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BIOTECNOLOGIA DE PHAGE DISPLAY APLICADA PARA O DESENVOLVIMENTO DE UMA VACINA CONTRA AS LEISHMANIOSES E NOVAS PLATAFORMAS DE DIAGNÓSTICO SOROLÓGICO

Belo Horizonte

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Tese apresentada junto 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 Doutor junto ao referido Programa.

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FOLHA DE APROVAÇÃO

"Biotecnologia de Phage Display aplicada para o desenvolvimento de uma vacina contra as Leishmanioses"

LOURENA EMANUELE COSTA

Tese submetida à Banca Examinadora designada pelo Colegiado, como requisito para obtenção do grau de Doutor em Ciências da Saúde pelo Programa de Pós-Graduação em CIÊNCIAS DA SAÚDE - INFECTOLOGIA E MEDICINA TROPICAL.

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Dedico este trabalho:

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Fonte de vida, consolo, sabedoria, inspiração e força.

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LISTA DE ABREVIATURAS E SIGLAS

Å	Angstrons
AC	Acurácia
AUC	Área sobre a curva
BSA	Albumina sérica bovina
°C	Graus Celsius
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Comissão de Ética no Uso de Animais
COLTEC	Colégio Técnico
DAT	Teste de aglutinação direta
DO	Densidade ótica
EBI	Instituto Europeu de Bioinformática
EDTA	Ácido etilenodiaminotetracético
ELISA	Ensaio de imunoadsorção por ligação enzimática
ESP	Especificidade
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
FML	Ligante fucose-manose
g	Grama
h	Horas
ICT	Teste imunocromatográfico
IDRM	Teste de intradermorreção de Montenegro
IFNγ	Interferon-gama
IgG	Imunoglobulina G
INCT	Instituto Nacional de Ciência e Tecnologia
IPTG	Isopropil β-D-tiogalactopiranosídeo
J	Índice *Youden*
kDa	Kilodálton
KMP-11	Proteína cinetoplástida de membrana de 11 kilodáltons
LACK	Homólogo em *Leishmania* do receptor da proteína quinase C ativada
LB	Luria-Bertani
LCD	Leishmaniose cutâneo-difusa
LMC	Leismaniose muco-cutânea
LRP	Proteínas ribossomais de *Leishmania*
LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
LVC	Leishmaniose visceral canina
M	Molar
mM	Milimolar

mg	Miligrama
m^2/g	Metro ao quadrado por grama
MS	Ministério da Saúde
M13	Fago filamentoso da classe M13
µm	Micrômetro
µL	Microlitro
mL	Mililitro
µg	Micrograma
MES	(Tampão) Ácido 2-morfolinoetanosulfónico monohidratado
Min	Minuto
N	Normal
nm	Nanômetro
ng	Nanograma
NAR	Normaliza Absorbance Ratio
NaCl	Cloreto de sódio
$NaHCO_3$	Bicarbonato de sódio
OMS	Organização Mundial de Saúde
Pb	Pares de bases
PBS	Tampão salina fosfato
PCLV	Programa de Controle da Leishmaniose Visceral
PCR	Reação em cadeia de polimerase
PDB	*Protein data bank*
PEG	Polietilenoglicol
PFU	Unidades formadoras de colônias
Ph.D-C7C	Biblioteca randômica contendo peptídeos de sete aminoácidos
pH	Potencial Hidrogeniônico
RIFI	Reação de imunofluorescência indireta
pmol	Picomol
PRONEX	Programa de Apoio aos Núcleos de Excelência
ROC	*Receiver operating characteristic*
SDS	Dodecil sulfato de sódio
SE	Sensibilidade
Seg	Segundo
SLA	Extrato solúvel de *Leishmania spp.*
TBS	Tampão Tris-HCl com sódio
TBS-T	TBS com Tween 20 0,05%
TMB	Tetrametilbenzidina
TRYP	Triparedoxina peroxidase
VPP	Valor Preditivo Positivo
VPN	Valor Preditivo Negativo
WHO	World Healthy Organization
X-gal	5-Bromo-4-cloro-3indolil- α-D-galactosideo

LISTA DE AMINOÁCIDOS

Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Ácido aspártico	Asp	D
Cisteína	Cis	C
Ácido glutâmico	Glu	E
Glutamina	Gln	Q
Glicina	Gly	G
Histidina	His	H
Isoleucina	Ile	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Fenilalanina	Fen	F
Prolina	Pro	P
Serina	Ser	S
Treonina	Thr	T
Triptofano	Trp	W
Tirosina	Tyr	Y
Valina	Val	V

CONSIDERAÇÕES INICIAIS

Esta tese de doutorado teve como objetivo geral selecionar peptídeos recombinantes miméticos de epítopos funcionais de antígenos parasitários que foram reconhecidos por anticorpos presentes nos soros de cães com leishmaniose visceral (LV) ativa, a partir da tecnologia de *phage display*; para serem utilizados como antígenos vacinais contra as leishmanioses, visceral e tegumentar e como plataforma de diagnóstico sorológico diferencial.

A escolha do tema ocorreu, primeiramente, por que a LV corresponde a um dos grandes problemas de Saúde Pública mundial, estando presente em 98 países e com uma incidência estimada de cerca de 500.000 novos casos. O Brasil é responsável por cerca de 90% dos casos registrados da doença nas Américas e a ocorrência da doença em cães tem aumentado significativamente. Também, a escolha do tema baseou-se na ineficácia do tratamento atual da doença e da observação da aquisição de imunidade duradoura após a cura da infecção por algumas espécies de *Leishmania spp*. contra a reinfecção pelo parasita; indicando a possibilidade do desenvolvimento de uma vacina protetora contra a doença.

Outro fator também relevante na escolha do tema baseia-se no fato do orientador deste trabalho atuar na pesquisa em leishmanioses desde o ano 2000, quando iniciou seu doutoramento. Desde então, vem desenvolvendo projetos de pesquisa nas linhas da prevenção, sob a forma de vacinas; no diagnóstico laboratorial e desenvolvimento de novos produtos terapêuticos para as leishmanioses tegumentar e visceral. Como aluna de iniciação científica por alguns anos em seu laboratório, tive a oportunidade de participar de algumas destas linhas, principalmente, àquelas voltadas para o diagnóstico laboratorial e para o desenvolvimento de vacinas contra as leishmanioses.

Durante meu mestrado no Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG; continuei a desenvolver minhas pesquisas na mesma linha, quando surgiu a possibilidade de uma parceria com o prof. Dr. Luiz Ricardo Goulart, da Universidade Federal de Uberlândia, considerado referência em *phage display* no Brasil. Tal fato possibilitou a publicação de um artigo no periódico "*Clinical and Vaccine Immunology*", intitulado: "Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic *Leishmania infantum* antigens with potential serodiagnosis applications", e no depósito de uma patente intitulada: "Método, Kit para teste de imunodiagnóstico para Leishmaniose Visceral canina e vacinas"; sob o número de registro (BR1020130130699).

No presente momento, meu trabalho visou identificar novos antígenos para futuramente permitir o desenvolvimento de uma vacina estável, de baixo custo e, principalmente, eficaz na prevenção das leishmanioses visceral e tegumentar. Nos dois anos de minha tese, obtivemos resultados promissores com o trabalho, que geraram o depósito de (3) três patentes em anexo (vide anexo 1, 2 e 3) e a publicação de artigos científicos publicados nos periódicos: *PLoS One*, intitulado - **"*Mimotope-based vaccines of Leishmania infantum antigens and their protective efficacy against visceral leishmaniasis*"**; na *Parasitology* - "***Phage-fused epitopes from Leishmania infantum used as immunogenic vaccines confer partial protection against Leishmania amazonensis infection***", na *Journal Immunol Methods* - "***New serological tools for improved diagnosis of human tegumentary leishmaniasis***" e *Diagnostic Microbiology Infection Disease* - "***Leishmania infantum mimotopes and a phage-ELISA assay as tools for a sensitive and specific serodiagnosis of human visceral leishmaniasis***" e ainda a submissão de um quinto artigo em andamento.

Esta tese está dividida em três partes. Na primeira parte, foi conduzida uma introdução e revisão da literatura onde está apresentada uma fundamentação teórica das leishmanioses e phage display. Nesta parte estão também presentes os objetivos geral da tese e os específicos de cada manuscrito. Na segunda parte da tese, foram apresentados os referentes manuscritos, onde se descreve o trabalho experimental. Na terceira e última parte esta inserida todo o fechamento da tese como a conlusão geral desta, suas perspectivas, referências bibliográficas e anexos importantes.

A apresentação deste trabalho foi realizada de acordo com a Resolução nº 02/2013, de 18 de setembro de 2013; que regulamenta o formato dos trabalhos finais e

de qualificação, estabelecendo condições para a marcação das defesas de teses e dissertações do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG.

O estudo está inserido na linha de pesquisa de "Leishmanioses".

RESUMO

As leishmanioses são doenças que apresentam uma elevada incidência no Brasil. Em modelos murinos, após a cura da doença causada por *Leishmania major*, os animais adquirem imunidade duradoura contra a reinfecção. Tal fato tem estimulado a realização de trabalhos objetivando o desenvolvimento de vacinas que possam atuar como uma medida efetiva de controle da doença. Com o avanço das ferramentas biotecnológicas, a pesquisa por novos antígenos, mais refinados, vem se mostrando como uma boa perspectiva para o desenvolvimento de novos produtos. A tecnologia de phage display baseia-se na expressão de peptídeos exógenos na superfície externa de clones de bacteriófagos (fagos) e permite a seleção daqueles que apresentam elevada especificidade para uma molécula alvo. Neste projeto, a técnica de phage display foi utilizada com a finalidade de se selecionar fagos que expressam peptídeos com elevada reatividade frente aos anticorpos IgG purificados de soros de cães com leishmaniose visceral (LV) sintomática e assintomática a fim de desenvolver uma vacina. Vinte clones de fagos foram selecionados após três ciclos de bio-pannings e foram avaliados por meio de ensaios *in vitro* de imunoestimulação de esplenócitos obtidos de camundongos BALB/c sadios ou cronicamente infectados com *Leishmania infantum*. Os clones que foram capazes de induzir a uma resposta imune Th1 polarizada, representada por níveis elevados de IFN-γ e baixos níveis de IL-4; foram selecionados. Por critérios de seletividade e especificidade, dois clones (B10 e C01) foram utilizados nos protocolos de vacinação. Camundongos BALB/c vacinados com os clones mais saponina apresentaram uma produção elevada de IFN-γ, IL-12, e GM-CSF após a estimulação específica *in vitro*. Após a infecção, esses animais apresentaram reduções significativas na carga parasitária no fígado, baço, medula óssea e linfonodos drenantes, em comparação aos grupos controles (PBS, saponina e fago selvagem mais saponina). Estes dois mimotopos de *Leishmania infantum* também foram testados contra a infecção por *L.amazonensis* a fim de verificar a proteção cruzada-heteróloga entre espécies. Os imunógenos B10 e C01 juntos e separados; com e sem adjuvantes típicos de resposta imune Th1 (saponina), foram usados no experimento. Ambos os clones induziram uma produção de IFN-γ, IL-12 e GMC-SF. Reduziram a carga parasitária das patas, fígado, baço, linfonodos drenantes e medula óssea. Em ambos os trabalhos a proteção foi associada com a produção de IFN-γ, dependente de IL-12, mediada principalmente por células T $CD8^{+}$. Estes animais apresentaram também a predominância de anticorpos IgG2a específicos dos parasitas. Dessa forma, o presente trabalho descreve a seleção e identificação de dois clones de bacteriófagos expressando peptídeos de interesse e sua aplicação como candidatos à vacina contra as leishmanioses.

PALAVRAS-CHAVE: Leishmaniose visceral, Leishmaniose tegumentar, phage display, peptídeos sintéticos, mimotopos, vacinas.

ABSTRACT

Leishmaniasis are diseases with a high incidence in Brazil. In murine models, after curing of the disease caused by *Leishmania major* species, animals acquire long-lasting immunity against re-infection. This fact has stimulated the development of studies aiming to develop vaccine candidates to be employed like an effective measure to control of the disease. With the advancement of biotechnology tools, the search for new antigens more refined and effective, is proving to be a good prospect for the development of new products. The phage display technology, based on expression of exogenous peptides on the outer surface of phage clones, allows the selection of those that present a high specificity for an individual target molecule. In this work, the phage display technology was employed with the purpose of selecting phage clones expressing peptides that present a high reactivity towards IgG antibodies purified from sera samples of dogs with symptomatic and asymptomatic visceral leishmaniasis (VL). Twenty phage clones were selected after three bio-pannings cycles, and they were evaluated in the *in vitro* immune stimulation of splenocytes obtained from naive or chronically infected with *Leishmania infantum* BALB/c mice. Clones able to induce a more polarized Th1 immune response; based on high levels of IFN-γ and low levels of IL-4, were selected. Adopting criteria of selectivity and specificity, two clones (B10 and C01) were selected and used in vaccination protocols. In the results, BALB/c mice vaccinated with the individual clones plus saponin showed high IFN-γ, IL-12, and GM-CSF levels after the *in vitro* specific stimulation. After challenge, these animals showed significant reductions in parasite load in the liver, spleen, bone marrow, and draining lymph nodes, when compared to control groups (PBS, saponin, wild-type phage plus saponin groups). Protection was associated with an IFN-γ production, which was IL-12 dependent, and mainly induced by $CD8^+$ T cells. These animals also showed increased levels of parasite-specific IgG2a antibodies. These two Leishmania infantum mimotopes were also tested against infection by L.amazonensis to check cross-protection between heterologous species. The immunogens B10 and C01 together and apart; with and without adjuvants typical Th1 immune response (saponin) were used in the experiment. Both clones induced production of IFN-γ, IL-12 and CM-SF. Reduced parasitic load of the footpads, liver, spleen, draining lymph nodes and bone marrow. These studies describes the selection and identification of two phage clones expressing target peptides, and its application as vaccine candidates against the leishmaniasis.

KEYWORDS: Visceral leishmaniasis, tegumentary leishmaniasis, phage display, synthetic peptides, mimotopes, vaccines.

INTRODUÇÃO

1. INTRODUÇÃO

As leishmanioses são um grupo de doenças infecto-parasitárias causadas por parasitas protozoários do gênero *Leishmania* e transmitidas durante o repasto sanguíneo por insetos vetores dos gêneros *Phlebotomus*, em países do Velho Mundo e *Lutzomyia*, nas Américas. Embora a manutenção do ciclo biológico dos vetores transmissores ocorra, principalmente, em ambientes silvestres; atualmente, pode-se observar uma expansão das leishmanioses em áreas periurbanas e urbanas.

As leishmanioses são endêmicas em 98 países no mundo, sendo que mais de 350 milhões de pessoas estão expostas ao risco de infecção. Estima-se que cerca de 2 milhões de novos casos ocorram por ano, sendo que 1,0 a 1,5 milhões correspondam a casos de leishmaniose tegumentar (LT), e 500.000 casos sejam de leishmaniose visceral (LV), a qual ocasiona cerca de 60.000 óbitos por ano (WHO, 2014). O Brasil é responsável por aproximadamente 90% dos casos registrados de LV no Continente Americano, fato que torna as leishmanioses um importante problema de Saúde Pública e que requer, dessa forma, atenção especial por nossas autoridades competentes (Alvar *et al.*, 2012).

As medidas profiláticas e de controle contra as leishmanioses visam principalmente à interrupção do ciclo biológico do parasito. Entretanto, o número de espécies de *Leishmania spp.*, o caráter zoonótico da doença e a manutenção do ciclo silvestre, dificultam a adoção de medidas efetivas de controle. A aplicação de inseticidas para a eliminação dos vetores pode ser realizada em ambientes domésticos e peri-domésticos, entretanto, em regiões florestais, apresenta eficácia reduzida (Tesh, 1995). A eutanásia de cães soropositivos constitui-se em uma medida profilática adotada por órgãos competentes, porém, devido ao fato de serem animais de estimação, e por tal procedimento não impactar significativamente na redução do número de novos casos; tal medida é considerada ineficaz e eticamente repreensível (Tesh, 1995; Gontijo & Melo, 2004). A eliminação de animais reservatórios silvestres também não se encaixa como uma medida executável e ecologicamente correta, além da possibilidade de adaptação do parasito a outros reservatórios existentes naquele dado ambiente (Grimaldi & Tesh, 1993; Gramiccia & Gradoni, 2005).

O tratamento de primeira escolha para as leishmanioses se baseia na utilização de antimoniais pentavalentes administrados por via parenteral, entretanto, tais produtos causam elevada toxicidade nos pacientes, normalmente não levam à cura parasitológica e um aumento do número de casos de recidivas pós-tratamento vem sendo observado

(Croft & Coombs, 2003; Minodier & Parola, 2007). A anfotericina B lipossomal tem sido considerada como um tratamento efetivo da doença, mas o seu custo elevado restringe sua utilização a uma minoria dos pacientes (Mondal *et al.,* 2010). Triagens clínicas utilizando miltefosina têm mostrado eficácia, porém, tal fármaco pode induzir teratogenicidade e, por conseguinte, não é recomendado em mulheres grávidas e em período fértil (Oliveira *et al*., 2011). Desta forma, o desenvolvimento ou melhoria de estratégias que possam atuar na prevenção das leishmanioses, como a busca por vacinas eficazes capazes de induzir imunidade protetora; tem se tornado prioridade e tratada como uma medida para o controle da doença. Entretanto, devido à diversidade epidemiológica da doença e de espécies que podem causar a infecção, o desenvolvimento de vacinas contra as leishmanioses torna-se uma tarefa complexa, embora seja considerada uma solução real para seu controle (Grimaldi & Tesh, 1993; Gonzalo *et al*., 2002; Sukumaran *et al*., 2003; Ramiro *et al*., 2003; Desjeux, 2004). Cabe ressaltar que uma vacina deve conter antígenos que sejam compartilhados por diferentes espécies dos parasitas, ser preferencialmente imunogênica contra a maioria destas espécies, além de não interferir na imunogenicidade induzida por outros antígenos (Coelho *et al.*, 2003).

A utilização de ferramentas biotecnológicas inovadoras tem levado à identificação de novos alvos com potencial biológico. Neste trabalho, utilizando uma dessas técnicas, a de phage display; identificamos mimotopos expressos na superfície externa de clones de bacteriófagos, antígenos esses que foram então avaliados como candidatos vacinais contra a infecção experimental de camundongos BALB/c com a espécie *Leishmania infantum e com a espécie Leishmania amazonensis*. Os clones foram selecionados por meio de experimentos *in vitro* e aqueles que induziram uma resposta Th1 mais polarizada (maiores níveis de IFN-γ e menores níveis de IL-4) foram avaliados nos experimentos *in vivo* de vacinação experimental.

2. REVISÃO DE LITERATURA

2.1. Epidemiologia das leishmanioses

As leishmanioses são doenças endêmicas em 98 países, os quais, em sua grande maioria, apresentam as menores taxas de desenvolvimento sócio-econômico mundial. Estima-se que 350 milhões de pessoas estejam expostas aos riscos de infecção e que 12 milhões sejam clinicamente afetadas pela doença. Segundo levantamentos da Organização Mundial da Saúde (OMS), de 1,5 a 2,0 milhões de novos casos de leishmanioses são registrados a cada ano, dos quais cerca de 1,0 a 1,5 milhões correspondem a casos de leishmaniose tegumentar (LT) e cerca de 500.000 casos de LV (WHO, 2003; Desjeux, 2004; WHO, 2010).

Mais de dois terços dos casos de LV com transmissão antroponótica no mundo concentram-se no nordeste da Índia, sudeste do Nepal e região central de Bangladesh; sendo que o segundo maior foco da doença ocorre em países do leste da África, como Etiópia e Sudão. Outro importante foco da doença, entretanto, com transmissão zoonótica, ocorre na bacia do Mediterrâneo, Oriente Médio e Ásia Ocidental (WHO, 2010). No Continente Americano, o Brasil é responsável por 90% dos casos de LV, dados que tornam as leishmanioses um importante problema de Saúde Pública e que requer, dessa forma, atenção especial pelas autoridades competentes (Alvar *et al*., 2012).

Em relação à Leishmaniose Tegumentar (LT), dos novos casos registrados a cada ano, um terço dos casos ocorre nas Américas, na bacia do Mediterrâneo e Ásia Ocidental, do Oriente Médio para a Ásia Central (Figura 1). Os dez países com maior número de casos são: Afeganistão, Argélia, Brasil, Colômbia, Costa Rica, Etiópia, Irã, Peru, Sudão e Síria, e que, juntos, são responsáveis por 70 a 75% da incidência global estimada de LC (Alvar *et al*.,2012, Pigott *et al*., 2014; WHO, 2014).

Em relação à LV, estima-se sua incidência em cerca de 500 mil novos casos e 60 mil mortes a cada ano, em todo o mundo (Figura. 2), sendo que mais de 90% dos casos de LV ocorrem em seis países: Bangladesh, Brasil, Etiópia, Índia, Sudão do Sul e Sudão (Ioset, 2008; Ameen, 2010; Mishra *et al*., 2011; WHO, 2014).

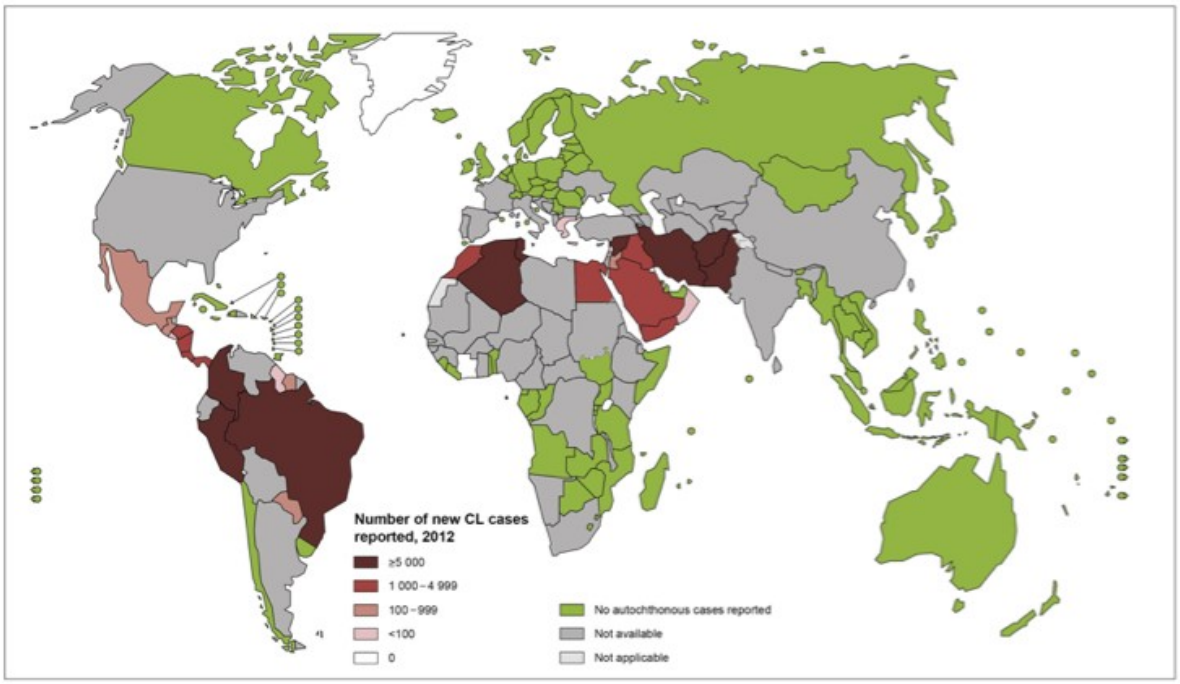

Figura 1. Incidência mundial da leishmaniose tegumentar (WHO, 2012).

