Bhowmick et al., 2007; Rosa et al., 2007; Iborra et al., 2008), *L. major* (Stober et al., 2007; Soto et al., 2015) and *L. amazonensis* (Chávez-Fumagalli et al., 2010). In addition, GM-CSF has been also related with macrophage activation and resistance in murine models against infection by different *Leishmania* species (Murray et al., 1995; Costa et al., 2015; Martins et al., 2015). In concordance, in the present study, vaccinated mice and lately challenged showed higher levels of this cytokine, when both rLiHyD and parasite extracts were used as stimuli.

In the infected and vaccinated animals, the partial protection was correlated with a decrease in the protein- and parasite-specific IL-4 and IL-10 levels. In BALB/c mice, high levels of IL-4 are related with the presence of anti-*Leishmania* IgG1 antibodies. The IgG isotypes expression in these animals is influenced by multiple factors, including the prevailing cytokine environment. It is also known that IFN-γ (as a Th1 cytokine) and IL-4 (as a Th2 cytokine) induce isotype switching to IgG2a and IgG1 production, respectively (Snapper and Paul, 1987). In the present study, rLiHyD/saponin vaccinated mice showed higher levels of protein- and parasite-specific IgG2a isotype antibodies before infection, which were maintained after challenge using both *Leishmania* species. This fact corroborated with the development of Th1 immune response in the vaccinated animals, which was also maintained after the challenge infections.

5. Conclusion

The present study investigated if a *Leishmania*-specific hypothetical protein, which was firstly identified and evaluated as protective against *L. infantum*, could cross-protect BALB/c mice against two parasite species causing TL. Taken together, our data indicate that LiHyD, when administered in a recombinant format associated with a Th1 adjuvant, was able to induce a partial cross-protection against challenges using *L. major* and *L. braziliensis*. In both cases, the protective immunity was correlated to the development of a Th1 immune response, which was primed by production of IFN-γ, IL-12, and GM-CSF, associated with low levels of IL-4, IL-10 and parasite-specific IgG1 isotype antibodies. Therefore, LiHyD could be considered to compose an effective pan-*Leishmania* vaccine to be applied worldwide to prevent TL.

Conflicts of interest

The authors hereby declare that they have no conflicts of interest.

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6. CONCLUSÕES

Considerando-se os resultados obtidos, pode-se concluir que:

- Em relação ao potencial antigênico, a proteína rLiHyD e seu epitopo conformacional (Peptídeo-3) mostraram-se extremamente eficazes, tendo sido reconhecidos com elevadas sensibilidade e especificidade nos testes sorológicos para o diagnóstico da LVC.

- A administração da proteína rLiHyD, associada com o adjuvante saponina, foi capaz de induzir proteção cruzada contra as infecções causadas pelas espécies *L. infantum*, *L. major* e *L. braziliensis*. Em todos os casos, a proteção foi correlacionada com o desenvolvimento de uma resposta imune Th1, caracterizada por elevados níveis de IFN-γ, IL-12 e GM-CSF e baixos níveis de IL-4 e IL-10 nos animais imunizados e/ou infectados.

- Os resultados apresentados neste trabalho colocam a proteína recombinante LiHyD como uma possível candidata para ser usada no sorodiagnóstico e/ou como uma candidata à vacina contra as leishmanioses.

7. PERSPECTIVAS

- Avaliar a proteína recombinante e seu epitopo conformacional frente a um maior número de amostras de soros de cães e de humanos, a fim de validar tais antígenos para o sorodiagnóstico das leishmanioses.

- Avaliar o potencial imunogênico e o grau de proteção induzido pela proteína recombinante LiHyD em modelo de hamsters e/ou cão para proteção contra a infecção pelo parasito *Leishmania*.

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ANEXOS

Certificado de aprovação do projeto junto ao Comitê de Ética em Experimentação Animal (CEUA) da UFMG.

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o Protocolo nº. 333 / 2015, relativo ao projeto intitulado "Proteínas selecionadas em Leishmania por imunoproteômica aplicadas como vacinas contra as leishmanioses tegumentar e visceral", que tem como responsável EDUARDO ANTONIO FERRAZ COELHO, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de 09/12/2015. Este certificado espira-se em 09/12/2020.

CERTIFICATE
We hereby certify that the Protocol nº. 333 / 2015, related to the Project entilted "Proteins selected in Leishmania by immunoproteomics applied as vaccines against tegumentary and visceral leishmaniasis", under the supervision of EDUARDO ANTONIO FERRAZ COELHO, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in 09/12/2015. This certificates expires in 09/12/2020.

Cleuza Maria de Faria Rezende
Coordenador(a) da CEUA/UFMG
Belo Horizonte, 09/12/2015.

Atenciosamente.

Sistema CEUA-UFMG
https://www.ufmg.br/bioetica/cetea/ceua/

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Patentes depositadas durante o mestrado

Patente intitulada “Proteína quimérica, composição vacinal contra leishmanioses e usos”.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 006121 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: Universidade Federal de Minas Gerais
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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54): “PROTEÍNA QUIMÉRICA, COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSES E USOS”
Resumo: A presente invenção refere-se a uma composição vacinal baseada em uma quimera polipeptídica recombinante composta por epitopos específicos de linfócitos T CD4+ e CD8+ de humano e de camundongo derivados de quatro proteínas (LiHyp1, LiHyp6, LiHyV e HRF) de Leishmania, que foi capaz de induzir proteção contra a leishmaniose visceral e tegumentar e seu uso.
Figura a publicar: 1

Patente intitulada “Composição vacinal contra as leishmanioses tegumentar e visceral, e uso”.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

22/03/2016 870160010093 09:53

00.000.2.2.16.0134320.0

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 006219 5

Dados do Depositante (71)

Depositante 1 de 1

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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): “COMPOSIÇÃO VACINAL CONTRA AS LEISHMANIOSES TEGUMENTAR E VISCERAL, E USO”

Resumo: A presente invenção trata de uma composição vacinal composta por duas proteínas imunogênicas, definidas pelas SEQ ID No 1 e 2, e seu uso no tratamento e/ou prevenção das leishmanioses tegumentar e visceral no cão e no homem.