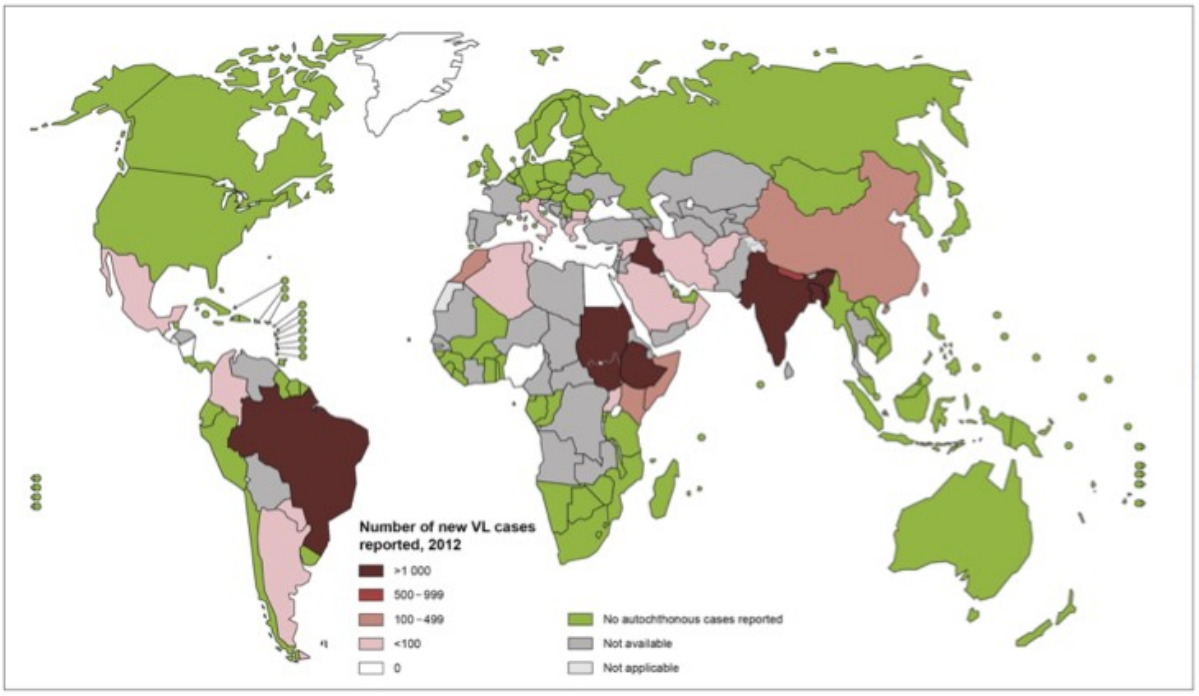

Figura 2 - Incidência mundial da leishmaniose visceral (WHO, 2012).

No Brasil, no período de 1988 a 2009, a LT apresentou média anual de 27.093 casos registrados e coeficiente médio de detecção de 16 casos por 100 mil habitantes. São observados picos de transmissão a cada cinco anos, apresentando tendência de aumento do número de casos registrados a partir de 1985, quando há a implantação das

ações de vigilância e controle epidemiológico da doença no país. No entanto, o número de casos continua a crescer em nosso país, segundo dados da Organização Mundial de Saúde (Figura 3 e 4), (Alvar *et al*., 2012; WHO, 2014; MS, 2011).

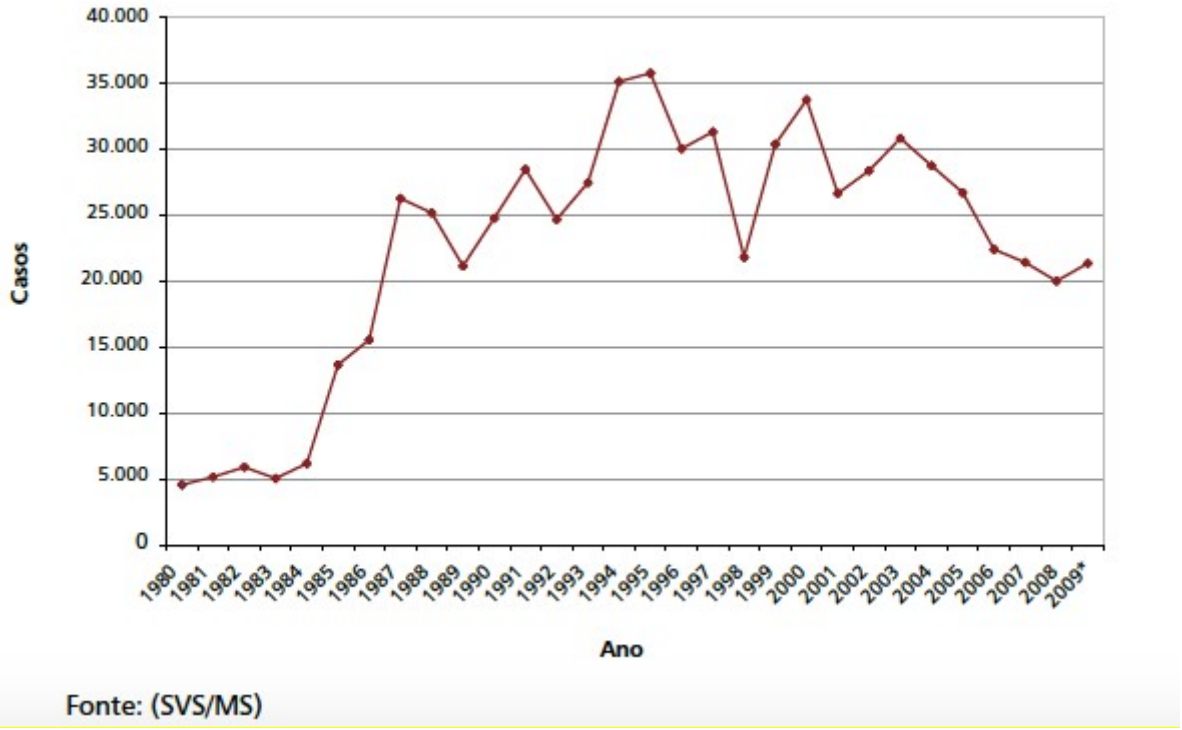

Figura 3 - Número de casos da leishmaniose tegumentar no Brasil (WHO, 2014).

Número de casos de leishmaniose cutânea reportados								
País	2012	2011	2010	2009	2008	2007	2006	2005
Brasil	23793	21356	22397	21989	20123	21530	22397	26685

Figura 4 - Casos de leishmaniose tegumentar (Secretária de Vigilância em Saúde, Ministério da Saúde do Brasil, 2011).

Observa-se ainda uma expansão geográfica da doença, visto que, no início da década de 1980, foram registrados casos em 19 unidades federadas e, em 2003, todos os estados apresentaram incidência da doença. Em 1994, houve registro de casos autóctones em 1.861 municípios, o que representava 36,9% dos municípios do País. Em 2005, houve expansão da doença para 2.068 municípios (37,2%) (Ministério as Saude do Brasil, 2011).

A LV apresenta comportamento epidemiológico cíclico, com elevação de casos em períodos médios de cinco anos. No período de 1998 a 2009, a média anual de casos foi de 3.349, e a incidência de 1,97 casos por 100 mil habitantes (Figura 5).

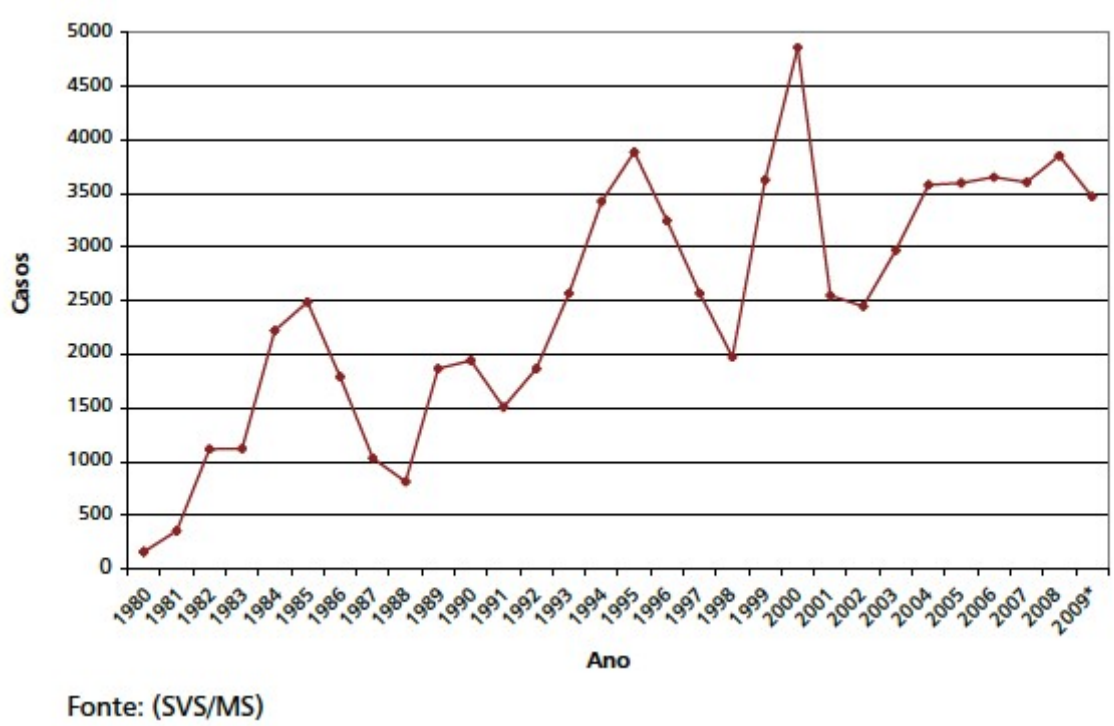

Figura 5 - Casos de leishmaniose visceral (Secretária de Vigilância em Saúde, Ministério da Saúde do Brasil, 2011).

Observa-se que a letalidade vem aumentando gradativamente, passando de 3,2% em 2000 para 5,7% em 2009 (Alvar *et al*., 2012). No entanto, segundo a Organização Mundial de Saúde, de 2007 a 2012, houve uma oscilação no número de casos de LV no Brasil (Figura 6).

Número de casos de leishmaniose visceral reportados								
País	2012	2011	2010	2009	2008	2007	2006	2005
Brasil	3118	3840	3716	3693	3852	3604	3651	3597

Figura 6 - Número de casos de leishmaniose visceral no Brasil (WHO, 2014).

Os dados epidemiológicos dos últimos anos revelam também a periurbanização e a urbanização da LV, destacando-se os surtos ocorridos no Rio de Janeiro (RJ), Belo Horizonte (MG), Araçatuba (SP), Santarém (PA), Corumbá (MS), Teresina (PI), Natal

(RN), São Luís (MA), Fortaleza (CE), Camaçari (BA) e, mais recentemente, as epidemias ocorridas nos municípios de Três Lagoas (MS), Campo Grande (MS) e Palmas (TO) (Passos *et al*., 1993).

As leishmanioses representam um grave problema de Saúde Pública devido à elevada morbidade e mortalidade observada nos casos agudos e não tratados, especialmente em relação à LV. Nos últimos anos, o número de mortes e a taxa de letalidade relacionadas à LV vêm aumentando gradativamente, o que torna a doença preocupante e demonstra a necessidade da busca por medidas de controle mais efetivas (Alvar *et al*., 2012).

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

As leishmanioses são doenças causadas por parasitas protozoários do gênero *Leishmania*, pertencente ao 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 a fêmea possui hábitos hematófagos e é capaz de transmitir o parasita ao hospedeiro mamífero (Grimaldi & Tesh, 1993).

O ciclo biológico do parasita compreende duas formas morfológicas principais: amastigota e promastigota. As formas amastigotas são arredondadas, com flagelo rudimentar, altamente infectivos e responsáveis pelo desenvolvimento da doença no hospedeiro mamífero. São parasitas intracelulares obrigatórios, sendo encontradas no interior de células fagocíticas, tais como macrófagos, neutrófilos, células dendríticas e células de Langehans, no hospedeiro vertebrado. As formas promastigotas são formas alongadas, com flagelo longo e encontradas no vetor transmissor da doença (Grimaldi & Tesh, 1993; Ashford, 2000). A passagem do parasita do hospedeiro mamífero para o vetor flebotomínio ocorre no momento em que o inseto realiza o repasto sanguíneo em um hospedeiro infectado e pode ingerir, juntamente com o sangue, formas amastigotas livres ou presentes dentro de células fagocíticas infectadas (Figura 7). Essas células, ao alcançarem o intestino do vetor, se rompem e liberam os parasitas, que se transformam em formas promastigotas procíclicas e migram para o trato digestório médio e anterior do vetor. Os parasitas passam por um processo de multiplicação e diferenciação celular, dando origem às formas promastigotas metacíclicas, que migram para a parte anterior do aparelho bucal do inseto. Quando o vetor, já infectado, realiza novo repasto

sanguíneo, o mesmo pode regurgitar formas promastigotas metacíclicas na derme do hospedeiro mamífero. Prontamente, células do sistema fagocítico-mononuclear do hospedeiro, migram para o local e podem fagocitar os parasitas. No interior dos fagolisossomos formados, ocorre a diferenciação das formas promastigotas em amastigotas, que passam a se replicar. A replicação dos parasitas por divisão binária pode resultar no rompimento das células e, consequentemente, na liberação dos parasitas, que podem ser fagocitados por novas células, levando ao desenvolvimento da doença no hospedeiro mamífero. Assim, um novo vetor não-infectado, poderá ingerir células fagocíticas parasitadas deste hospedeiro mamífero, completando o ciclo biológico do parasita (Pessoa & Martins, 1988; Medeiros *et al.*, 2005).

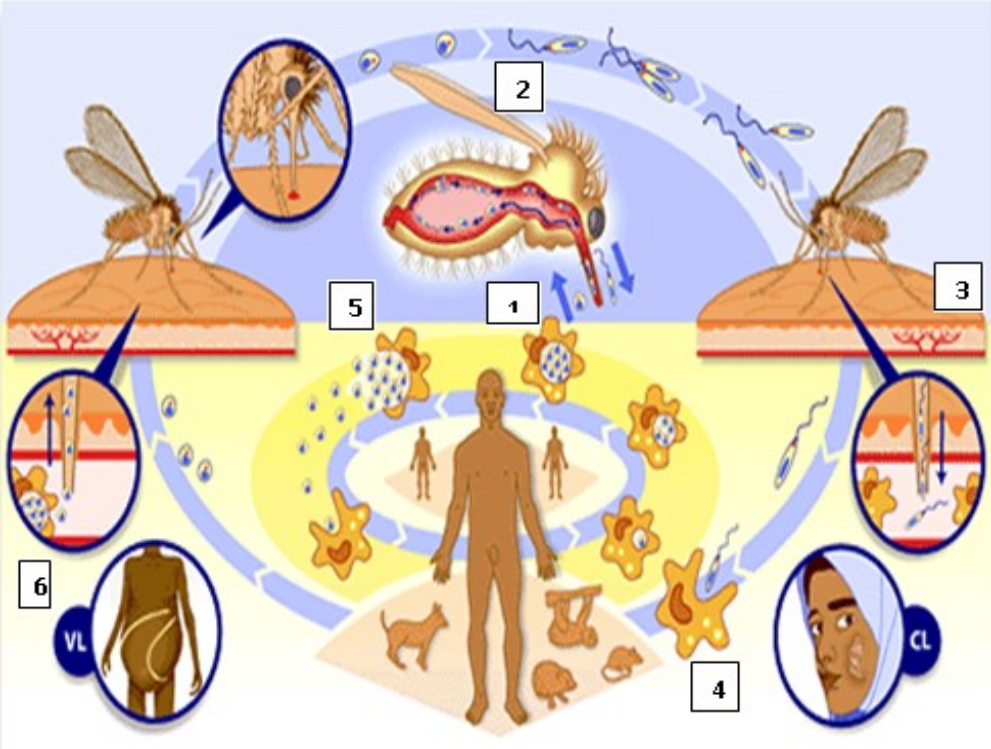

Figura 7. Ciclo biológico do parasita *Leishmania spp*. 1- Macrófagos infectados sendo ingeridos pelo vetor durante seu repasto sanguíneo. 2- No intestino do vetor, as amastigotas se diferenciam em promastigotas procíclicas e, posteriormente, em promastigotas metacíclicas, que migram para a porção anterior do intestino do mesmo. 3- Introdução de formas promastigotas metacíclicas na derme do hospedeiro mamífero. 4-Fagocitose das formas promastigotas metacíclicas por macrófagos do hospedeiro mamífero. 5- Rompimento do macrófago infectado liberando formas amastigotas, que poderão ser fagocitadas por novas células. 6- Exemplo de manifestação clínica da leishmaniose visceral no homem. (Adaptado de WHO, 2003).

2.3. Manifestações clínicas das leishmanioses

As leishmanioses apresentam dois grandes espectros clínicos de doença: LT e LV (Ashford, 2000; Desjeux, 2004). A LT, por sua vez, pode apresentar três diferentes tipos clínicos: a leishmaniose cutânea (LC), leishmaniose cutâneo-difusa (LCD) e a leishmaniose muco-cutânea (LMC). Tais patologias podem causar desde uma lesão cutânea única, no local da picada do vetor e que pode apresentar cura espontânea, até lesões debilitantes, disseminadas por várias partes e órgãos do corpo do paciente e que não apresentam cura espontânea, necessitando de tratamento quimioterápico (Grimaldi & Tesh, 1993). Na LT temos as seguintes formas clínicas:

• Forma cutânea localizada (LC) – ocorre pela lesão única situada, geralmente, no local da picada do vetor. Muitas vezes é ulcerosa, na qual há tendência à cura espontânea. Apresenta boa resposta ao tratamento, podendo acompanhar-se de linfoadenopatia local, linfangite ascendente e ulceração de alguns nódulos, reproduzindo as lesões iniciais.

• Forma cutâneo-difusa (LCD)– constitui manifestação rara e grave da LT, ocorrendo em pacientes considerados anérgicos, ou seja, com deficiência na resposta imune celular aos antígenos do parasito. De início insidioso, com lesão única sem resposta significativa ao tratamento, evolui lentamente, com formação de placas infiltradas e nodulações não ulceradas múltiplas, que recobrem grandes extensões cutâneas. O tratamento é difícil ou ineficaz (Castes *et al*., 1983; Okelo *et al.,* 1991; Barral-Neto *et al*., 1995).

As formas LC, normalmente, são causada pelas espécies *L. braziliensis*, *L. amazonensis*, *L. mexicana*, *L. lainsoni* e *L. guyanensis* nas Américas e por *L. major*, *L. tropica* e *L. aethiopica* em países do Velho Mundo (Ashfoord, 2000). Já as LCD são, normalmente, causadas pelas espécies *L. mexicana*, *L. venezuelensis* e *L. amazonensis* (Grimaldi & Tesh, 1993).

A LMC é, geralmente, causada pela disseminação hematogênica dos parasitos para as mucosas nasais, orofaringe, palato, lábios, língua, laringe e, excepcionalmente, traquéia e árvore respiratória superior. Espécies como *L. braziliensis*, *L. panamensis*, *L. guyanensis* e *L. mexicana* podem causar essa doença (Weigle & Saravia, 1996).

De acordo com a classificação clínica proposta por Marzochi & Marzochi (1997), e considerando como parâmetros o tempo decorrido entre as lesões cutâneas e mucosas, o local de inoculação e a localização das lesões, a forma mucosa pode receber as seguintes classificações:

• Forma mucosa tardia – caracteriza-se pelo aparecimento da lesão mucosa após alguns anos o surgimento da lesão cutânea, fato que costuma ocorrer dentro dos dois primeiros anos, mas há relatos de sua ocorrência décadas após a erupção da lesão na pele.

• Forma mucosa indeterminada – acometimento mucoso, sem identificação da porta de entrada, supondo-se que as lesões mucosas sejam originadas de infecção, sem manifestação cutânea clínica prévia.

• Forma mucosa primária – situação em que a lesão de mucosa é causada pela picada do vetor diretamente na mucosa, restrita às mucosas labial e genital.

Indivíduos com LV, comumente permanecem assintomáticos e apenas uma pequena parcela das infecções podem conduzir, de fato, às manifestações clínicas severas da doença. Porém, nesses casos, a LV é frequentemente letal, se não tratada a tempo (Kedzierski e Krystal, 2014). Pode ser causada pelas espécies *L. donovani, L. infantum* e *L. chagasi*; entretanto, as duas últimas espécies foram consideradas genotipicamente idênticas (Roberts *et al*., 2000). Após um período de incubação que geralmente varia entre dois e seis meses, indivíduos sintomáticos apresentam sinais de uma infecção sistêmica e persistente, incluindo febre, fadiga, fraqueza, perda de peso e evidências da presença e disseminação dos parasitas, como hepatoesplenomegalia e inchaço dos gânglios linfáticos (Chappuis *et al.*, 2007).

A LV caracteriza-se por um amplo espectro clínico, que pode variar desde as manifestações clínicas assintomáticas, passando pelas oligossintomáticas, até às formas sintomáticas graves, que, quando agudas e não tratadas, podem levar o paciente ao óbito (Chappuis *et al.*, 2007):

• Forma Assintomática – é aquela na qual não há evidência de manifestações clínicas, porém, os exames laboratoriais são positivos.

• Forma Oligossintomática – caracteriza o início da sintomatologia, que pode variar de paciente para paciente, mas na maioria dos casos inclui febre com duração inferior a quatro semanas, palidez cutâneo-mucosa e hepatoesplenomegalia. Frequentemente evolui para cura espontânea. A combinação de manifestações clínicas e das alterações laboratoriais, que melhor parece caracterizar a forma oligossintomática; são: febre, hepatomegalia, hiperglobulinemia e velocidade de hemossedimentação elevada.

• Forma Moderada – caracteriza-se por febre irregular, geralmente associada ao emagrecimento progressivo, palidez cutâneo-mucosa e hepatoesplenomegalia.

Apresenta um quadro clínico lento, geralmente com mais de dois meses de evolução, na maioria das vezes associado ao comprometimento geral do paciente.

• Forma Grave – caso não seja feito o diagnóstico e tratamento, a doença evolui de forma rápida, com febre contínua e comprometimento intenso do estado geral do paciente. Instala-se a desnutrição e edema dos membros inferiores, que pode evoluir para anasarca. Outras manifestações importantes incluem hemorragias (epistaxe, gengivorragia e petéquias), icterícia e ascite. Nesses pacientes, o óbito é geralmente determinado por infecções bacterianas e/ou hemorragia.

Abreu-Silva *et al.* (2004) demonstraram que camundongos BALB/c infectados experimentalmente com *L.amazonensis* desenvolveram manifestações patológicas similares às encontradas na LV. Caso similar foi também descrito por Barral *et al*. (1991) em pacientes humanos com LV, no estado da Bahia, onde estudo de genotipagem revelaram que 41 indivíduos acometidos pela doença estavam infectados por *L. amazonensis*, demontrando assim a gravidade da doença que pode ser gerada por tal espécie de *Leishmania*.

A LV canina (LVC) apresenta manifestações clínicas diversas, que dependem de vários fatores, tais como: a imunidade inata do cão, a espécie infectiva do parasita e a resposta imune gerada pelo animal. 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 infectivos durante tal época (Lanotte *et al*., 1979; Keenan *et al*., 1984).

As manifestações clínicas mais comuns da LVC são: linfoadenomegalia, enfraquecimento crônico, alopécia, úlceras, dermatite esfoliativa, onicogrifose, anemia, hepatoesplenomegalia, disfunção renal severa, hipergamaglobulinemia e colites (Abranches *et al*., 1991; Ferrer *et al*., 1991; Ciaramella *et al*., 1997; Font & Closa, 1997; Tafuri *et al*., 2001).

2.4. Diagnóstico, tratamento e medidas profiláticas das leishmanioses

O diagnóstico da LT é realizado com base na avaliação de parâmetros clínicos e na realização de exames laboratoriais. Para um diagnóstico definitivo, há a necessidade de exames parasitológicos para a detecção e identificação da espécie infectante. Análises microscópicas de aspirados de lesões ou de cortes histológicos em lâminas, a inoculação de aspirados de tecidos infectados em hamsters e a cultura de fragmentos de tecidos estão entre os métodos utilizados (Tavares *et al*., 2003).

O diagnóstico clínico da LV é dificultado pela semelhança dos sintomas clínicos com outras doenças, como a malária e tuberculose (Kemp, 1997). Testes parasitológicos, através da cultura de aspirados do baço e da medula óssea, são utilizados para o diagnóstico da LV, embora se apresentem como métodos invasivos em relação à coleta das amostras. O diagnóstico imunológico é baseado na detecção de anticorpos específicos aos antígenos do parasita ou de antígenos do próprio parasita em amostras de soros dos pacientes (Tavares *et al*., 2003). Nos casos de sorodiagnóstico da doença, a Análise de Imunoadsorção por Ligação Enzimática (ELISA) e o teste de aglutinação direta (DAT) são utilizados; todavia, os antígenos empregados não têm a capacidade de diferenciar os pacientes com a doença assintomática, na forma ativa, ou já curada. Outros testes, como a imunofluorescência, o Western-Blot e a Reação em Cadeia da Polimerase (PCR) são empregados no diagnóstico, embore possam apresentar problemas de sensibilidade, especificidade, alto custo e/ou logística de execução, necessitando de técnicos qualificados e equipamentos de custo elevado (Manson-Bahr, 1987; Reed, 1996).

O tratamento clínico das leishmanioses deve ser realizado para se evitar a mortalidade causada pela LV e reduzir a morbidade provocada pelas lesões desfigurantes nas formas mais graves de LT. A aplicação sistêmica de compostos antimoniais pentavalentes, como o N-metil antimoniato de meglumina (Glucantime®), é o método mais utilizado (Franke *et al*., 1990). 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. Altas doses diárias, necessárias no curso do tratamento, podem causar fadiga, artralgias, mialgias além de toxicidade renal, hepática e cardíaca. Há, também, a dificuldade de transporte dos pacientes, normalmente residentes em áreas rurais, até os centros mais especializados de saúde, para aplicação endovenosa ou intramuscular dos produtos; além do custo elevado dos fármacos, ainda que no Brasil estejam sendo custeados pelo governo (Grogl *et al*., 1991; Carvalho *et al*., 2002).

Pelas dificuldades inerentes ao tratamento das leishmanioses e pelo aumento do número de casos de recidiva da doença, o controle da transmissão do parasita constitui-se relevante. As medidas profiláticas visam geralmente à interrupção do ciclo biológico dos parasitas, entretanto, seu caráter zoonótico, o número variado de espécies de *Leishmania spp.* e a manutenção do ciclo silvestre; dificultam a adoção de medidas de controle eficazes (Tesh, 1995). O controle do inseto vetor pode ser feito através da

administração de inseticidas nos ambientes doméstico e peri-doméstico, porém, sua utilização apresenta eficácia reduzida em áreas abertas (Tesh, 1995). No que se refere à LVC, o cão é considerado um importante hospedeiro mamífero reservatório do parasita e representa uma fonte considerável de infecção para o vetor transmissor, agindo como elo na manutenção da transmissão da infecção entre o vetor e o homem. A eliminação de cães infectados constitui-se em uma medida profilática adotada pelos órgãos de Saúde competentes, embora não seja efetiva para a redução do número de casos da doença (Tesh, 1995; Gontijo e Melo, 2004). O tratamento de casos humanos e a adoção de medidas de proteção individual, tais como uso de redes e repelentes, também constituem medidas de prevenção e controle, porém, não representam impacto significativo na redução do número de casos de leishmanioses (Grimaldi e Tesh, 1993).

2.5. Imunologia das leishmanioses

A resistência à infecção contra o parasita *Leishmania spp.* é usualmente relacionada à indução de imunidade celular representada, principalmente, pela produção de IFN-γ nos hospedeiros infectados (Scott, 2003). A importância do desenvolvimento de linfócitos T $CD4^+$ do tipo Th1 e linfócitos T $CD8^+$ no balanço entre as linhagens celulares Th1/Th2 foi demonstrada em modelos murinos infectados com *L. major* (Sacks e Noben-Trauth, 2002). Os estudos mostraram a associação entre a resistência à infecção por tal espécie com o desenvolvimento de uma resposta Th1, pela produção elevada de IFN-γ e IL-12. Já a susceptibilidade foi associada com o desenvolvimento de uma resposta Th2, mediada pela produção de citocinas como IL-4, IL-10 e TGF-β. Em camundongos resistentes à espécie *L. major*, células dendríticas produtoras de IL-12 migram rapidamente para o local da infecção. Tais células têm a capacidade de interagir com os parasitas nos linfonodos drenantes e com linfócitos Th1 que expressam o receptor funcional de IL-12 (Scott, 2003).

Algumas linhagens de camundongos, como C57BL/6, C57BL/10, C3H e CBA são resistentes à infecção com *L. major*, mesmo quando um grande número de parasitas (10^7) é inoculado pela via subcutânea nos animais; enquanto outras linhagens, como BALB/c e SWR/J, apresentam um perfil de susceptibilidade. Camundongos BALB/c são utilizados como modelo de infecção para o estudo de candidatos vacinais contra várias espécies de *Leishmania spp.*, tais como *L. infantum* e *L. amazonensis* (Sacks & Noben-Trauth, 2002; Wilson *et al*., 2005).

2.5.1. Resposta imune na leishmaniose tegumentar

Diferenças no desenvolvimento clínico da doença causada por *L. major* ou *L. amazonensis* sugerem que diferentes aspectos da resposta imune podem estar envolvidos nas infecções por tais espécies. Nesse sentido, os mecanismos responsáveis pela susceptibilidade e patogênese da infecção por *L. amazonensis* ainda não estão completamente elucidados (Soong *et al.,* 1997; Campbell *et al*., 2004).

A fim de identificar o grau de envolvimento da resposta celular à *L. amazonensis*, Soong *et al*. (1997) demonstraram que a ativação de linfócitos T CD4+, além de facilitar o recrutamento de células para o sítio de infecção, foi essencial para a patogênese verificada em modelos murinos. Tais células foram capazes de produzir citocinas que regulam o recrutamento local de linfócitos e, uma vez que a multiplicação de *Leishmania* spp. ocorre preferencialmente em macrófagos imaturos, tal estratégia poderia beneficiar o parasita, em detrimento do hospedeiro (Soong *et al*., 1997).

A susceptibilidade à infecção com *L. amazonensis* tem sido associada à produção de baixos níveis de IFN-γ (Afonso & Scott, 1993). Entretanto, a inoperância dos mecanismos efetores, com prejuízo para a ativação de macrófagos, conduz à progressão da doença nos hospedeiros infectados com tal espécie do parasita. Aparentemente, as populações de linfócitos T CD4+ e CD8+ não são ativadas, sugerindo que possa estar ocorrendo uma evasão da resposta imune do hospedeiro (Ji *et al*., 2002). Assim, estudos envolvendo a infecção com *L. amazonensis* sugerem que a doença progressiva, verificada no modelo murino, seja devido à falha na indução inicial da produção de citocinas e quimiocinas com perfil de proteção, ou seja, importantes para o desenvolvimento da resposta Th1; o que acaba por conduzir o sistema imune do animal a um estado de inoperância frente ao parasita (Ji *et al*., 2002). Camundongos C57BL/10, embora resistentes à infecção com *L. major* são susceptíveis à infecção com *L. amazonensis*. O perfil de susceptibilidade é similar ao perfil desenvolvido em camundongos BALB/c, nos quais o desenvolvimento da resposta Th2 não parece ser essencial ao fenótipo de susceptibilidade ao parasita. A ausência da expressão de IL-10, verificada em camundongos *knock-out* para esta citocina, aparentemente não influencia na resolução das lesões crônicas, uma vez que não é suficiente para aumentar a produção de IFN-γ. Esses resultados sugerem que, durante a infecção com *L. amazonensis,* mecanismos alternativos à produção de IL-10 sejam desencadeados e capazes de limitar a produção de IFN-γ (Jones *et al.*, 2002).

Dessa forma, tais estudos indicam peculiaridades inerentes à resposta imune contra as diferentes espécies de *Leishmania* e a necessidade de outros estudos que objetivam elucidar os mecanismos envolvidos na proteção contra tal relevante parasita.

2.5.2. Resposta imune na leishmaniose visceral

O modelo murino tem sido utilizado para caracterizar a patogênese e a resposta imune associada à LV. A dicotomia Th1 *versus* Th2, evidente no modelo de infecção com *L. major,* não tem sido, entretanto, verificada em camundongos infectados com outras espécies do parasita (Ahmed *et al*., 2003). A capacidade de camundongos BALB/c resolverem a infecção por espécies viscerotrópicas, mesmo com carga parasitária presente e após período prolongado de tempo, faz com que estes camundongos sejam o melhor modelo para estudo da infecção visceral subclínica nas leishmanioses. Por outro lado, pelo desenvolvimento de infecção de caráter progressivo em hamsters, tal espécie animal torna-se um bom modelo parasitológico para estudo dos casos de LV (Wilson *et al*., 2005).

A infecção experimental com *L. chagasi* em camundongos BALB/c evolui usualmente no fígado, baço e medula óssea (Marques-da-Silva *et al*., 2005). Características como a hepatoesplenomegalia são observadas em camundongos infectados pela via endovenosa, sendo um pico de crescimento parasitário observado no fígado entre 4 e 6 semanas após a infecção, embora a evolução da infecção no baço ocorra mais lentamente, atingindo o pico por volta de 8 semanas após a infecção (Melby *et al*., 1998). A via utilizada para a infecção (intradérmica ou endovenosa) não altera o perfil da mesma, o que indica que o tropismo pelos órgãos internos corresponde às características inerentes do parasita (Ahmed *et al*., 2003; Wilson *et al*., 2005).

A imunidade protetora ou resistência à LV relaciona-se com o desenvolvimento de uma resposta Th1, e ocorre pela presença de citocinas ativadoras de macrófagos, como IFN-γ e IL-12, com a formação de granulomas, estruturas capazes de conter a infecção pela ação de fagócitos ativados por IFN-γ (Murray *et al*., 2002). Outros estudos indicam que o fenótipo de susceptibilidade relaciona-se mais à incapacidade do desenvolvimento de uma resposta Th1 à elaboração de uma resposta Th2 exacerbada nos animais (Miralles *et al*., 1994). A existência de agentes solúveis, como TGF-β, que reduz os níveis de IFN-γ no local de crescimento parasitário, parece exercer este efeito

independente da expressão de IL-10, o que sugere que a modulação negativa da resposta Th1 na infecção visceral possa causar susceptibilidade à doença (Wilson *et al.*, 2005).

No que se refere à resposta humoral, alguns autores consideram que os anticorpos não desempenham papel de proteção contra a infecção por várias espécies de *Leishmania spp.* podendo, inclusive, contribuir para progressão da doença (Bohme *et al.*, 1986). Todavia, verifica-se uma tendência para o perfil de resistência quando ocorre a elevação da produção de anticorpos do isotipo IgG2a; enquanto que a progressão da infecção se relaciona com o predomínio do isotipo IgG1 específico aos parasitas (Martins *et al*., 2013; Costa *et al*., 2014).

Ao contrário do que ocorre na infecção experimental, em que é possível prever e analisar o curso do estabelecimento da infecção em órgãos específicos, a LV humana se manifesta de forma heterogênea, com diferenças marcantes das fases aguda e crônica da infecção no baço, fígado e medula óssea (Wilson *et al*., 2005). Além disso, vale ressaltar que estudos clínicos destinados à elucidação da resposta imune na LV em humanos são limitados devido à dificuldade de analisar diretamente os tecidos e órgãos afetados dos pacientes. Dessa forma, utiliza-se como alternativa a investigação da resposta sistêmica, através do estímulo de células mononucleares do sangue periférico (PBMCs) *in vitro* e na análise dos níveis séricos de diversas citocinas. Todavia, essa metodologia não reflete necessariamente os mecanismos imunes órgão-específicos, que são de extrema relevância para o entendimento de fatores determinantes da defesa do hospedeiro ou para o estabelecimento da infecção; dentre os quais se destacam os padrões de tropismo do parasita, bem como as diferentes respostas desenvolvidas no fígado e baço (Engwerda e Kaye, 2000).

Nesse contexto, considerando que a fase aguda da infecção se estabelece no fígado, a geração de uma resposta protetora pelas células de Kupffer torna-se importante e dependente da formação de granulomas, que contribuem para manutenção de um microambiente favorável à destruição do parasita (Murray, 2001). As células T $CD4^+$ desempenham papel predominante na resposta protetora durante a infecção, através da produção de citocinas características do perfil de resposta Th1, incluindo IL-12, IFN-γ, TNF-α, GM-CSF e IL-2, que contribuem para formação do granuloma e morte do parasita, por meio da ativação de macrófagos e produção de óxido nítrico e espécies reativas de oxigênio e nitrogênio (Kedzierski e Evans, 2014). As células T $CD8^+$ também possuem importante papel na resposta protetora, contribuindo principalmente na geração de uma resposta imune de memória (Stager *et al.* 2000). Citocinas como IL-17 e IL-22 complementam o papel de proteção exercido controle da LV, porém, os

mecanimos de atuação que lhes são inerentes permanecem ainda desconhecidos (Faleiro *et al.*, 2014).

Por outro lado, a evolução da LV em humanos está fortemente associada ao aumento da produção de IL-10, bem como aos níveis elevados de anticorpos específicos aos parasitas, que podem contribuir para a formação de complexos imunes que se ligam a receptores Fc em macrófagos, conduzindo à produção de IL-10 e contribuindo para patogênese da doença (Caldas *et al.*, 2005, Kedzierski e Evans, 2014).

No que se refere ao tropismo do parasita, enquanto a resolução da fase aguda recebe destaque no fígado, o estabelecimento da infecção parasitária crônica ocorre no baço, que é um importante local de ativação de células dendríticas e células T, que constituem fonte de IL-12 e contribuem na polarização da resposta Th1 (Gorak *et al*., 1998). Os neutrófilos parecem também desempenhar importante função na fase inicial de resposta no baço, uma vez que sua ausência resulta em um decréscimo da produção de IFN-γ por células T $CD4^+$ e $TCD8^+$, culminando no aumento da carga parasitária. Esse efeito, provavelmente, é específico para o baço, uma vez que a ausência de neutrófilos produz menos efeitos na carga parasitária nos hospedeiros infectados (McFarlane *et al*., 2008). Karp *et al*. (1993) encontraram níveis elevados de IFN-gama e IL-10 em aspirados da medula óssea de indivíduos com LV ativa. Essas observações sugerem que um resultado clínico desfavorável não está estritamente relacionado à ausência de resposta Th1 e à presença de uma resposta Th2, mas sim que outros mecanismos imunológicos podem também contribuir para a patogênese ou proteção contra a infecção (Faleiro *et al*., 2014).

Sendo assim, enquanto o paradigma Th1/Th2 é estabelecido para determinação dos perfis de resistência/susceptibilidade na LC, ainda não está claro seu papel no resultado da LV. Nesse contexto, embora a ênfase da resposta imune protetora resida nas citocinas características da resposta Th1, a co-expressão de citocinas Th2, como IL-4, pode contribuir para a proteção contra a infecção (Stager *et al.*; 2003).

2.6. **Desenvolvimento de vacinas para prevenção da leishmanioses**

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). Ao longo dos anos, diversas preparações 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.

A leishmanização foi uma das primeiras estratégias vacinais utilizadas a partir de 1908 em combate às leishmanioses no Oriente Médio, União Soviética e Israel. Esta técnica consiste na inoculação de uma pequena quantidade de parasitos vivos em uma região não exposta do corpo, com o objetivo de que uma lesão cutânea local se desenvolva e cure espontaneamente, 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; tendo dado 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). Enquanto no Oriente Médio a vacinação com organismos mortos falhou em proteger os indivíduos, no Brasil, os ensaios clínicos mostraram uma excelente proteção. Esta instabilidade na eficácia de uma vacina com parasitos mortos também foi percebida em estudos realizados com camundongos na década de 1980. Os ensaios clínicos demonstraram que havia uma diferença na eficácia vacinal dependente da via de administração utilizada. Animais inoculados por via intravenosa ou intraperitoneal apresentaram uma excelente proteção contra a doença, em contrapartida, esta proteção não foi observada quando a via subcutânea foi utilizada (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 mais doses do imunógeno fossem administradas, a fim de se manter o antígeno presente, ou que vacinas de parasitos vivos atenuados tornassem 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 testada 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. A presença deste parasito permite que o sistema imune do hospedeiro monte uma resposta específica contra os antígenos e que células efetoras e de memória geradas venham 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 destes genes de virulência, permitindo que tais candidatos vacinais ainda 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 e atentar 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).

Como alternativa vacinal mais segura, frações proteicas de Leishmania spp. têm demonstrado excelentes 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 sendo testadas com diferentes sistemas de entrega.

No Brasil uma formulação vacinal baseada em extrato bruto de *L. brasiliensis* adicionada de saponina, denominada de LBSap, foi administrada em cães e demonstrou ser capaz de induzir uma resposta humoral com anticorpos específicos anti-Leishmania, além de induzir um perfil celular baseado em LT CD4+ e LT CD8+. Uma elevada produção de IFN-ɣ e IL-12 e a redução na carga parasitária do baço demonstraram a imonogenicidade e proteção contra a infecção experimental por *L. infantum* (Roatt *et al.*, 2012; Resende *et al.*, 2013). Uma vacina muito parecida 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 LTCD8+ e LB, demonstrando um elevado potencial imunogênico contra as leishmanioses (Araújo *et al.*, 2008).

Na França, a utilização de proteínas secretadas de *L. infantum* 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 e 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), além de dar origem a uma vacina denominada Leishmune®, composta por FML-saponina, que foi capaz de induzir proteção na maioria dos animais vacinados (Palatnik-de-Sousa, 2012).

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).

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 dourados (Joshi *et al.*, 2014). Outras proteínas recombinantes ainda foram testadas e demonstraram ser boas candidatas à vacina, como a F14 e as 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).

Antígenos expressos nas formas amastigotas dos parasitos têm sido menos explorados como candidatos à vacina contra a LV, haja vista a dificuldade de cultivo destas formas. Assim, a maioria dos estudos em relação às vacinas contra as leishmanioses tem-se centrado em antígenos expressos nas formas promastigotas de Leishmania spp. No entanto, as formas amastigotas parecem ser o alvo mais apropriado para o sistema imune dos hospedeiros, uma vez que poucas horas após a infecção inicial e durante a doença ativa, esta fase do parasita encontra-se em contato com as células do sistema imune dos hospedeiros. Além disso, as formas amastigotas residem no interior das células fagocíticas e são alvos para LT CD4+ e LT CD8+, elementos envolvidos na imunidade protetora contra Leishmania spp (Fernandes *et al.*, 2012)

Uma nova estratégia, denominada 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).

Devido aos polimorfismos genéticos encontrados no sistema imune de mamíferos, uma vacina multiantigênica, composta por antígenos definidos e que leve a uma resposta imune do tipo Th1, pode apresentar uma melhor imunogenicidade e proteção do que os imunógenos utilisados isoladamente (Goto *et al.*, 2011). Algumas preparações multiproteicas vêm sendo testadas e têm apresentado bons resultados, como a proteína Q (Lip2a, Lip2b, H2A e P0), a Leish111f – MPL – SE (TSA, LmSTI-1, LeIF) e a KSAC (KMP11, SMT, A2 e CPB) (Joshi *et al.*, 2014).

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 e evitar os inconvenientes de 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 qualquer 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, cisteíno-proteinases, dentre outras, vêm sendo utilizados para compor vacinas, uma vez que têm apresentado resultados satisfatórios em camundongos (Joshi *et al.*, 2014). Mais recentemente nosso grupo, utilizou-se a técnica de *phage diplay* onde 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.

Devido ao fato de que alguns indivíduos não infectados, mas expostos ao inseto vetor, apresentarem uma resposta protetora contra a infecção pelo parasito, 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).

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 a variação na utilização dos diferentes adjuvantes vacinais (Vitoriano-Souza *et al.*, 2012; Pereira *et al.*, 2015; Thakur *et al.*, 2015). Desta forma, a escolha dos antígenos, do veículo de entrega, do adjuvante e da via de administração da vacina são importantes ao se definir a estratégia vacinal a ser utilizada.

2.7. Tecnologia de phage display

A tecnologia de phage display, proposta por Smith (1985), apresentou um grande impacto na imunologia e biologia celular, possibilitando a descoberta de novos fármacos, vacinas e melhoria dos testes diagnósticos em geral, sendo uma ferramenta útil por permitir a identificação e caracterização de peptídeos ligantes frente a uma variedade de moléculas, como as imunoglobulinas; envolvidas diretamente na resposta

imune aos agentes infecciosos (Blank *et al*, 1999). A técnica pode ser utilizada para revelar os diversos tipos de interações que existem na relação antígeno-anticorpo, definir epítopos para anticorpos monoclonais, selecionar substratos para enzimas, dentre outras aplicações (Kay *et al,* 1996).

Essa tecnologia utilizadifenretes classes de bacteriófagos (ou fagos), filamentodos ou não. No caso deste trabalho, fagos filamentosos da classe M13, capazes de infectar bactérias gram-negativas, como *Escherichia coli*, foram utilizados. A partícula de fago é formada por uma fita de DNA simples, envolta por uma capa proteica constituída por cinco proteínas: pIII, pVI, pVII, pVIII e pIX, conforme ilustrado na figura 2 (Housmand *et al*, 1999).

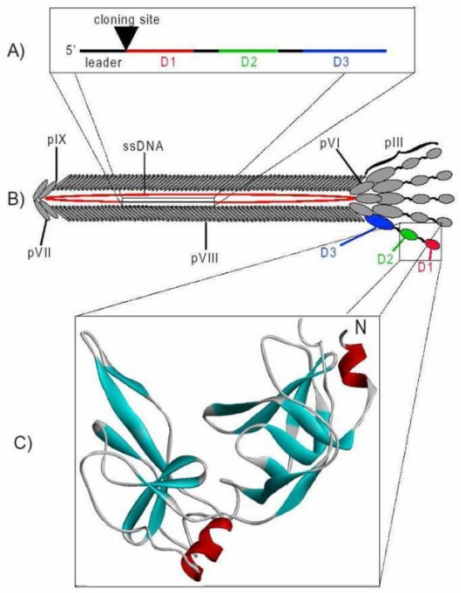

Figura 8- Representação da estrutura de um fago filamentoso. A) Composição do gene *III* mostrando o sítio de ligação de clonagem para introdução do gene adicional. B) Partícula viral com as proteínas pIII, pVI, pVII, pVIII e pXI. C) Cristalografia dos domínios D1 e D2 da proteína III (Holliger & Williams, 1999).

A técnica utiliza o princípio de que polipeptídeos podem ser expressos na superfície de bacteriófagos pela inserção de um segmento de DNA codificante no genoma dos mesmos, de modo que o peptídeo ou a proteína expressa fique exposta na superfície da partícula viral, fusionada a uma proteína endógena do vírus, como é o caso das proteínas pIII e pVIII (Barbas *et al*, 2001; Benhar, 2001).

A exposição de um peptídeo aleatório na superfície do fago possibilita a sua atuação como um ligante a uma enzima, um imunógeno ou desempenhando qualquer outra atividade em processos biológicos. A inserção de oligonucleotídeos e a subsequente construção de bibliotecas de peptídeos tornam possível a seleção de

proteínas com afinidades específicas (Makowski, 1994). Esta ligação entre o genótipo e fenótipo permite o enriquecimento de fagos selecionados, por exemplo, usando processos de bio-seleção (ou bio-pannings) frente a um determinado alvo. Dessa forma, fagos que exibem um ligante que seja relevante são retidos em virtude de sua ligação com o alvo, enquanto que os fagos não-aderentes são retirados por processo por meio de sucessivas lavagens (Benhar, 2001). Tal processo de seleção é realizado artificialmente, no qual a molécula alvo é imobilizada em um suporte sólido, geralmente, uma placa de ELISA de fundo chato, entretanto, *beads*, resinas ou membranas podem também podem ser utilizados.

O fato do fago M13 ter a capacidade de infectar bactérias *E. coli*, pela ligação da proteína pIII ao *pillus* F da célula bacteriana (Azzazy & Highsmith, 2002), permite a amplificação do número desses clones selecionados utilizando a maquinaria celular das bactérias (Barbas *et al*, 2001). As partículas de fago são processadas no espaço periplasmático da bactéria (Benhar, 2001), sendo que o gene lacZ no bacteriófago permite a distinção entre as colônias bacterianas infectadas com os fagos que carregam sequências exógenas, representadas pela coloração azul, daquelas não-infectadas, que permanecem de cor branca (Messing, 1983).

Outra característica importante em phage display é que tal metodologia utiliza protocolos curtos de seleção, uma vez que o sobrenadante das culturas infectadas pode ser utilizado diretamente nos processo de seleção. Tal fato dispensa a transferência para membranas que, além de limitar o número de clones, poderia ser um processo mais trabalhoso (Brigido & Maranhão, 2002).

O avanço tecnológico no campo das microtecnologias permitiu a abertura de novos campos na bioquímica analítica e no desenvolvimento de plataformas de detecção. A vantagem de se explorar técnicas baseadas em microrreações é o uso de pequenos volumes de reagente, menor produção de resíduos tóxicos, aumento do número de análises por unidade de volume, análises mais rápidas e, por fim, uma relação custo-eficácia efetiva (Härmä *et al.*, 2000). Muitos ensaios e testes diagnósticos têm utilizado partículas de tamanho de submicron ou em microesferas (*beads*), em substratos ou suportes como base para as reações imunológicas com vistas à pesquisa diagnóstica. Microesferas ou *beads* são partículas de polímeros esféricos de diferentes tamanhos e compostos por diversos materiais, tais como poliestireno ou sílica. Por apresentarem superfícies ativadas com diversos grupamentos químicos, tornam essas partículas adequadas para a ligação a uma infinidade de compostos. Depois de ativadas, podem ser preparadas para o acoplamento de ligantes. A adsorção de um ligante é um

processo em que vários fatores físico-químicos estão envolvidos. A quantidade e o modo no qual o ligante é adsorvido dependem da natureza do mesmo, das características da superfície sólida e das condições da solução tamponante (Peula-Garcia *et al.,* 2002). Microesferas magnéticas têm sido amplamente utilizadas em diagnóstico e pesquisa básica para captura de biomoléculas e células. Tipicamente, exibem propriedades super-paramagnéticas, em que há magnetização apenas na presença de um campo sem, contudo, permanecerem magnetizadas quando há a remoção do mesmo (Camilo, 2006).

A utilização de *beads* magnéticos acoplados a microesferas permite ampla variedade de separação e manipulação biomagnética. A superfície hidrofílica garante excelente habilidade de dispersão e baixas interações inespecíficas. Por apresentarem um pequeno tamanho (2,8 micrômetros - μm) e uma ampla área de superfície (2-5 metro ao quadrado por grama - m^2/g), tornam-se particularmente adequados para o isolamento de proteínas de uma amostra, para a realização de bioensaios e para a seleção de ligantes de afinidade (Wheatley; Schmidt Jr, 1999).

Já o acoplamento do bacteriófago M13 nos *beads* ocorre na região N-terminal da proteína do capsídeo. De acordo com Straus (2007), o bacteriófago possui aproximadamente 60 angstrons (Å), com diâmetro e 1 a 2 micrômetros (μm) de comprimento. O capsídeo proteico apresenta estrutura de uma concha helicoidal, formada por milhares de subunidades contendo, cada qual, 50 resíduos idênticos formando a principal proteína que protege o DNA central, sendo que a parte N-terminal encontra-se para fora do capsídeo e a porção C-terminal é voltada para o interior do mesmo, interagindo com o material genético. Dessa forma, a possibilidade de se desenvolver associações múltiplas através da interação entre antígenos selecionados por phage display, quando de sua conjugação às microesferas magnéticas, seguido da seleção por uso de anticorpos séricos; poderia possibilitar o achado de resultados com rapidez, efetividade e baixo custo.

A originalidade deste projeto está apoiada na associação da técnica de phage display a um sistema de *beads*, ainda não usado em estudos biológicos em leishmanioses; e na validação de epitopos selecionados por phage display ocmo candidatos à vacina contra a infecção por *L. infantum*.

3. OBJETIVOS

3.1. OBJETIVO GERAL DA TESE

Identificar clones de bacteriófagos, por meio da técnica de phage display, que expressam epítopos de interesse em seus capsídeos virais, para serem avaliados como candidatos à vacina em camundongos BALB/c contra as leishmanioses e também como plataforma de diagnóstico sorológico diferencial.

3.2. OBJETIVOS ESPECÍFICOS DO MANUSCRITO 1

- Purificar anticorpos da classe IgG de cães com leishmaniose visceral (LV) sintomática e assintomática, e de animais não-infectados; e acoplar tais anticorpos às microesferas de proteína-G composto por um sistema de *beads* magnéticos.
- Realizar os ciclos de bio-pannings para a seleção dos clones de bacteriófagos expressando, em sua superfície, epitopos que forem reconhecidos apenas pelos anticorpos purificados dos soros de cães com LV.
- Estimular culturas de esplenócitos de camundongos BALB/c sadios ou infectados com *L. infantum* com os clones de bacteriófagos anteriormente selecionados, a fim de identificar aqueles que fossem capazes de induzir à maior proporção entre IFN-γ e IL-4 nos sobrenadantes das culturas celulares;
- Extrair e sequenciar o DNA dos clones de bacteriófagos selecionados nos experimentos de imunoestimulação *in vitro*, com vistas à identificação das sequências dos epitopos exógenos.
- Analisar a homologia dos peptídeos selecionados frente às sequências primárias de proteínas do parasita *Leishmania spp.*
- Imunizar camundongos BALB/c com os clones de fagos selecionados, associados à saponina, como adjuvante; e avaliar a resposta imune celular e humoral induzida após a imunização, por meio da dosagem das citocinas IFN-γ, IL-12, GM-CSF, IL-4 e IL-10, e determinação dos níveis de anticorpos IgG1 e IgG2a específicos aos clones.

- Infectar os animais imunizados com formas promastigotas em fase estacionária de crescimento de *L. infantum* e avaliar o grau de proteção por meio da determinação da carga parasitária no baço, fígado, linfonodo drenante e medula óssea dos animais.
- Avaliar a resposta imune celular e humoral nos animais após o desafio, por meio da dosagem de citocinas IFN-γ, IL-12, GM-CSF, IL-4 e IL-10 e determinação dos níveis de anticorpos IgG1 e IgG2a específicos aos parasitas.

3.3. OBJETIVOS ESPECÍFICOS DO MANUSCRITO 2

- Utilizar os clones obtidos através do trabalho descrito no manuscrito 1 e testá-los frente a outras espécies causadoras de leishmanioses, a fim de avaliar a proteção cruzada (heteróloga).
- Imunizar camundongos BALB/c com os clones de fagos selecionados, em separaodos, juntos, e associados ou não à saponina, como adjuvante; e avaliar a resposta imune celular e humoral induzida após a imunização, por meio da dosagem das citocinas IFN-γ, IL-12, GM-CSF, IL-4 e IL-10, e determinação dos níveis de anticorpos IgG1 e IgG2a específicos aos clones.
- Infectar os animais imunizados com formas promastigotas em fase estacionária de crescimento de *L. amazonensis* e avaliar o grau de proteção por meio da determinação da carga parasitária no baço, fígado, linfonodo drenante, medula óssea e patas dos animais.
- Avaliar a resposta imune celular e humoral nos animais após o desafio, por meio da dosagem de citocinas IFN-γ, IL-12, GM-CSF, IL-4 e IL-10 e determinação dos níveis de anticorpos IgG1 e IgG2a específicos aos parasitas

3.4. OBJETIVOS ESPECÍFICOS DO MANUSCRITO 3

- Desenvolver novos marcadores de diagnóstico de Leishmaniose Tegumentar Humana LTH,
- Descrever uma plataforma simples para melhorar o sorodiagnóstico diferencial da LTH.
- Realizar o Phage-ELISA usando soros de pacientes com leishmaniose visceral (n = 20), doença de Chagas (n = 10), mucosa (n = 30) e leishmaniose cutânea (n = 20); bem como de indivíduos saudáveis que vivem em áreas endêmicas (n = 20) e não endêmicas (n = 30) da doença.
- Avaliar estatisticamente a acurácia do testes e eficácia diagnóstica dos refericos clones/fagos.

3.5. OBJETIVOS ESPECÍFICOS DO MANUSCRITO 4

- Descrever uma estratégia de seleção de fagos empregando sequencialmente IgG de indivíduos saudáveis e pacientes que desenvolveram doença de Chagas ou LV.
- Selecionar novos epítopos fundidos com bacteriófagos (mimotopos) para que possam ser aplicados no sorodiagnóstico da LV humana.
- Testar através de Phage-ELISA, soros de pacientes humanos com LV (n = 30), doença de Chagas (n = 15), LTH nas formas: mucosa (n = 34) e cutânea (n = 10), bem como soros de indivíduos saudáveis vivendo em áreas endêmicas (n = 27) ou não endêmicas (n = 30) de leishmaniose.
- Avaliar estatisticamente a acurácia do testes e eficácia diagnóstica dos refericos clones/fagos.

3.6. OBJETIVOS ESPECÍFICOS DO MANUSCRITO 5 (***EM SUBMISSÃO***)

- Avaliar a antigenicidade dos fagos e seus respectivos peptídeos sintéticos frente a soros de cães com LVC e de pacientes humanos com LV e LT nas formas mucosa e cutânea a fim de ver diferenciação sorológica heteróloga em cães e humanos.

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Mimotope-Based Vaccines of *Leishmania infantum* Antigens and Their Protective Efficacy against Visceral Leishmaniasis

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MANUSCRITO 1

4. MANUSCRITO 1

4.1. Full title

Protective efficacy of epitopes that mimic *Leishmania infantum* antigens selected by phage display against visceral leishmaniasis

4.2. Authors

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4.5. Abstract

Background: The development of new and cost-effective alternative therapeutic and prophylactic strategies to treat or prevent leishmaniasis has become a high-priority. In the present study, the phage display biotechnology was used for the identification of new antigens, represented by mimotopes expressed in the foreign surface of phage clones, which were specifically recognized by antibodies from sera of dogs with asymptomatic and symptomatic visceral leishmaniasis (VL).

Results: Twenty phage clones were selected after bio-pannings cycles, and they were evaluated in *in vitro* experiments of immune stimulation of splenocytes obtained from BALB/c mice naive and chronically infected with *Leishmania infantum*; in order to select those able to induce a polarized Th1 immune response, represented by high levels of IFN-γ and low levels of IL-4. Two phage clones (B10 and C01) were selected and employed in *in vivo* vaccination protocols in BALB/c mice. Vaccinated mice with B10 or C01 clones plus saponin showed a high and specific production of IFN-γ, IL-12, and GM-CSF after *in vitro* stimulation with the individual phages or *Leishmania* antigenic extract. Immunized and infected mice, as compared to the control groups (saline, saponin and wild-type phage clone plus saponin), showed significant reductions in the parasite burden in liver, spleen, bone marrow and in the paws' draining lymph nodes. Protection was associated with an IL-12-dependent production of IFN-γ, produced mainly by CD8 T cells. In these mice, a decrease in the parasite-mediated IL-4 and IL-10 response was also observed.

Conclusions: The present study showed that two new antigens, selected by a yet non-described phage display technique, could be employ associated to a Th1-type adjuvant, as candidate to compose a vaccine against VL.

Keywords: Phage display; Vaccine; Visceral leishmaniasis; IFN-gamma; phage clones; mimotopes.

4.6. Background

Leishmaniasis is a disease with a wide spectrum of clinical manifestations caused by different species of protozoa belonging to the *Leishmania* genus [1]. The disease presents a high morbidity and mortality throughout the world, where 350 million people in 98 countries at risk of contracting the infection, and approximately 1.0 to 1.5 million cases of Tegumentary Leishmaniasis, and 200,000 to 500,000 cases of Visceral Leishmaniasis are registered annually [2, 3]. Visceral Leishmaniasis (VL) caused by *Leishmania donovani* and *L. infantum/L. chagasi* represents an important disease in the world. The first choice of treatment is still based on the use of the parenteral administration of pentavalent antimonials compounds; however, several side effects reported by patients and increased parasite resistance have been important problems [4, 5]. In addition, other options, like liposomal amphotericin B, miltefosine and others, although effectives, present problems related with side effects, high-cost and teratogenicity, how is the case of miltefosine [6, 7]. Therefore, the development of new strategies to prevent leishmaniasis has become a high priority [8]. The evidence of life-long immunity to leishmaniasis has inspired the development of vaccination protocols against the disease, but few have progressed beyond the experimental stage. In studies involving murine models and evaluating new vaccine candidates, the immunogens are administered in mice, associated with Th1-type adjuvants, and the animals are lately infected with *Leishmania* [9, 10, 11, 12]. After a few weeks following the infection, besides of the evaluation of parasitological parameters; spleen cells are recovered, *in vitro* cultured and stimulated with the own immunogen or *Leishmania* extract, in order to evaluate the immunogenicity induced by vaccine. In this case, cytokines with a Th1 profile, such as IFN-γ and IL-12, and others, taken with a Th2 profile, such as IL-4 and IL-10; have their determined levels and a comparison is done to, together with the parasitological results, evaluate the effectiveness of the vaccine candidate [9, 13]. Thus, antigens that are capable of stimulate the development of a Th1 immune response, primed by production of IFN-γ and IL-12 in *in vitro* spleen cells cultures

of infected, could be considered like promising vaccine candidates for use against *Leishmania* infection [14, 15].

Phage display is a technology based on DNA recombination, resulting in the expression of foreign peptide variants on the outer surface of phages. Using an *in vitro* selection process, based on binding affinity and so-called bio-panning cycles, peptides exposed in the selected phage clones are analyzed by DNA sequencing, and identified [16, 17, 18]. Phage display has been used to study antigen-antibody binding aiming to identify mimotopes (peptides that mimic linear, discontinuous, and even non-peptide epitopes [19]), which are recognized by specific antibodies, and applied to the diagnoses and/or vaccine candidate against some diseases, such as malaria [20, 21, 22], leishmaniasis [23], toxoplasmosis [24, 25], and Chagas´ disease [26]. In the present study, phage display technology was used for the identification of new antigens, represented by mimotopes expressed in the foreign surface of phage clones, to be applied in a yet non-described research to new vaccine candidates against *Leishmania* infection. Initially, phage clones from a library were selected by antibodies present in sera of dogs with asymptomatic and symptomatic VL, and lately used in the *in vitro* stimulation of spleen cells obtained from BALB/c mice naive and chronically infected with *L. infantum*; in order to select those able to stimulate a high production of IFN-γ and low levels of IL-4. Two phage clones presenting the best results were selected and tested in the vaccination protocols of BALB/c mice. Both clones, namely B10 and C01, when associated with saponin as adjuvant, were able to induce an *in vivo* specific-Th1 immune response, primed by high production of IFN-γ, IL-12 and GM-CSF, and low levels of IL-4 and IL-10; and the both phage clones were protective against *L. infantum*. In this context, these two new antigens could be considered like vaccine candidates, when in association with a Th1-type adjuvant, to compose a vaccine against VL.

4.7. Results

Selection of the phage clones

The phage clones´ selection was performed by excluding those that were recognized by antibodies present in sera samples of healthy dogs. After, a positive selection was performed by recovering the phages that were recognized by antibodies present

in the sera of dogs with asymptomatic and symptomatic VL. The technical protocol is summarized in the Figure 1.

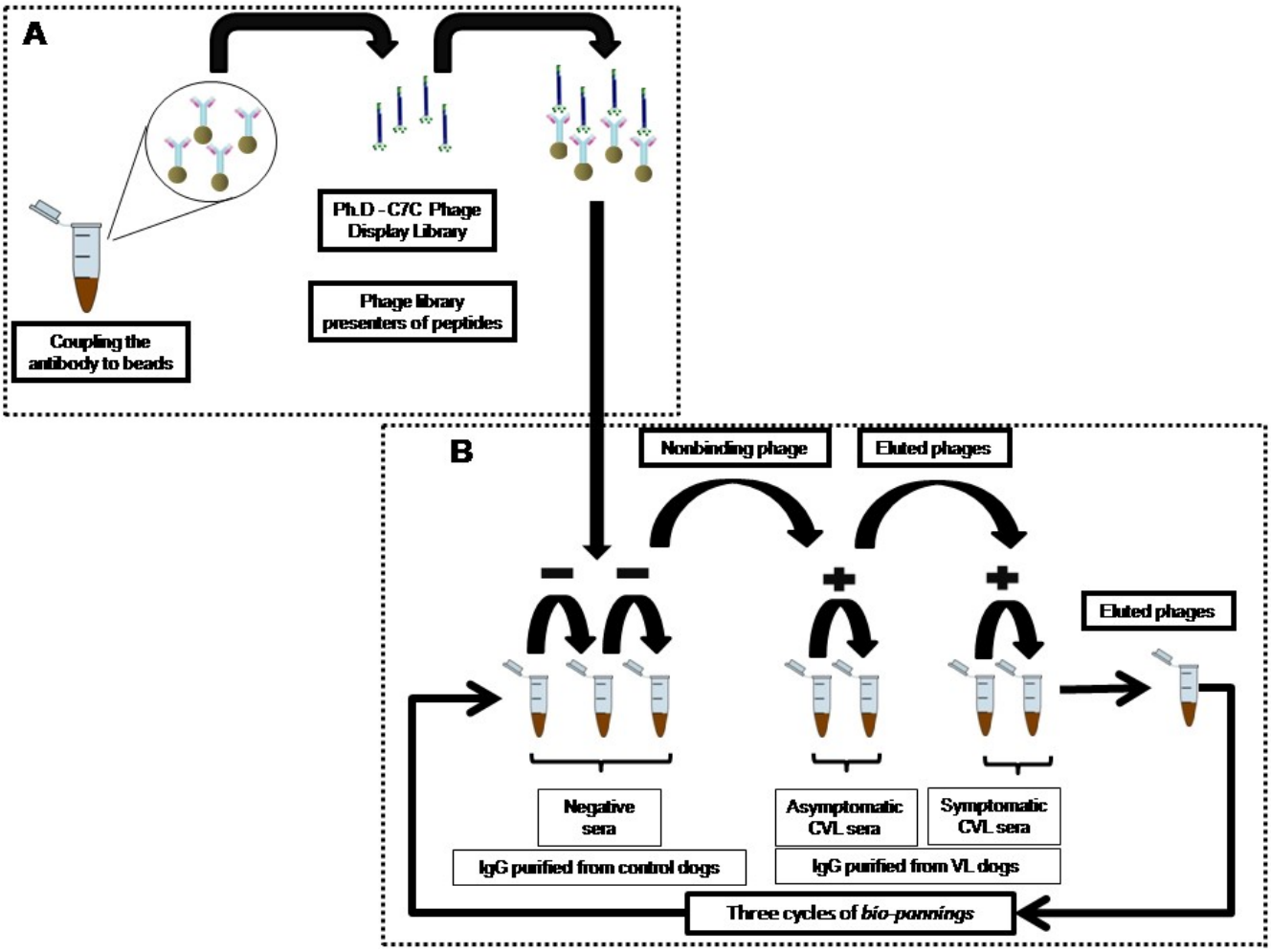

Figure 1. Schedule of technical protocol used in the bio-panning cycles of phage display.

Approximately 96 clones were randomly selected from individual colonies, and their DNA sequences were PCR amplified and sequenced. Twenty clones had their sequences clearly identified (Table 1), and an alignment showed that no identical consensus motif could be detected between them (data not shown).

Clone	Sequence
A10	LLSSKTL
B10	LSFPFPG
C1	FTSFSPY
C2	QATHFHS
D9	LASLPFR
E3	THVFSWI
E10	ACDPSPN
E11	TPSLHRS
F1	TAMARSA
F3	VALLPHH
F4	QSPPALL
F8	FSLLGSL
F9	VLLGPFP
F11	YPFSLLH
G2	SLGPQIK
G5	MSPTYLL
G9	DRAALSL
G11	ALTPQLL
G12	QTSPPLA
H5	FPLFGLS

Table 1. Amino acid sequences from target peptides present in the selected phage clones.

Spleen cells of BALB/c mice naive or chronically infected with *L. infantum* were *in vitro* cultured, and separately stimulated with each one of the 20 phage clones, when the IFN-γ and IL-4 levels were determined in the cultures supernatants. A wild-type phage clone, identical to the other phage clones presents on the library, but not expressing foreign peptides; was used as phage control. In addition, a random non-specific phage clone (NMSDFLRIQLRS) was also used as mimotope control.

In order to determine the specificity and selectivity of the selected phage clones to be applied like vaccine candidates against *Leishmania* infection, two different

approaches were performed. First, to evaluate the specificity of them, the cytokines levels found after stimulation of spleen cells derived from naive mice were normalized with the results obtained the data obtained after stimulus with the wild-type phage clone. In a second approach, to determine the selectivity of the phage clones, the levels of IFN-γ and IL-4 were normalized using the data obtained from the spleen cells obtained from infected mice, after stimulus with the non-relevant phage clone. In this context, the phage clones that presented a ratio between IFN-γ and IL-4 lesser than 1 were discarded (Figure 2). So, two phage clones were selected and lately evaluated in an *in vivo* murine model against *Leishmania*.

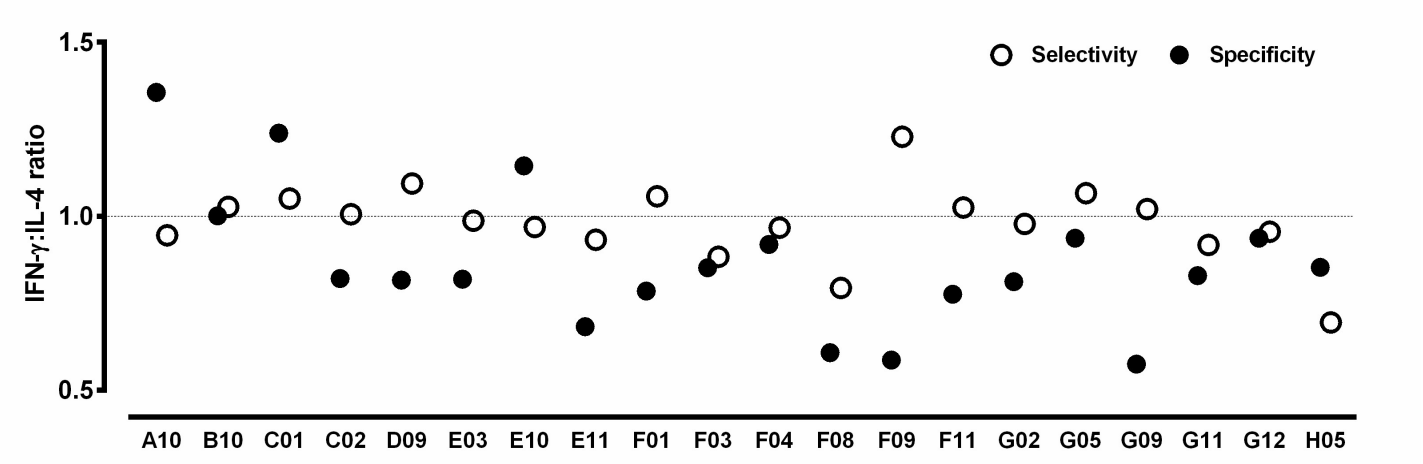

Figure 2. Evaluation of the specificity and selectivity of the selected phage clones by a phage display approach.

Immunogenicity and protective efficacy of the phage clones against *L. infantum*

The immunogenicity of the B10 and C01 phage clones was evaluated in BALB/c mice, 4 weeks after the last vaccine dose (Figure 3). Following *in vitro* stimulation with individual clones, spleen cells from vaccinated mice significantly produced higher levels of IFN-γ and IL-12 than those secreted by spleen cells from control mice (saline, saponin and wild-type phage plus saponin groups). No significant production of IL-4 and IL-10 could be observed in any experimental groups, after stimulation with individual clones (Figure 3A). The ratios between the IL-12/IL-4 and IL-12/IL-10 (Figure 3B), and between IFN-γ/IL-4 and IFN-γ/IL-10 (Figure 3C) levels showed that vaccinated animals presented an elevated Th1 immune response after B10 or C01-stimulus. In addition, mice vaccinated with either B10 or C01

clone plus saponin presented an specific humoral response, with the higher predominance of IgG2a isotype in relation to IgG1 (Figure 3D).

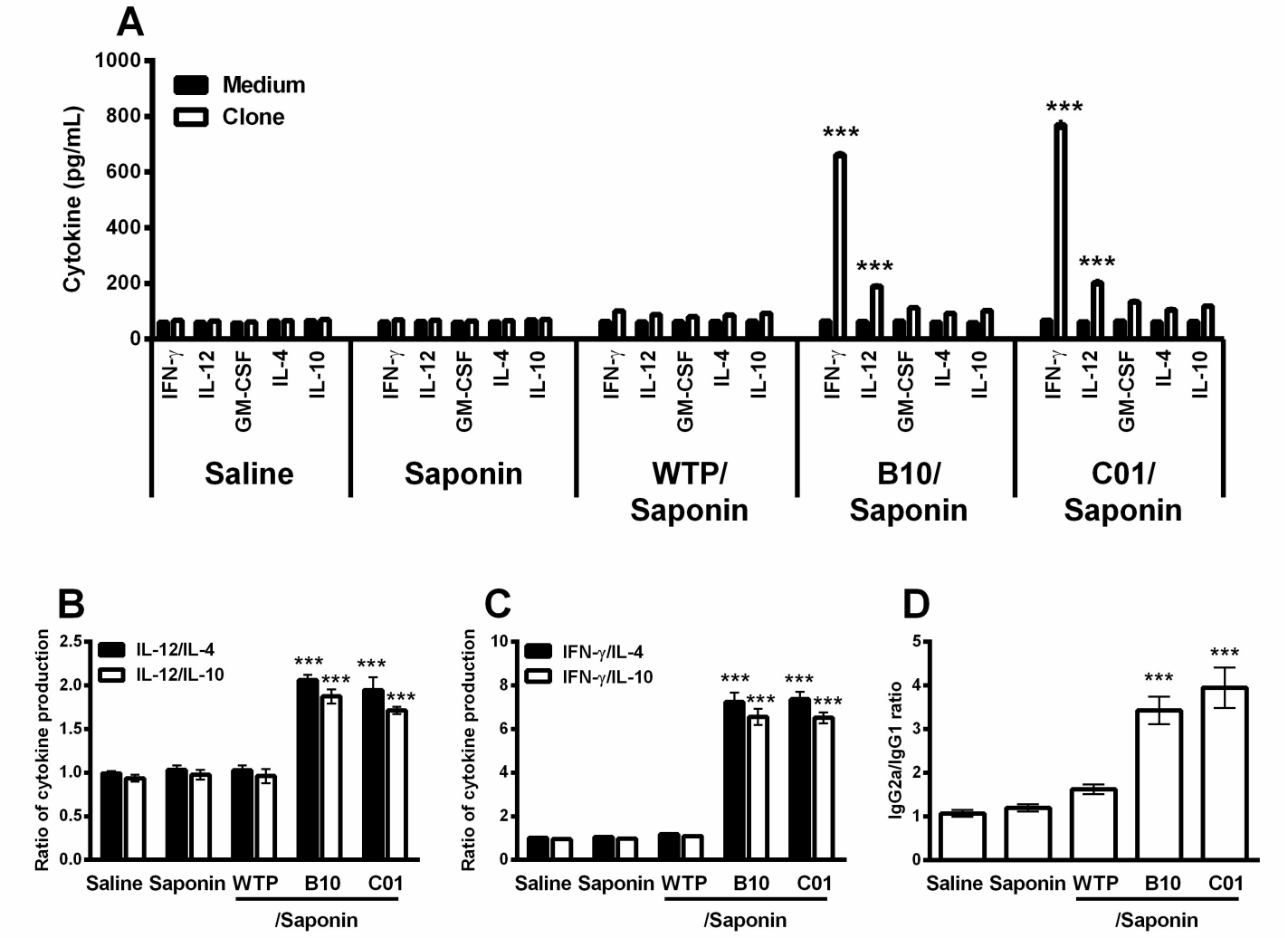

Figure 3. Cellular and humoral response induced in BALB/c mice by immunization with B10 or C01 phage clones plus saponin. Single cells suspensions were obtained from the spleen of mice, four weeks after vaccination. Cells were non-stimulated (medium; background control) or stimulated with individual clones (1 x 10^{11} phages mL^{-1}, each one) or SLA (20 μg mL^{-1}) for 48 h at 37°C, 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured in culture supernatants by capture ELISA (A). Each bar represents the mean ± standard deviation (SD) of data from four individual mice per group. Statistically significant differences in the IFN-γ and IL-12 levels between the B10 or C01 clone plus saponin groups and control mice (saline, saponin and wild-type phage plus saponin groups) were observed (***$P < 0.0001$). The ratio between IL-12/IL-10 and IL-12/IL-4 levels (B), and between IFN-γ/IL-10 and IFN-γ/IL-4 levels (C) are also showed. Statistically significant differences in the ratios

between the B10 or C01 clone plus saponin groups and control groups were also observed (***$P < 0.0001$). The ratio between phage-specific IgG1 and IgG2a antibodies was obtained for sera of each individual mouse within their respective vaccination group, and statistically significant difference between the B10 or C01 clone plus saponin groups and control groups were also observed (***$P < 0.0001$) (D).

Next, the present study analyzed whether the immunization with the B10 or C01 phage clones plus saponin was able to induce protection against *L. infantum*. The infection was followed up over a 10-weeks period, when the parasite burden in the liver, spleen, paws' draining lymph nodes (dLN), and bone marrow (BM) was determined. Significant reductions in the number of parasites were observed in the different evaluated organs of vaccinated mice, as compared with those that received only saline or that were immunized with saponin or wild-type phage clone plus saponin (Figure 4). In this context, vaccinated mice with B10 or C01 clones plus saponin showed significant reductions in the parasite load in liver (2.5- and 5.3-log reductions, Figure 4A), spleen (2.8- and 4.4-log reductions, Figure 4B), dLN (2.5- and 4.0-log reductions; Figure 4C), and BM (2.5- and 5.3-log reductions, Figure 4D); in relation to the saline group, respectively.

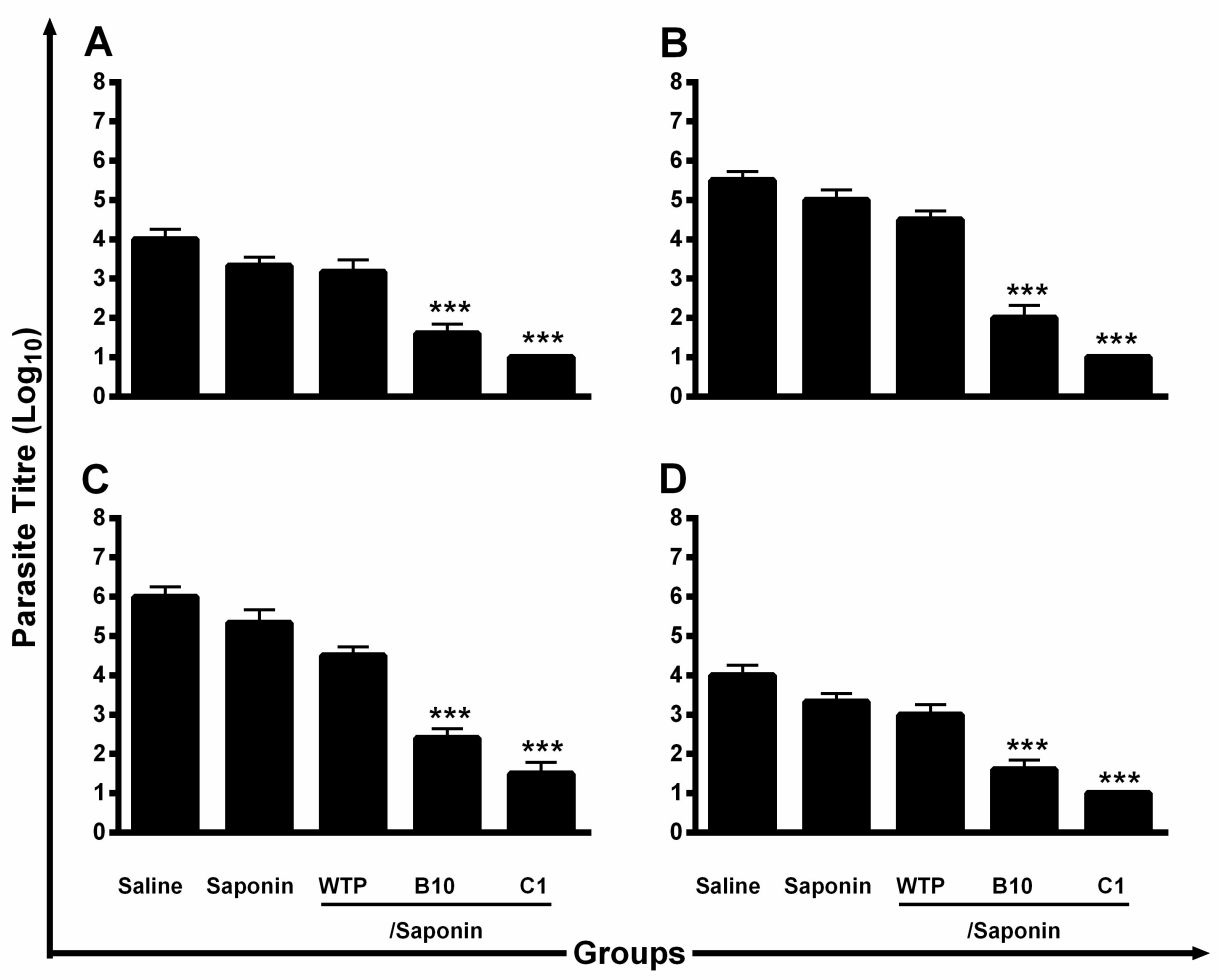

Figure 4. Protection of BALB/c mice vaccinated with phage clones plus saponin against *L. infantum.* Mice inoculated with saline, saponin, wild-type (WTP), B10 or C01 phage clones plus saponin were subcutaneously infected with virulent 1 x 10^7 stationary-phase promastigotes of *L. infantum.* The number of parasites in the liver (A), spleen (B), paws′ draining lymph nodes (C), and bone marrow (D) was measured, 10 weeks after challenge by a limiting-dilution technique. Mean ± standard deviation (SD) of four mice in each group is shown. Statistically significant differences in the parasite load in all evaluated organs between the B10 and C01 clones plus saponin group and control mice (saline and saponin groups) are showed (***$P < 0.0001$). Data shown in this study are representative of two independent experiments, which presented similar results.

Cellular responses elicited after L. infantum *challenge infection*

The production of cytokines in the supernatants of spleen cells cultures stimulated with B10 or C01 clones and SLA after challenge, was analysed to determine the immunological correlates of protection induced by the both phage clones. The

spleen cells from mice vaccinated with B10 or C01 clones plus saponin produced higher levels of SLA-specific IFN-γ, IL-12 and GM-CSF than those secreted by spleen cells from both control groups, 10 weeks after infection (Figure 5A). In contrast, the SLA-driven production of IL-4 and IL-10 showed that vaccination with both phage clones plus saponin induced no production of these cytokines in the vaccinated and infected animals. As observed before challenge, the ratios between the IL-12/IL-4 and IL-12/IL-10 (Figure 5B), and between IFN-γ/IL-4 and IFN-γ/IL-10 (Figure 5C), indicated that vaccinated mice developed an elevated Th1 response, which was maintained after infection in these animals. It was also possible to observe that vaccinated and infected mice presented SLA-specific IgG2a antibodies that were significantly higher than the IgG1 levels (Figure 5D).

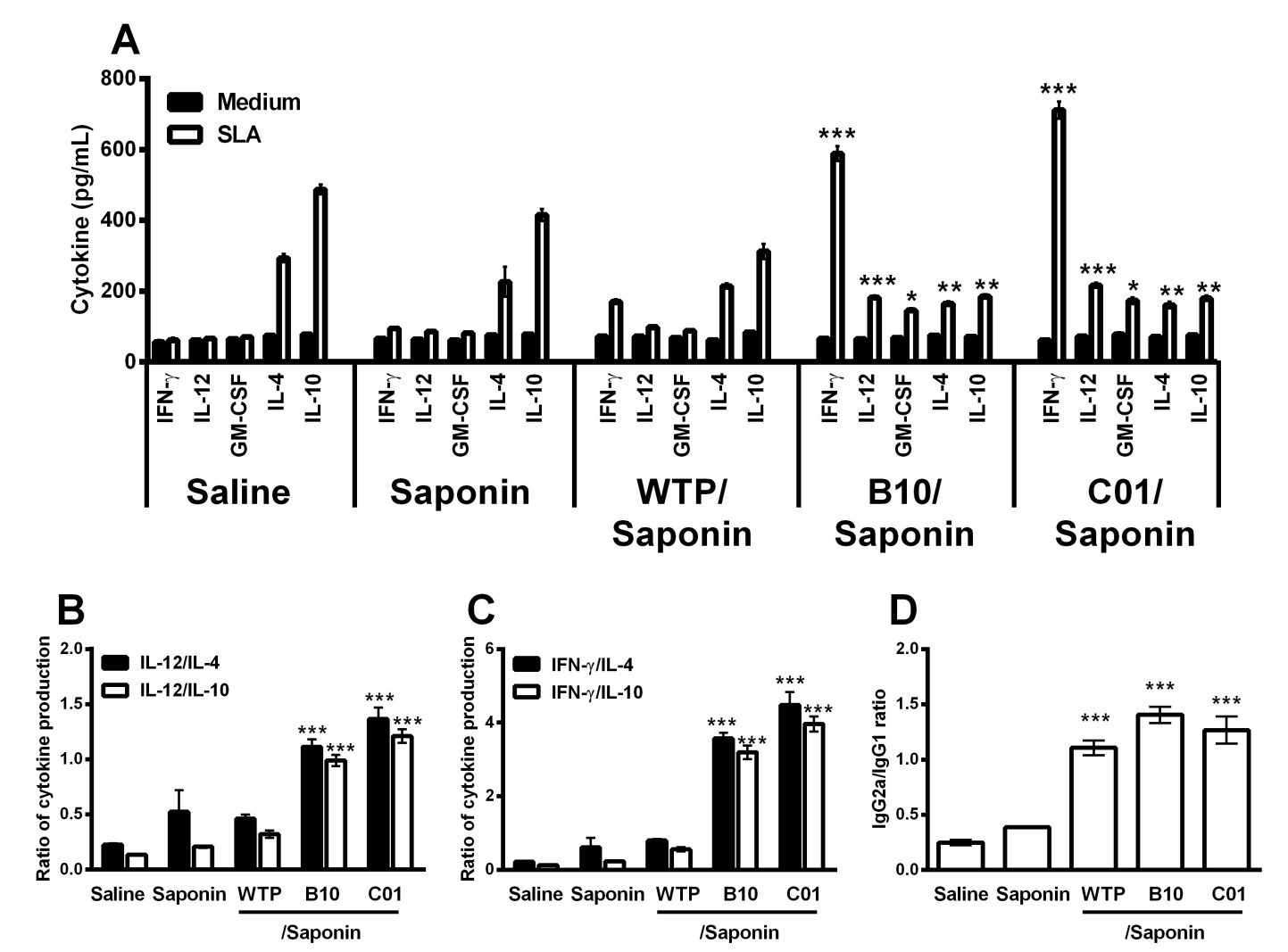

Figure 5. Analysis of the cellular and humoral response in BALB/c mice after *L. infantum* challenge infection. Single cells suspensions were obtained from the spleens of mice, 10 weeks after infection. Cells were non-stimulated (medium; background

control) or stimulated with SLA (20 μg mL^{-1}) for 48 h at 37°C, 5% CO_2. Levels of IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 were measured in culture supernatants by capture ELISA. Mean ± standard deviation (SD) of the cytokines levels determined in four individual mice per group is shown (A). Statistically significant differences between the B10 and C01 clones plus saponin group and the control mice (saline, saponin and wild-type phage plus saponin groups) were observed (***$P < 0.0001$). The ratio between IL-12/IL-10 and IL-12/IL-4 levels (B), and between IFN-γ/IL-10 and IFN-γ/IL-4 levels (C), are also showed. Statistically significant differences between the B10 and C01 clones plus saponin group and the control groups were observed (***$P < 0.0001$). The ratio between SLA-specific IgG1 and IgG2a antibodies levels were calculated for sera of each individual mouse within their respective vaccination group and statistically significant difference between the phage clones groups and the control (saline and saponin) groups was also observed (*$P < 0.005$) (D).

The contribution of CD4 and CD8 T cells and the dependence of IL-12 production for the SLA-specific IFN-γ response from the spleen cells of mice immunized with B10 or C01 phage clones plus saponin and challenged with *L. infantum* was evaluated (Figure 6). The IFN-γ production was significantly suppressed using anti-CD8 monoclonal antibody in the spleen cells cultures. Addition of anti-CD4 or anti-IL-12 antibodies to the cultures also decreased the production of IFN-γ, as compared to the control cells culture without treatment; however, the production of this cytokine proved to be higher than that produced by the use of the anti-CD8 monoclonal antibody.

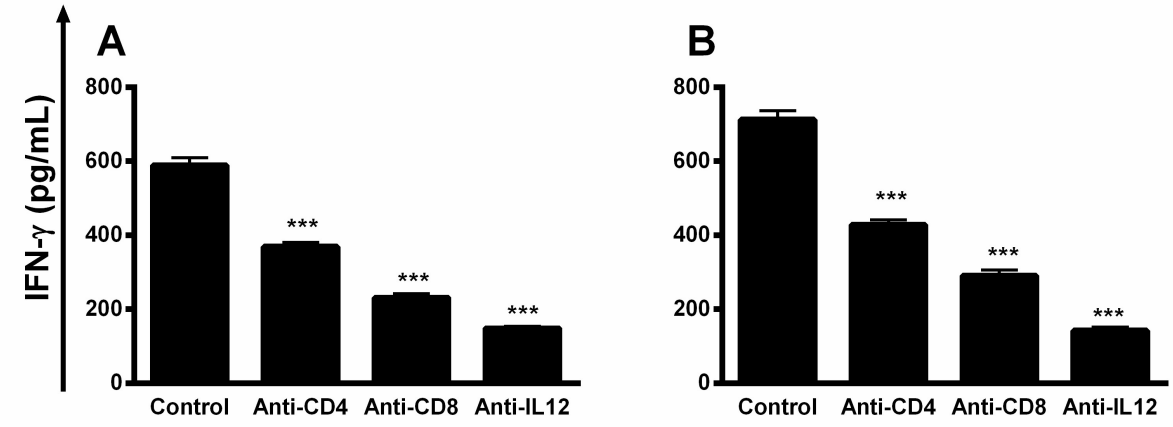

Figure 6. Involvement of IL-12, CD4 and CD8 T cells in the IFN-γ production after *L. infantum* challenge. Single cells suspensions were obtained from the spleens of mice vaccinated with B10 clone plus saponin (A) or C01 clone plus saponin (B), 10 weeks after infection. Cells were non-stimulated (medium; background control) or stimulated with SLA Levels of IFN-γ were measured in culture supernatants by capture ELISA of spleen cells cultures stimulated with SLA (20 μg mL^{-1}) for 48 h at 37°C, 5% CO_2. Cultures were incubated in the absence (positive control) or in the presence of anti-IL-12, anti-CD4, or anti-CD8 monoclonal antibodies. Statistically significant differences between non-treated control cells and cultures incubated with anti-CD4, anti-CD-8 and anti-IL-12 monoclonal antibodies were observed (***$P < 0.0001$). Mean ± standard deviation (SD) of the IFN-γ levels determined in four individual mice per group is shown.

4.8. Discussion

In the present study, the phage display technology was applied for the identification of antigens, represented by mimotopes expressed in the foreign surface of phage clones, which could be used as new vaccine candidates against murine VL. The selected phage clones expressing the target mimotopes were selected after rigorous bio-panning cycles of negative and positive selections, followed by *in vitro* immune stimulation experiments using spleen cells derived from BALB/c mice naive and chronically infected with *L. infantum*. After definition of the selectivity and specificity of the phage clones using results obtained after wild-type and non-redundant phage clones, two clones, namely B10 and C01, were selected based on their ability of inducing a significantly higher production of IFN-γ and low levels of IL-4, in the both models. These clones were then evaluated in BALB/c mice, according their ability to induce protection against the infection with *L. infantum*.

The use of murine models in vaccination study to leishmaniasis allowed the identification of two distinct subtypes of T cells producing cytokines with different effector functions. In this context, studies based on the BALB/c model and *L. major* infection defined the Th1 or Th2 paradigm of resistance or susceptibility, respectively, to the infection; as well as the role of IFN-γ and IL-4, respectively, in the development of a Th1 or Th2 immune response [27]. In the murine VL, the Th1

response (IFN-γ production) could be suppressed by production of cytokines, such as IL-4 and IL-10, which could be related to the deactivation of macrophages and the onset of the disease in the infected animals [14, 28, 13]. In studies evaluating vaccine candidates in the murine LV, immunogens are administered in mice, associated with Th1-type adjuvants and, after few weeks, they are infected and follow-up by a couple of months. In this time, usually, spleen cells of the animals are collected and *in vitro* stimulated with the antigen used in the vaccination, or *Leishmania* extract, in order to evaluate the induced immunogenicity. In this point, cytokines such as IFN-γ and IL-12, markers of Th1 response, and IL-4 and IL-10, indicators of Th2 response, have their levels determined and a comparison between them is performed, which together with the results of the parasite levels, determine the efficacy or not of the vaccine [9, 13]. Thus, antigens capable of stimulating the development of a Th1-type immune response, based on the production of high levels of IFN-γ, in mice infected with *Leishmania*, could be considered as promising vaccine candidate for use against leishmaniasis.

The antigens currently used as vaccine candidates normally include complete proteins, in a recombinant proteins format [29, 30, 31]. Such antigens, however, can present epitopes that posses a beneficial effect to protection, but others that could have a deleterious effect in the immune system of the immunized animals, compromising thus the effectiveness of whole vaccine [9, 14]. The use of peptides as vaccine candidates tends to allow the design of a more specific vaccine, so that the immunized animals will develop an immune response only against this antigen, specific to it and to the disease to which it was selected. In general, these antigens also present a simpler and faster production, besides of a low-cost, in comparison to the production of the recombinant proteins.

Phage display, since its first description [32] and introduction into laboratory practice [33], has proven to be useful in selecting specific peptides that are highly reactive against many pathogen targets. The biological applications ranging from vaccine candidates for rabies [34], hepatitis C [35], *Escherichia coli* [36], *Plasmodium vivax* [22], and *Schistosoma japonicum* [37]; to diagnostic antigens to pneumonia [38], leishmaniasis [23], neurocysticercosis [39, 40, 41], equine arteritis virus [42], rabies [43], and anaplasmosis [44].

In the present study, the immunization using B10 or C01 phage clones associated with saponin was able to induce a specific type 1 immune response in the animals,

caracterized by an *in vitro* specific production of IFN-γ, IL-12 and GM-CSF, combined with the presence of low levels of IL-4 and IL-10, as well as a predominance of IgG2a antibodies. After infection, mice immunized, when compared to control groups, displayed significant reductions in the parasite burden in several evaluated organs (liver, spleen, dLN, and BM), which also correlated a high clones- and SLA-dependent production of IFN-γ in the spleen cells, one of the main cytokines implicated in the acquired immunity against infection with *Leishmania* [21, 22, 24]. The CD8 T cells proved to be the major source of IFN-γ in the protected mice, since depletion of these cells in cultures of splenic cells stimulated with SLA, significantly abrogated this response. Although previous reports have shown that the activation of both CD4 and CD8 cell subsets may be important for the killing of parasites in mice vaccinated with different parasite recombinant antigens [25, 26, 13] the present study's data suggest that CD4 cells may contribute in a less extension to the induction of IFN-γ mediated responses elicited by immunization using phage clones. The CD8 T cells proved be the main resource of IFN-γ, and these cells may also contribute to infection control by their direct cytotoxic effect on infected cells, as previously demonstrated in other experimental models [24]. In addition, the immunization using phage possibility the exposition of their mimotopes to CD8 and CD4 T cells, due to the fact that phages can be taken up and processed efficiently by mechanisms of major histocompatibility complex (MHC) class I and II patways, inside of the phagocityc cells [45, 46].

The present study also demonstrates that protection in BALB/c mice against *L. infantum* was associated with a significant decrease in the production of the macrophage deactivating cytokines, IL-4 and IL-10. Very low levels of parasite-specific IL-10 were detected after the stimulation of spleen cells from vaccinated mice, 10 weeks after infection. In contrast, spleen cells from both control mice groups showed a significantly higher production of this cytokine. Indeed, control of the parasite-mediated IL-10 response in vaccinated mice may be critical for protection, since this cytokine is considered to be the most important factor for VL progression after infection with viscerotropic *Leishmania* species in IL-10 deficient mice [47, 48, 49] or in mice treated with an anti-IL-10 receptor antibody [50]. In BALB/c mice, the IL-4-dependent production of IgG1 antibodies is associated with disease progression due to some *Leishmania* species, including *L. amazonensis* and

L. infantum [51, 28]. Here, immunized mice with the phage clones presented higher levels of SLA and clones-specific IgG2a antibodies, in comparison to IgG1 levels, demonstrating the developed Th1 immune response found in these animals.

Spleen cells from vaccinated mice, as compared to the control groups, produced higher levels of SLA and clones-specific GM-CSF, a cytokine related with macrophage activation and resistance in murine models against different intracellular pathogens, including *L. major* [52], *L. donovani* [53], and *L. infantum* [47, 13]. It has also been shown that the administration of a therapeutic vaccine containing some *Leishmania* antigens plus GM-CSF could be correlated with the curing of tegumentary leishmaniasis patients [33].

4.9. Conclusion

The present study describes a subtractive phage display strategy that led to the identification of novel antigens to be evaluated like vaccine against *Leishmania* infection. The results indicated that two selected phage clones expressing target mimotopes confered protection in BALB/c mice against *L. infantum*. Protection correlated with CD4 and, mainly, by CD8 T cells response, which caracterized by high IFN-γ, IL-□□□□and GM-CSF, and low IL-4 and IL-10 levels. These data permits infers that the B10 and C01 phage clones could constitute new vaccine candidates against VL. Studies are in progress in order to cloning the native proteins expressing these mimotopes, with the purpose to compare its effectivity of them in relation to the selected mimotopes selected here.

4.10. Methods

Ethics Statement

Experiments were performed in compliance with the National Guidelines, as set forth by the Institutional Animal Care (Law number 11.794, 2008), and the Committee on the Ethical Handling of Research Animals from the Federal University of Minas Gerais (UFMG), who approved this study under protocol number 043/2011.

Mice and parasites

Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), UFMG; and were maintained under specific pathogen-free conditions. Experiments were carried out using the *L. infantum* (MOM/BR/1970/BH46) strain. Parasites were grown at 24ºC in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 10% heat-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 *L. infantum* antigenic (SLA) extract was prepared from 1 x 10^{10} stationary-phase promastigotes, as described [16]. Parasites were kindly provided by Prof. Maria Norma Melo (Department of Parasitology, ICB, UFMG).

Sera samples

The sample size of this study consisted of 77 domestic dogs (*Canis familiaris*), made up of males and females of different breeds and ages, collected from an endemic area of Belo Horizonte, Minas Gerais, Brazil. Sera of CVL were selected on the basis of two serological tests (IFAT [IFAT- LVC Bio-Manguinhos kit] and ELISA [EIE-LVC Bio-Manguinhos kit], both from Biomanguinhos, Fiocruz, Brazil) for *Leishmania spp*. Dogs with an IFAT titre < 1/40 or ELISA reactivity below the cut-off value indicated by the manufacturer were considered to be seronegative. Animals with an IFAT titre > 1/40 and an ELISA value over the cut-off were considered to be seropositive. Thus, symptomatic dogs (n=20) were those positive by IFAT and ELISA, but also positive parasitological results by PCR-RFLP (restriction fragment length polymorphism) in blood samples, and presenting more than three clinical symptoms (weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis; and exfoliative dermatitis on the nose, tail, and ear tips). Asymptomatic dogs (n=20) presented also positive serological (IFAT and ELISA) and parasitological (PCR-RFLP in blood samples) results, but they did not present any clinical signals or symptoms of leishmaniasis. Healthy dogs (n=20) were selected from an endemic area of Belo Horizonte, but they presented negative serological (IFAT and ELISA) and parasitological (PCR) results, as well as they were free of any clinical signs or symptoms of leishmaniasis. Sera samples were provided by Prof. Maria Norma Mello (Department of Parasitology, UFMG, Belo Horizonte, Brazil), and Prof. Ana Paula Fernandes (Department of Clinical and Toxicological Analysis, UFMG, Belo Horizonte, Brazil).

Purification of antibodies by coupling in protein G microspheres

The purification of IgG antibodies from sera samples was performed by their coupling to magnetic microspheres (magnetic beads) conjugated to protein G (Dynabeads, Invitrogen). To this end, 2 x 10^9 particles of microspheres were washed 3 times in 1 mL of 0.1 M MES buffer pH 5.0, and were added to them: 375 µL of a pool of sera samples of healthy dogs, 195 µL of a pool of sera samples of asymptomatic CVL or 300 µL of a pool of sera samples of symptomatic CVL. An incubation of 40 min was performed under constant stirring, at room temperature. The microspheres coupled with the antibodies were washed 3 times using 1 mL of 0.1 M MES buffer pH 5.0, in order to remove the non-adhered antibodies. Next, the beads-antibody system was washed twice with 1 mL of 0.2 M triethanolamine buffer pH 8.2, and resuspended in 1 mL of covalent coupling buffer (containing 20 mM dimethy pimelimidate/HCl diluted in triethanolamine buffer) for 30 min, under constant stirring, at room temperature. The neutralization of unbound reactive sites was made by incubating 1 mL of 50 mM Tris-base pH 7.5, for 15 min at room temperature. The microspheres were washed 3 times with 1 mL TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20), blocked by addition of 2 mL of blocking solution (5% BSA diluted in TBS-T) for 1 h at 37°C, and then resuspended in 200 µL of TBS buffer. To verify the coupling, 5 µL of beads coupled using IgGs were incubated for 1 h at 37°C with an anti-dog IgG peroxidase antibody (1:5,000 dilution), when they were washed 3 times with 1 mL of TBS-T, and the reaction was revealed by adding TMB substrate. The reaction was stopped by adding 25 µL H_2SO_4 2 N, and the optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 450 nm.

Bio-panning cycles

To carry out a negative selection process in the bio-panning cycles, 1 x 10^{11} viral particles from a phage library containing random seven-peptides fused to a minor coat protein of M13 filamentous phages (Ph.D.®-C7C library, New England BioLabs, USA) were diluted in 190 µL of TBS-T buffer. The mixture was incubated for 30 min at room temperature with the microspheres coupled to IgGs that had been purified from healthy dogs, and then precipitated by magnetic attraction to produce a Dynal Biotech support (12020). The supernatant containing the clones that were not adhered to the IgGs was recovered and transferred to a new tube, been this procedure repeated for three times.

After this, the previously recovered phage clones were transferred to a tube containing the microspheres coupled to IgGs purified from asymptomatic CVL and incubated for 30 min at room temperature. After, the supernatant was removed, and the remained bound phages to the IgGs were washed 10 times with 1 mL of TBS-T and eluted in 500 µL of 0.2 M glycine buffer, pH 2.0. Next, 75 µL of 1 M Tris-base pH 9.0 was added to neutralize the acid pH. Subsequently, the recovered phage clones were transferred to a new tube containing IgGs that had been purified from symptomatic CVL. The process was repeated three times with asymptomatic and symptomatic CVL sera, when the selected phage clones were then recovered and titrated.

Titration of phage clones

Phage clones were diluted 10^{-1} to 10^{-11} in 500 µL of sterile PBS, mixed with an *E. coli* culture (OD_{600nm} ~ 0.5), and plated on LB agar plates containing 1 mL IPTG/X-gal (1.25 g IPTG, 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and 25 mL DMSO). Colonies were individually quantified, and the titration was performed for each bio-panning cycle. After the 3rd of positive selection, using IgGs from CVL sera, 96 colonies were selected from the plate were added in 200 µL of LB in a sterile culture plate (BD Falcon TM clear, 96-well microtest TM plate), when the plate was sealed and incubated for 5 h, under constant stirring, at 37ºC. After incubation, the plate was centrifuged for 20 min by 2,250 x *g*, and the supernatant was transferred to a new plate, where a PEG/NaCl (20% PEG 8.000 and 2.5 M NaCl) solution was added (1/6 of the total volume of the supernatant), and the plate was incubated for 16 h at 4ºC. After, the plate was centrifuged for 1 h, the supernatant was removed, and the pellet was resuspended in 500 µL of a solution composed by 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 4 M NaI. The plate was shaken vigorously for 5 min, and 250 µL of a 70% ethanol solution was added. After having been incubated for 10 min, the plate was centrifuged (2,250 x *g* at 4ºC, for 10 min), and the supernatant was discarded. The pellet containing the DNA of interest of each phage clone was washed with 500 µL of 70% ethanol, and again centrifuged. Finally, the DNA was diluted in 20 µL of ultra-pure water and its quality was evaluated in a 1% agarose gel, which was stained with an ethidium bromide solution (10 µg mL^{-1}). The individual DNA of clones was used for the sequencing and identification of target peptides.

DNA sequencing and bioinformatics

The sequencing reaction was performed using 500 ng of DNA for each selected phage clone, 5 pmol primer 96 gIII (5'-OH CC TCA TAG TTA GCG TAA CG-3', Biolabs), plus a pre-mix (Dye Terminator Cycle ET Journal Kit®, Amersham Biosciences). Thirty-five cycles were performed in a thermocycler under the following conditions: denaturation at 95ºC for 20 sec, ringing at 58ºC for 15 sec, and extension at 60ºC for 60 sec. The generated amplicons were precipitated with 1 µL of ammonium acetate and 27.5 µL of ethanol PA. The plate was centrifuged for 45 min to 2,432 x *g*, the supernatant was discarded, and 150 µL of 70% ethanol was added to the pellet. The resuspended DNA was centrifuged for 10 min at 2,432 x *g*, and the supernatant discarded. The plate was inverted on a paper towel and, in this position, was centrifuged at 486 x *g* for 1 min. Next, the plate was covered for 5 min until the complete evaporation of the remaining ethanol had been achieved. The pellet was resuspended in dilution buffer, and the sequencing was performed in a MegaBace 1000 automatic sequencer (Amersham Biosciences). Peptide sequences of 20 valid sequences were deduced using the Expasy server (www.expasy.org), and the sequences were analyzed using NCBI (www.ncbi.nlm.nih.gov/Blast) and UniProt databases.

Evaluation of the *in vitro* immune stimulation

BALB/c mice (n=8) were subcutaneously infected with 1 x 10^7 stationary-phase promastigotes of *L. infantum*, and were monitored by 10 weeks. Naive mice were also used in the immune stimulation experiments. Liver, spleen, dLN and BM of the animals infected were collected for parasite quantification, following a limiting-dilution protocol [17]. The parasite load was performed to confirm that the animals were chronically infected (data not shown). After this, spleen cells were collected and *in vitro* cultured in duplicate in 24-well plates (Nunc, Nunclon®, Roskilde, Denmark), at 5 x 10^6 cells per mL. Cells were incubated in RPMI 1640 medium (Sigma; background control), or separately stimulated with each phage clone (20 individual clones, using 1 x 10^{10} phages per well), at 37^0C in 5% CO_2 for 48 h. The same experimental conditions were performed using spleen cells of naive mice. A wild-type phage clone was used as control. The IFN-γ and IL-4 levels were then determined in the culture supernatants, using commercial kits (BD OptEIA™, Pharmingen, San Diego, CA, USA), according to manufacturer´ instructions.

Immunization and challenge infection

After the *in vitro* immunogenicity experiments, two from 20 phage clones were selected, namely B10 and C01, based on their polarized Th1 immune response, and they were used to *in vivo* experiments. For this, BALB/c mice (n=8, per group) were vaccinated subcutaneously in their left hind footpad with two selected clones (B10 and C01) or wild-type phage (1 x 10^{11} phages, each one), associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma), or only adjuvant or diluent (saline). Three doses were administered at 2-week intervals. Four weeks after the final immunization, animals (n=4, per group) were euthanized for the analysis of the immune response elicited by vaccination. At the same time, the remaining animals were infected subcutaneously in the right hind footpad, with 1 x 10^7 stationary promastigotes of *L. infantum*, and they were follow-up for 10 weeks. Data showed in this study are representative of two independent experiments, which presented similar results.

Estimation of parasite load

The liver, spleen, BM and dLN were collected of the euthanized animals for parasite quantification, using a limiting-dilution protocol. Briefly, the organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at 150 × *g*, and cells were concentrated by centrifugation at 2,000 × *g*. Pellets were resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Two hundred and twenty microliters were plated onto 96-well flat-bottom microtiter plates (Nunc) and diluted in log-fold serial dilutions in supplemented Schneider's medium with a 10^{-1} to 10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the culture at 24°C. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per microgram of tissue.

Cytokine production

Splenocyte cultures and cytokine assays were performed before infection and at 10^{th} week after challenge, as described [16]. Briefly, single-cell preparations from spleen tissue were plated in duplicate in 24-well plates (Nunc) at 5 x 10^6 cells per mL. Cells were incubated in RPMI 1640 medium (background control), or separately stimulated with SLA (25 μg mL^{-1}), or individual phage clones (1 x 10^{11} phages, each one), at 37^0C in 5% CO_2 for 48 h. The IFN-γ, IL-4, IL-10, IL-12, and GM-CSF levels were

determined in the culture supernatants, using commercial kits (Pharmingen), according to manufacturer´ instructions. In order to block IL-12, CD4, and CD8 mediated T cell cytokine release, spleen cells of mice vaccinated and challenged were *in vitro* stimulated with SLA (25 μg mL^{-1}), and incubated in the presence of 5 μg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12 (C017.8), CD4 (GK 1.5), or mouse 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).

Analysis of the humoral response

SLA and phage-specific IgG1 and IgG2a antibodies were measured by ELISA, as described elsewhere [9]. Peroxidase-labeled antibodies specific to mouse IgG1 or IgG2a isotypes (Sigma) were diluted at 1:5,000 and incubated for 1 h at 37^0C, then incubated with H_2O_2 and *o*-phenylenediamine. Optical densities were read at 492 nanometers in an ELISA microplate spectrophotometer.

Statistical analysis

The statistical analysis was done using the GraphPad Prism software (version 5.0 for Windows). Statistical analysis with the vaccinated and/or infected mice was performed by one-way analysis of variance (ANOVA), using the Bonferroni´s post-test for multiple comparisons between the groups. Differences were considered significant when $P < 0.05$.

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Phage-fused epitopes from *Leishmania infantum* used as immunogenic vaccines confer partial protection against *Leishmania amazonensis* infection

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5. MANUSCRITO 2

5.1. Full title

Phage-fused epitope immunogens from *Leishmania infantum* confer cross-protection against *Leishmania amazonensis* infection

5.2. Running title

A mimotope-based vaccine against *Leishmania amazonensis*

5.3. Authors

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5.6. Summary

Two mimotopes of *Leishmania infantum* identified by phage display were evaluated as vaccine in BALB/c mice against *Leishmania amazonensis*. The epitope-based immunogens, B10 and C01, and presented as phage-fused peptides; were used without association of a Th1 adjuvant, and clones were administered isolated or in combination into animals. Both clones showed a specific production of IFN-γ, IL-12, and GM-CSF after *in vitro* spleen cells stimuli and were able to induce protection. Significant reductions of parasite load of infected footpads, liver, spleen, bone marrow, and paws' draining lymph nodes were observed in the immunized mice, in comparison to the control groups (saline, saponin, wild-type and non-relevant clones). Protection was associated with an IL-12-dependent production of IFN-γ, mediated mainly by $CD8^+$ T cells, against parasite proteins. Protected animals also presented low levels of IL-4 and IL-10, as well as increased levels of parasite-specific IgG2a antibodies. The association of both antigens resulted in an improved protection in relation to their individual use. Besides, the absence of adjuvant did not diminish the cross-protective efficacy against infection. This study describes for the first time two epitope-based immunogens selected by phage display against *L. infantum* infected dogs sera, which cross-protected mice infected with *L. amazonensis*.

Key words: Phage display, mimotopes, vaccine, leishmaniasis, Th1 immune response.

Key findings: ⇒ Subtractive phage display selection using sera of visceral leishmaniasis dogs. ⇒ Phage clones were selected and evaluated as immunogens

against *Leishmania infantum*. ⇒ Cross-protective efficacy was evaluated against *Leishmania amazonensis*. ⇒ The absence of an adjuvant did not diminish the cross-protective efficacy. ⇒ Two phage-fused epitope immunogens protected mice against *L. amazonensis*.

5.7. Introduction

Leishmaniasis presents a high morbidity and mortality throughout the world, where about 350 million people in 98 countries are at risk of contracting the infection (WHO, 2010). Moreover, approximately 1.0 to 1.5 million new cases of tegumentary leishmaniasis (TL), and 200,000 to 500,000 new cases of visceral leishmaniasis (VL) have been registered annually (Alvar *et al*. 2012). Several geographical regions are endemic for different *Leishmania sp.* species, being this the case of the South America, where the disease is caused by at least eight different species of the parasites (Grimaldy and Tesh, 1993; Coelho *et al*. 2003; Reinthinger *et al.* 2007; Lainson and Shaw, 2010). Among them, *Leishmania amazonensis,* presents particular importance, since it is able to cause human disease (Garcez *et al*. 2002), as well as a broad spectrum of clinical manifestations, from cutaneous to visceral leishmaniasis (Barral *et al*. 1991). The treatment of the disease is hampered due to the side effects registered in the patients, by increased parasite resistance and high cost of the conventional drugs (Croft and Coombs, 2003; Minodier and Parola, 2010). Recently, Bacon *et al*. (2013) evaluated the potential economic value of a cutaneous leishmaniasis (CL) vaccine in endemic countries localized in the Americas, and the study showed that a protective vaccine could provide at least 5 years of protection, being less costly than new treatment approach. Therefore, the development of improved strategies to prevent leishmaniasis could be considered a priority (Costa *et al*. 2011; Coelho *et al*. 2012).

The evidence of life-long immunity against infection with some *Leishmania sp.* species has inspired the development of prophylactic vaccination models, but few have progressed beyond the experimental stage (Fernandes *et al*., 2008; Chávez-Fumagalli *et al*., 2010; Modabber, 2010; Das and Ali, 2012; Ramírez *et al*., 2013; Costa *et al*., 2014; Ramírez *et al*., 2014). Using murine models, it has been showed that an important challenge for the development of an effective vaccine is to find a formulation able to induce a Th1-type immunity, based on the production of cytokines, such as IFN-γ, IL-12, and GM-CSF, produced by $CD4^+$ and/or $CD8^+$ T cells; as well as by controlling the

disease associated, IL-4 mediated, humoral response (mainly in TL models), and IL-10 deactivating response (Afonso and Scott, 1993; Noben-Trauth *et al*., 2003). From these experimental models; vaccine candidates, such as whole parasite (Mizbani *et al*. 2009; Dey *et al*. 2013), parasite fractions (Rosa *et al*. 2007; Iborra *et al*. 2008; Chávez-Fumagalli *et al*. 2010), recombinant proteins (Coelho *et al*. 2003; Fernandes *et al*. 2008; Martins *et al*. 2013), poly-proteins (Bertholet *et al*. 2009; Goto *et al*. 2011), DNA-based vaccine (Zanin *et al*. 2007; Carrión *et al*., 2008), and synthetic peptides (Spitzer *et al*. 1999; Basu *et al*. 2007); have been shown to be immune stimulatory, and induce variable degrees of protection against some *Leishmania sp.* species. It appears that a live or killed parasite vaccine will not be feasible for TL, since results from clinical trials using whole parasites (using either live or killed parasites) can reach from 0 to 75% efficacy (Coler *et al*. 2007). Moreover, difficulties of standardization and production in large-scale are additional disadvantages of such vaccines (Handman, 2001). Protein-based vaccines, although offering considerable advantages in terms of safety and production´ costs, are less immunogenic, and usually the association of adjuvants is necessary (Cerpa-Cruz *et al*. 2013).

Notwithstanding, the adjuvants regulation for human use are far more rigorous than those applied to veterinary vaccines. In addition to preclinical studies on the adjuvant itself, the combined antigen-adjuvant formulation also requires toxicological evaluation for entering phase 1 clinical trials (Goldenthal *et al*. 1993). Amongst the biggest regulatory hurdles is the required population size that needs to be tested to prove efficacy and particularly safety of a new adjuvant or vaccine. So, one could speculate that an effective vaccine candidate should not be associated with an adjuvant, being the own composition able to induce an effective immune response before infection; aiming to reduce adverse effects as well as their production costs (Bazan *et al*. 2012). An interesting approach towards the discovery of new products to be evaluated in biotechnological applications has been based on phage display technology (Clark and March, 2004). This technology is focused on DNA recombination, resulting in the expression of foreign peptide variants, namely mimotopes, on the outer surface of phage clones (Smith and Petrenko, 1997). Using an *in vitro* selection process, based on binding affinity, so-called bio-panning cycles; these mimotopes (that correspond to peptides that mimic linear, discontinuous, and even non-peptide epitopes) exposed on surface of phages are selected, analyzed by DNA sequencing and identified (Barbas *et al*., 2001; Wang and Yu, 2004). Phage display has been used to select mimotopes to be applied as vaccine candidates against cysticercosis (Manoutcharian *et al*. 2004),

trichinellosis (Gu *et al*. 2008), VL (Costa *et al*. 2014), and Alzheimer´s disease (Frenkel *et al*. 2000); as well as therapeutic agents (Manoutcharian *et al*. 2001).

Recently, our group used this technology to identify mimotopes that were highly reactive against 100% of dogs sera infected by *L. infantum*. Two clones, B10 and C01, were selected and evaluated in vaccination experiments in BALB/c mice. Both clones, when associated with saponin, were able to induce a Th1 immune response before infection, which was primed by high production of IFN-γ, IL-12, and GM-CSF; as well as by low levels of IL-4 and IL-10 and, after challenge, they were protective against infection (Costa *et al*. 2014). In the present study, these two phage clones were tested as cross-protective immunogens against *L. amazonensis* infection, and without the association of adjuvants; in order to evaluate the immune stimulatory potential of own phage capsid, as described (García *et al*. 2005; Manoutcharian, 2005). Cross-protection against infection raised by both clones was demonstrated with and without saponin association, suggesting that elicitation of the protective Th1 immune response was phage-dependent. Thus, we present evidences of a simple vaccine formulation composed by the combination of two epitope-based immunogens to protect against *L. amazonensis* infection.

5.8. Materials and Methods

Mice

Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), UFMG; and were maintained under specific pathogen-free conditions. Experiments were performed in compliance with the Brazilian Guidelines for Animal Care (Law number 11.794, 2008), and under the approval of the Ethics Committee on the Handling of Research Animals from the Federal University of Minas Gerais (UFMG), protocol number 043/2011.

Parasites

Experiments were carried out using the *L. amazonensis* (IFLA/BR/1967/PH-8) strain. Parasites were grown at 24ºC in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic (SLA) extract was prepared

from stationary-phase promastigotes, as described (Coelho *et al*. 2003). Briefly, 1 x 10^9 promastigotes of *L. amazonensis* per mL, in a volume of 5 mL, were washed 3 times in 5 mL of cold sterile phosphate-buffered saline (PBS). After seven cycles of freezing (-196ºC) and thawing (+37ºC), the suspension was centrifuged at 8,000 x *g* for 20 min at 4ºC, and the supernatant containing SLA was collected in 500 μL aliquots and stored at -80ºC, until use. The protein concentration was estimated by Bradford method (Bradford, 1976).

Selection of the phage clones

The technical procedures used to select the B10 and C01 phage clones were performed as described (Costa *et al*. 2014).

Immunization and challenge infection

BALB/c mice (n=8, per group) were vaccinated subcutaneously in their left hind footpad with B10, C01, wild-type (WTP) or non-relevant phage (NRP) clones (1 x 10^{11} phages, each one), or with the association between B10 and C01 clones (5 x 10^{10} phages, each) administered with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma Aldrich). An additional group of animals received only diluent (PBS). Three doses were administered at two-week intervals. Four weeks after the last immunization, animals (n=4, per group) were euthanized to analyze the immune response elicited by vaccination. At the same time, the remaining animals were infected subcutaneously in their right hind footpad with 1 x 10^6 stationary-phase promastigotes of *L. amazonensis*. The course of the disease was monitored at weekly intervals by measuring footpad thickness with a metric calliper, and expressed as the increase in thickness of the infected footpad compared to the uninfected footpad. Ten weeks post-challenge, animals were euthanized and their sera samples, infected footpads and some organs were harvested for immunological analysis and parasite quantification. Experiments were repeated twice and presented similar results.

Estimation of parasite load

The infected footpad, liver, spleen, bone marrow (BM) and infected paws´ draining lymph nodes (dLN) were collected for parasite quantification, using a limiting-dilution technique (Coelho *et al*. 2003). Briefly, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by

centrifugation at 150 × *g*, and cells were concentrated by centrifugation at 2,000 × *g*. Pellets were resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Two hundred and twenty microliters were plated onto 96-well flat-bottom microtiter plates (Nunc), and diluted in log-fold serial dilutions using supplemented Schneider's medium, to a 10^{-1} to 10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the cultures, at 24°C. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per microgram of tissue.

Cytokine response and NO production

Spleen cells cultures and cytokine assays were performed before infection and at 10^{th} week after challenge, as described (Coelho *et al*. 2003). Briefly, single-cell suspensions from spleen tissue were plated in duplicate in 24-well plates (Nunc), at 5 x 10^6 cells per mL. Cells were incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4; or separately stimulated with individual B10, C01, WTP or NRP clones (1 x 10^{11} phages, each one), or with SLA *L. amazonensis* (25 µg mL^{-1}); for 48 h at 37^0C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12, and GM-CSF levels were determined in the culture supernatants, using commercial kits (Pharmingen), according to manufacturer´ instructions. In order to block IL-12, $CD4^+$, and $CD8^+$ mediated T cells cytokine release, spleen cells of mice vaccinated with B10/C01, added or not with saponin, and lately infected with *L. amazonensis*, were *in vitro* stimulated with SLA *L. amazonensis* (25 µg mL^{-1}), and incubated in the presence of 5 µg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12 (C017.8), CD4 (GK 1.5), or mouse 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 NO production was evaluated in all groups, at 10^{th} week after challenge, for which 100 µL of SLA-stimulated culture supernatants were mixed with an equal volume of Griess reagent (Sigma). After 30-min incubation at room temperature, nitrite concentration was calculated using a standard curve of known concentrations. Data were expressed as µM per 5 x 10^6 cells.

Analysis of the humoral response

The B10- and C01-specific IgG1 and IgG2a antibodies, as well as SLA *L. amazonensis*-specific IgG1 and IgG2a antibodies were measured by an ELISA technique. Briefly, previous titration curves were performed to determine the appropriate antigens concentration and antibodies dilution. Microtiter immunoassay plates (Biofil®, Belo Horizonte, Minas Gerais, Brazil) were coated with either B10 or C01 clone ($1x10^{10}$ phages per well), or with SLA *L. amazonensis* (1.0 µg per well) diluted in 100 µL coating buffer (50 mM carbonate buffer, pH 9.6) for 18 h at 4°C. After incubation, free binding sites were blocked using 200 µL of TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) buffer containing 5% casein for 1 h at 37°C. After having been washing three times using PBS-T, the plates were incubated with sera samples diluted 1:100 in TBS-T containing 0.5% casein solution for 1 h at 37°C. Then, plates were washed 7 times using TBS-T, and incubated with the peroxidase-labeled antibodies specific to mouse IgG1 or IgG2a isotypes (Sigma Aldrich) diluted at 1:5,000 and 1:10,000, respectively; and incubated for 1 h at 37^0C. Plates were again washed 7 more times with TBS-T, and the reaction was 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. The reaction was stopped by adding 25 µL H_2SO_4 2 N, and optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada), at 492 nanometers.

Statistical analysis

Results were entered into Microsoft Excel (version 10.0), and analyzed using GraphPad Prism™ (version 6.0 for Windows). Results were expressed by mean ± standard deviation (SD). Outliers were evaluated using ROUT′ test, and excluded from statistical analyses. The normality analysis of the data was performed using the D'Agostino & Pearson test. Statistical analysis with the data from vaccinated and/or infected mice was performed by one-way analysis of variance (ANOVA), using Tukey's post-test for multiple comparisons among groups. Differences were considered significant with $P < 0.05$. Data showed in this study are representative of two independent vaccination experiments, which presented similar results.

5.9. Results

Immunogenicity of selected phage clones in BALB/c mice

Two phage clones (B10 and C01) isolated by their antigenicity in CVL, and tested as protective against *L. infantum* (Costa *et al*. 2014), were analyzed for their protective role against *L. amazonensis* infection. Clones were administered independently in the absence of any adjuvant and in combination, in this case, associated or not with saponin. The immunogenicity of the clones was evaluated in the immunized mice four weeks after the last vaccine dose (Fig. 1). Spleen cells' cultures derived from vaccinated mice with the B10 and/or C01 clones significantly secreted higher levels of IFN-γ and IL-12 than cells from control groups (WTP, NRP, and saline) after specific stimuli with each phage clone. In addition, very low levels of IL-4 and IL-10 were observed in all experimental groups, after stimulation with specific clones employed in the vaccination regimens (Fig. 1A). Also, spleen cells cultured from the saline group were separately stimulated with the individual clones; however, no significant cytokines production was observed (data not shown). The ratios between the IL-12/IL-4 and IL-12/IL-10 levels (Fig. 1B), as well as between the IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 1C), were calculated and the results showed that vaccinated animals with B10, C01, B10/C01, and B10/C01/saponin were able to induce a specific Th1 response before infection, when the specific phage-stimulus was employed in culture cells. The association between B10 and C01 clones was able to increase these cytokines´ ratios. In addition, when the humoral response was evaluated, mice vaccinated with B10 and/or C01 clones presented higher predominance of the phage-specific IgG2a isotype in comparison to the phage-specific IgG1 levels (Fig. 1D), corroborating with the higher production of IFN-γ and lower levels of IL-4 in these vaccinate animals, representing a typical and specific Th1 immune response mounted in these vaccinated animals.

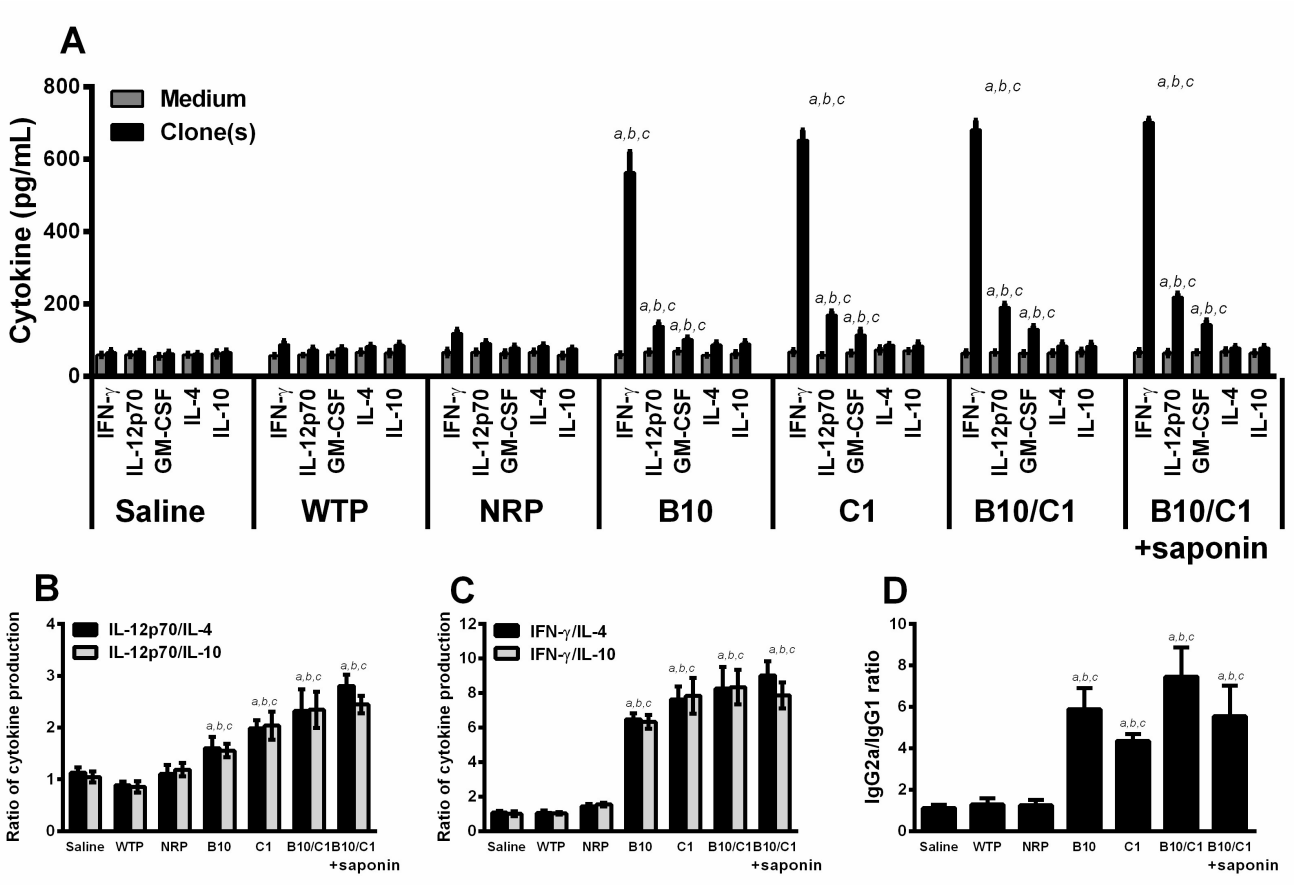

Figure 1. Immune response induced in BALB/c mice by immunization with B10 and/or C01 phage clones. Single cells suspensions were obtained from the spleen of mice, four weeks after the last immunization. Cells were non-stimulated (negative control), or separately stimulated with the B10, C01, wild-type (WTP) or non-relevant phage (NRP) clones (1 x 10^{11} phages, each one), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured in culture supernatants by capture ELISA (A). Each bar represents the mean ± standard deviation (SD) from groups. The ratios between the IL-12/IL-10 and IL-12/IL-4 levels (B), as well as between the IFN-γ/IL-10 and IFN-γ/IL-4 levels (C); are showed. Also, the ratio between the levels of specific phage-IgG2a and IgG1 antibodies were calculated, and are also showed (D). (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0.001$).

Protective efficacy of the phage clones against L. amazonensis

The protective effect of immunization of BALB/c mice with the B10 and C01 clones against *L. amazonensis* infection was evaluated by measuring lesion development in

infected footpads, as well as by determination of the parasite burden in infected tissues and some organs of the animals (Fig. 2). Animals vaccinated with B10, C01, B10/C01 and B10/C01/saponin presented partial protection and significant reductions in edema in the infected footpads when compared with control groups (saline, WTP, and NRP groups). The reduction in the lesion development observed in these groups correlated with a 3 mm decrease in the infected footpad swellings, when compared to control groups (Fig. 2A). No significant differences were observed in lesion development among immunized groups with B10, C01, B10/C01 or B10/C01/saponin, corroborated by the diminished footpad swellings and parasite burden in the infected tissue in comparison to the control groups (Fig. 2B).

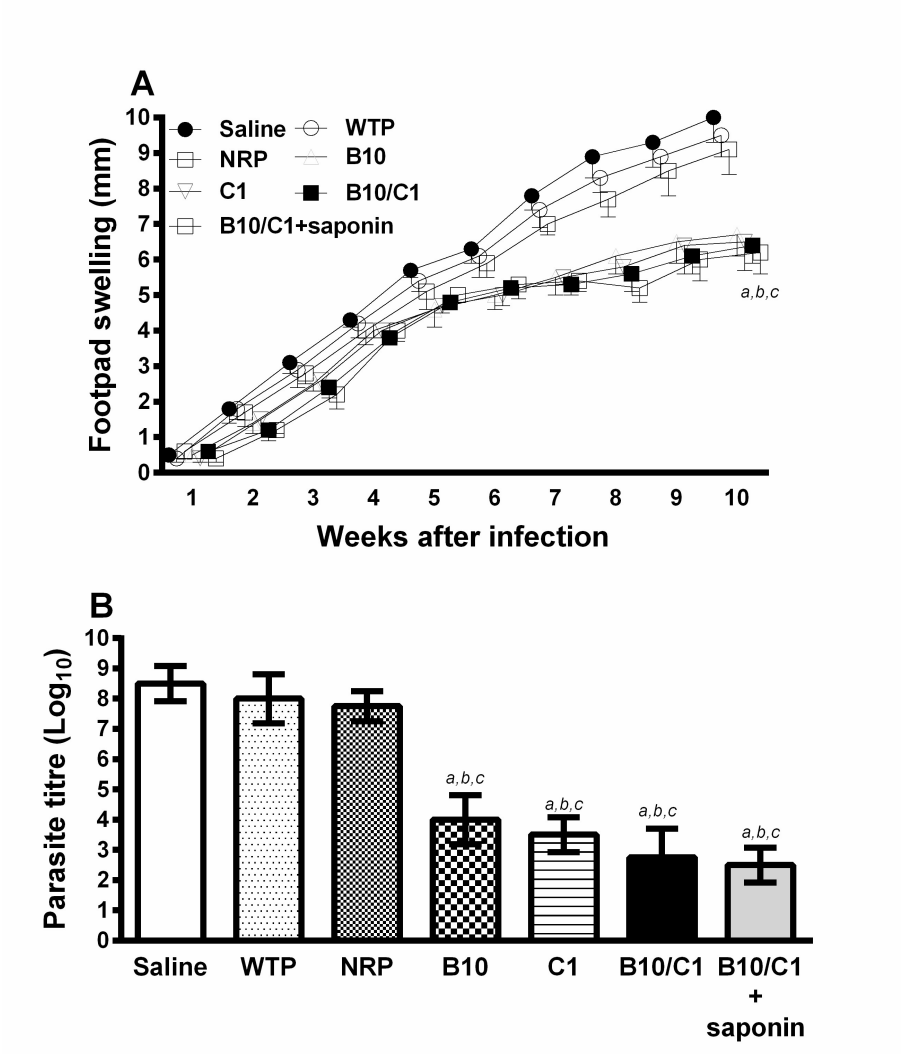

Figure 2. Protection of BALB/c mice vaccinated with B10 and C01 phage clones against *Leishmania amazonensis* infection. Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1 x 10^{11} phages, each one), or with the association between B10 and C01 clones (5 x 10^{10} phages, each), with or without 25 µg of saponin (*Quillaja saponaria* bark saponin; Sigma Aldrich). Three doses were

administered at 2-week intervals, and four weeks after the last immunization; animals were subcutaneously infected with 1 x 10^6 stationary-phase promastigotes of *L. amazonensis.* The course of the disease was monitored at weekly intervals and expressed as the increase in thickness of the infected footpad compared to the uninfected footpad (A). The number of parasites in the infected footpads was also measured (B), 10 weeks after challenge, by a limiting-dilution technique. Each bar represents the mean ± standard deviation (SD) from groups. Data shown are representative of two independent experiments, which presented similar results. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0.001$).

Also, vaccinated mice with B10 and/or C01 phage clones presented significant reductions in the number of parasites in the liver, spleen, dLN, and BM in comparison to controls (saline, WTP or NRP) (Fig. 3). Specifically, vaccinated mice with B10, C01, B10/C01 or B10/C01/saponin compared to the saline group showed significant reductions in the parasite load in the liver (1.9-, 2.1-, 3.0, and 3.8-log reductions, respectively; Fig. 3A), spleen (2.0-, 2.3-, 3.0-, and 3.5-log reductions, respectively; Fig. 3B), dLN (2.5-, 2.7-, 4.5-, and 5.4-log reductions; respectively; Fig. 3C), and BM (2.0-, 2.4-, 3.0-, and 4.0-log reductions, respectively; Fig. 3D). In the same context, immunized mice with B10, C01, B10/C01 and B10/C01/saponin compared to the NRP clone (non-relevant mimotope control) presented significant reductions in the parasite load in the liver (1.8-, 2.3-, 2.8-, and 3.5-log reductions, respectively; Fig. 3A), spleen (1.9-, 2.4-, 2.7-, and 3.2-log reductions, respectively; Fig. 3B), dLN (2.3-, 2.5-, 2.8-, and 3.1-log reductions; respectively; Fig. 3C), and BM (1.7-, 1.7-, 2.5-, and 3.3-log reductions, respectively; Fig. 3D). The analysis of the protective efficacy of individual clones against infection demonstrated that the C01 clone presented greater protection, although no significant difference has been observed between clones. On the other hand, the combination of B10 and C01 clones improved the protection against infection in comparison to individual clones. Also, the use of saponin as adjuvant to the B10/C01 vaccine did not induce any significant alteration in the protection, when compared to the group without adjuvant.

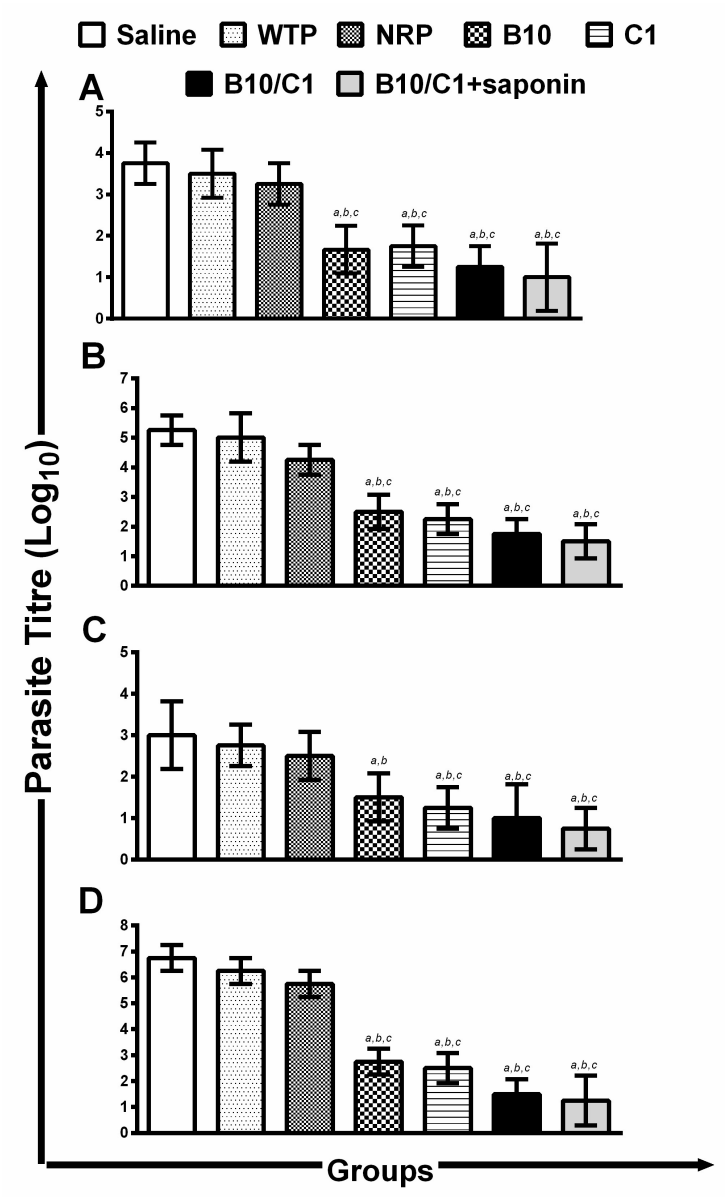

Figure 3. Parasite burden in different organs of the immunized animals with B10 and C01 phage clones, and challenged with *Leishmania amazonensis.* Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1 x 10^{11} phages, each one), or with the association between B10 and C01 clones (5 x 10^{10} phages, each), with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma Aldrich). After the immunization schedules, animals were infected with 1 x 10^{6} stationary-phase promastigotes of *L. amazonensis*, and 10 weeks after challenge, the parasite burden was determinate in the liver (A), spleen (B), bone marrow (C) and infected paws′ draining lymph nodes (D), by a limiting-dilution technique. Each bar represents the mean ± standard deviation (SD) from groups. Data shown are representative of two independent experiments, which presented similar results. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$).

(*b*) indicates statistically significant difference in relation to the WTP group ($P < 0.001$).
(*c*) indicates statistically significant difference in relation to the NRP group ($P < 0.001$).

Cellular response elicited after L. amazonensis infection

The production of cytokines in the supernatants of spleen cell cultures stimulated with specific phages or SLA *L. amazonensis*, 10 weeks after infection, was analyzed in order to evaluate the immunological correlates of protection induced by previous immunization (Fig. 4). The spleen cells derived from mice vaccinated with B10, C01, B10/C01 or B10/C01/saponin produced higher levels of phages- and SLA-specific IFN-γ, IL-12, and GM-CSF than did those secreted by spleen cells from control groups (saline, WTP and NRP groups). In contrast, the phages- and SLA-driven production of IL-4 and IL-10 showed that vaccination with both clones induced no significant production of these cytokines in the vaccinated and infected animals (Fig. 4A). The ratios between the IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 4B), as well as between the IL-12/IL-4 and IL-12/IL-10 levels (Fig. 4C) obtained after the SLA *L. amazonensis* stimulus were calculated, and showed that vaccinated and infected animals mounted a typical Th1 immune response against parasites, which has possibly contributed to the protection observed against the challenge.

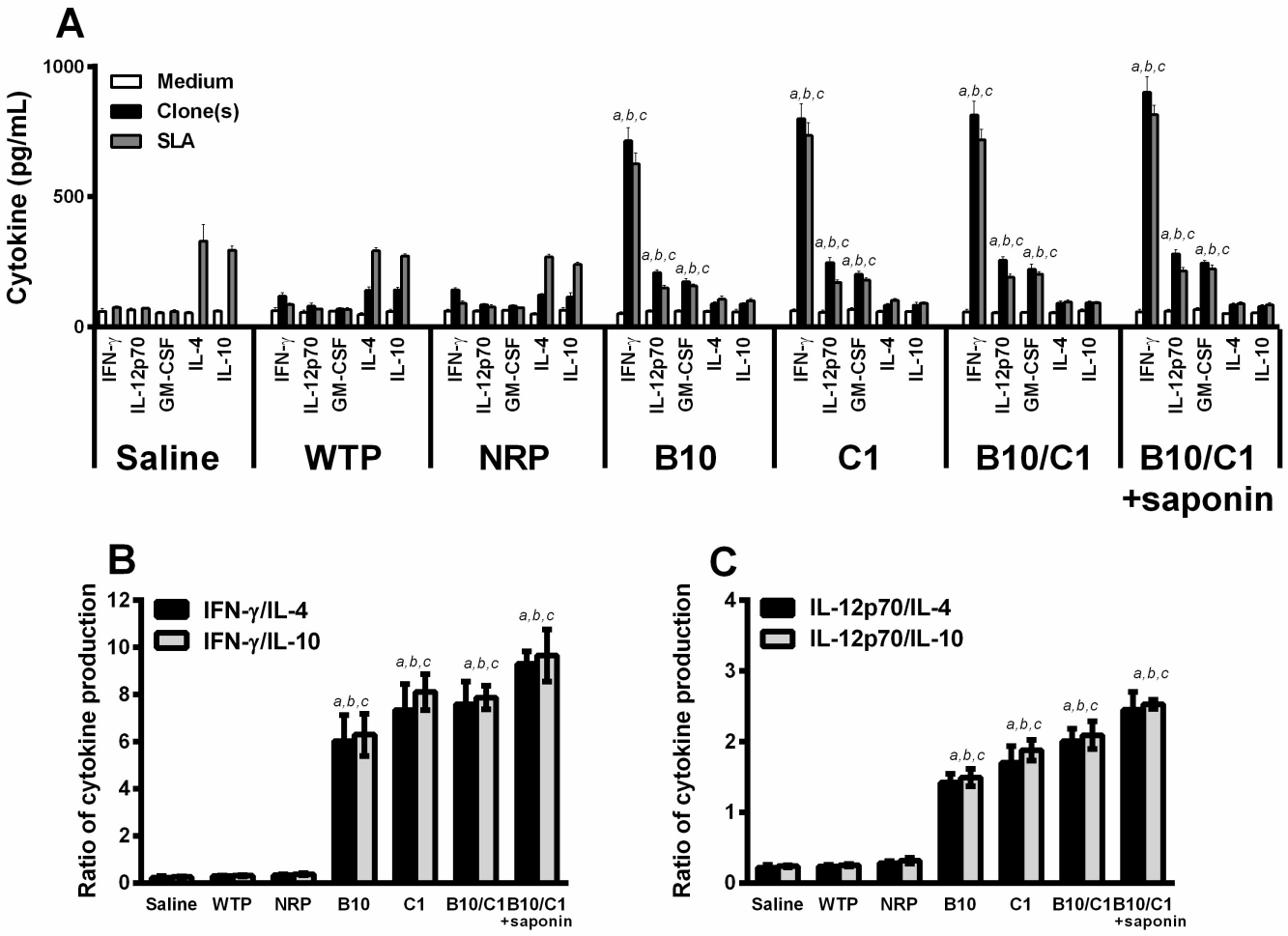

Figure 4. Analysis of the cellular response in BALB/c mice after *Leishmania amazonensis* infection. Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1 x 10^{11} phages, each one), or with the association between B10 and C01 clones (5 x 10^{10} phages, each), with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma Aldrich). Four weeks after the last immunization, animals were subcutaneously infected with 1 x 10^6 stationary-phase promastigotes of *L. amazonensis*, and they were monitored until 10 weeks after challenge. Then single-cells suspensions were obtained from the spleens of animals, and they were non-stimulated (negative control), or separately stimulated with SLA *L. amazonensis* (25 μg mL^{-1}), or with the B10, C01, WTP or NRP clones (1 x 10^{11} phages, each one); for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in the culture supernatants by capture ELISA. Each bar represents the mean ± standard deviation (SD) from groups (A). The ratios between the IFN-γ/IL-10 and IFN-γ/IL-4 levels (B), as well as between the IL-12/IL-10 and IL-12/IL-4 levels (C); are showed. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the WTP

group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0.001$).

The involvement of $CD4^+$ and $CD8^+$ T cells, as well as the dependence of IL-12 production for the SLA *L. amazonensis*-specific IFN-γ response from the spleen cells of mice immunized with B10/C01 or B10/C01/saponin, and lately infected with *L. amazonensis*, was evaluated (Fig. 5). The IFN-γ production was significantly suppressed using the anti-$CD8^+$ monoclonal antibody in the spleen cell cultures in both B10/C01 (Fig. 5A) and B10/C01/saponin (Fig. 5B) groups. On the other hand, the addition of anti-$CD4^+$ or anti-IL-12 antibodies to the cultures also decreased the production of this cytokine when compared to the control cells culture without treatment; however, this production proved to be greater than that occurred by use of anti-$CD8^+$ monoclonal antibody (Fig. 5).

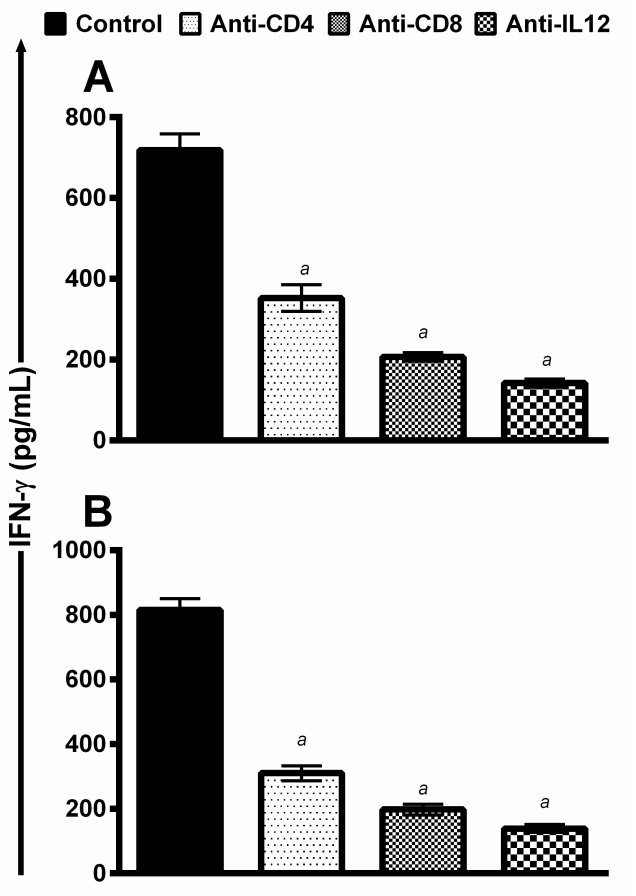

Figure 5. Involvement of $CD4^+$ and $CD8^+$ T cells, as well as IL-12, in the IFN-γ production after challenge infection. Single-cells suspensions were obtained from the spleens of mice that were immunized with B10/C01 (A) or B10/C01/saponin (B), at 10 weeks after infection. Levels of IFN-γ were measured in the culture supernatants by

capture ELISA of spleen cells cultures stimulated with SLA *L. amazonensis* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. Cultures were incubated in the absence (positive control) or in the presence of 5 μg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12 (C017.8), CD4 (GK 1.5), or mouse CD8 (53-6.7). (*a*) indicate statistically significant differences between non-treated control cells and cultures incubated with anti-CD4, anti-CD-8 or anti-IL-12 monoclonal antibodies ($P < 0.001$). Each bar represents the mean ± standard deviation (SD) of the IFN-γ levels from groups.

Humoral response and nitrite production after infection

Evaluating the humoral response induced after challenge, it was also possible to observe that mice vaccinated with B10, C01, B10/C01 or B10/C01/saponin and infected presented a significant predominance of SLA *L. amazonensis*-specific IgG2a antibodies, which was significantly higher than the observed SLA-specific IgG1 levels. The ratio between IgG2a and IgG1 levels (Fig. 6A) corroborated with the Th1 profile found in the vaccinated and protected animals.

In an attempt to evaluate the influence of the phage clones on the *L. amazonensis* specific killing effectors functions in the spleen of vaccinated and infected mice; the nitrite concentration was evaluated as an indicator of nitric oxide (NO) production in the spleen cell cultures, using either specific phage- or SLA-stimulus (Fig. 6B). The nitrite production was significantly higher in mice vaccinated with the B10, C01, B10/C01, and B10/C01/saponin after stimulation with phages or SLA, when compared to the control groups (saline, WTP and NRP groups).

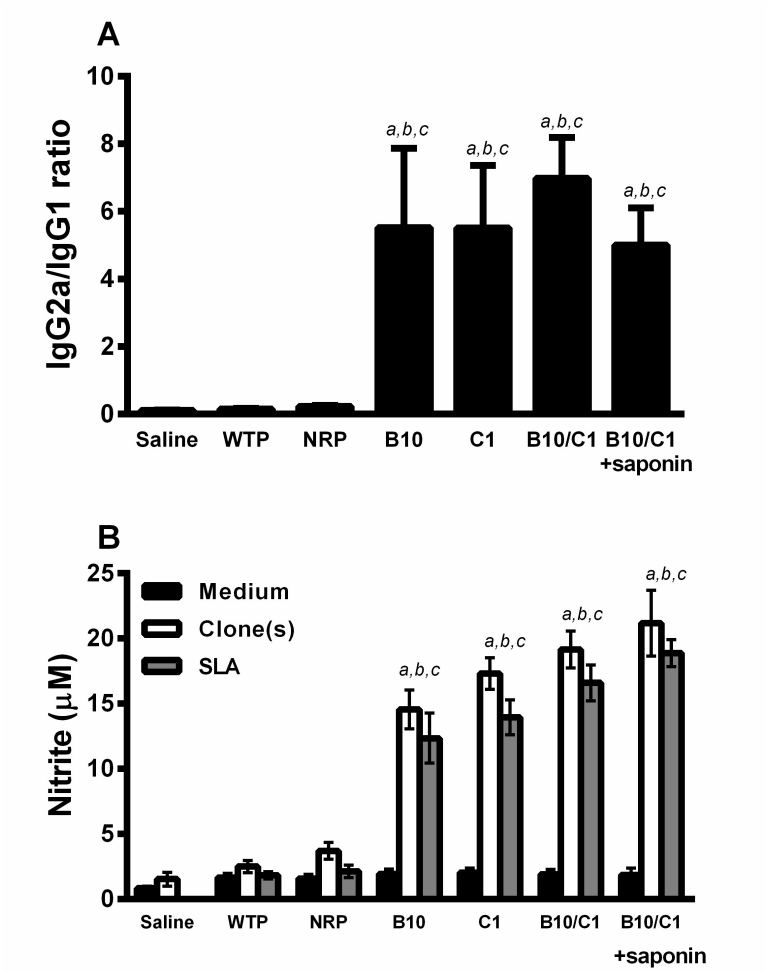

Figure 6. Humoral response and nitrite production in BALB/c mice after *L. amazonensis* infection. Sera samples of the immunized and infected animals were collected, at 10 weeks after infection. The levels of SLA *L. amazonensis*-specific IgG1 and IgG2a antibodies levels were determined, and a ratio between the values (IgG2a/IgG1) was calculated (**A**). The NO production was also evaluated in all groups, at 10 weeks after challenge; when single-cells suspensions were obtained from the spleens of animals, and they were non-stimulated (negative control), or separately stimulated with SLA *L. amazonensis* (25 μg mL^{-1}), or with the B10, C01, WTP or NRP clones (1 x 10^{11} phages, each one); for 48 h at 37°C in 5% CO_2. Then, 100 μL of the individual culture supernatants were mixed with an equal volume of Griess reagent. After an incubation of 30 min at room temperature, nitrite concentration was calculated using a standard curve of known concentrations (**B**). Each bar represents the mean ± standard deviation (SD) from groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0.001$).

5.10. Discussion

We have recently identified novel antigens based on a subtractive phage display strategy, represented by *L. infantum* mimotopes fused to the phage capsid; which were strongly reactive against positive sera from VL dogs, and their immunoprotective role was investigated. The subtraction against sera from non-infected animals, followed by a positive selection against asymptomatic and symptomatic VL dogs, led to the selection of two immunodominant antigens, represented by the phage clones B10 and C01, which were successfully employed in the protection of BALB/c mice against *L. infantum* infection (Costa *et al*. 2014). Due to their immunodominance and protective activity, we hypothesized that such immunogens could be cross-protective against other *Leishmania sp.* species, including those causing TL. Interestingly, besides proving that such mimotopes could protect against *L. amazonensis*, we also showed that a simple vaccine formulation is possible just by using the bacteriophage as immunogenic carrier, which was able to generate antibodies against the two recombinant peptides displayed at the amino terminus of phage coat proteins without the need of additional adjuvants.

Two experimental controls were used in this study: the wild-type phage (WTP) and a non-relevant peptide (NRP) fused to the phage capsid. The WTP is the same filamentous bacteriophage derived from the Ph.D.-C7C Phage Display Peptide Library Kit, without exogenous peptides fused to the pIII protein. The WTP was used to demonstrate the specific immune activation in the host, and how this could interfere in the target response. The NRC was used as a mimotope control, in order to verify if the protection induced by clones was due to the specific mimotopes. No protection was observed in both controls, confirming that protection induced by both B10 and C01 clones was due to the foreign antigens exposed in the selected phage particles.

Despite the fact that the science behind the treatment of infectious diseases through vaccination was first shown by Edward Jenner more than three centuries ago (Riedel, 2005), and that use of vaccine in treatment of diseases is widespread now, there are still problems and limitations in preparing vaccines by traditional means. This set the stage to explore other approaches for producing better vaccines (O´Hagan and Rappuoli, 2004). In this way, the phage display technology has proven to be useful in selecting antigens based on exposed foreign peptides on phages, either using their own clones or their synthetic mimotopes, such as Burkitt's lymphoma (Hardy and Raiter, 2005), melanoma (Wagner *et al*. 2005), colorectal cancer (Coomber *et al*. 2001), hepatitis B

virus (Wan *et al*. 2001), VL (Costa *et al*. 2014), and rotavirus (Van der Vaart *et al*. 2006).

Whole phage particles possess many intrinsic characteristics that make them ideal as vaccine delivery vehicles. Phage vaccines are cheap and can be easily produced on a large scale. Phages are highly stable and are even stable within the pH range 3.0 to 11.0, over a 24 hours period. Because the displayed protein is fused with a coat protein, it is not susceptible to nuclease degradation under the protective protein matrix (Gao *et al*. 2010). The safety issue of phage vaccine is an important matter and should be outlined. Phage vaccine has been shown to be safe in mice (Hashemi *et al*. 2010), pigs (Gamage *et al.* 2009), and human (Bruttin *et al*. 2005). Hence, phage itself acts as a strong adjuvant and thus enhances excellent immune response against any antigens present along with phage (Frenkel *et al.* 2000; Manoutcharian *et al*. 2004). Additionally, vaccination with phage particles also induces a highly immunogenic signal against phage coat protein, which provides an easily detectable marker to confirm the vaccination effect in animals (Margonari *et al*. 2006).

The B10 and C01 phages evaluated in the present study are expressed fused to pIII proteins of the phage´ structure. The minor coat protein pIII is presented in five copies per virion, of which all five can be fused to short peptides, without interfering in the molecule´ infectivity. In contrast, the major coat protein pVIII is presented as 2,700 copies per virion, of which 10% can be reliably fused to peptides. As a result, peptides expressed as pIII fusions are present at lower valency, whereas pVIII fusions are present at higher valency (Gu *et al*. 2008). Therefore, one could speculate that the protection found here could be improved by using a construction based on the hybrid phage fusion of B10 and C01 epitopes to the pVIII molecules from filamentous phages. In addition, the incorporation of other immunogenic mimotopes could be considered also as a strategy to develop a phage-displayed multi-epitope based vaccine protective to a wider range of species of *Leishmania spp*.

There is an emerging trend in vaccinology that focuses more on multi-strain or multi-species rather than strain-specific vaccines (Hamad, 2011). The concept of universal vaccines is chiefly based on the presence of common antigens among pathogens and on the ability of properly formulated vaccines to elicit cross-protective adaptive immunity. Support for antigenic commonality among pathogens derives from both indirect and direct evidence (Barbour and Restrepo, 2000; Kyes *et al*. 2007). In this sense, genomics is fast expanding the list of common proteins among organisms (Pizza *et al*. 2000; Kanduc *et al.* 2008; Real et al. 2013), and has proven to be useful in selecting common

antigens for vaccine development against diseases, such as brucellosis (Cherwonogrodzky *et al.* 2014), salmonellosis (Heithoff *et al*. 2015), and encephalitis (Lobigs and Diamond, 2012). Since multiple *Leishmania sp*. species are distributed in the same or adjacent geographical regions (Duthie *et al*. 2012), it would be desirable to develop vaccines containing candidates capable of inducing protection against the infection caused by different parasite species. Thus, to be effective as a human vaccine against leishmaniasis its components should be shared by different parasite species and, prior to its use in humans, the protective efficacy of these candidates should be analyzed in different models of experimental leishmaniasis (Coelho *et al*. 2003; Chávez-Fumagalli *et al*. 2010; Nico *et al*. 2014).

In the murine leishmaniasis, the development of an IL-4 or IL-10 mediated immune response inhibits the protective effects from the IFN-γ response, which may well be related to the deactivation of macrophages and the onset of the disease in the infected animals (Gumy *et al*. 2004). In studies evaluating vaccine candidates against leishmaniasis, immunogens are usually administered in mice and, after only a few weeks, they are infected and followed-up for a couple of months. In this time, spleen cells are collected and cultured *in vitro* with the antigens used in the immunization process, and/or with *Leishmania* extracts, in order to evaluate their immunogenicity. In this point, cytokines, such as IFN-γ and IL-12, markers of a Th1 response; and IL-4 and IL-10, indicators of a Th2 response, have their levels determined and, together with the results of the parasite burden, the efficacy of immunogens is evaluated (Martins *et al.* 2013). Thus, antigens capable of stimulate the development of a Th1 response, based on the production of high levels of IFN-γ and IL-12; could be considered a promising candidate for use against *Leishmania sp*. In the present study, the immunization using the B10 and/or C01 clones was able to induce a robust and phage-specific Th1 immune response in the immunized animals, which was primed by high levels of IFN-γ, IL-12, combined with the presence of low levels of IL-4 and IL-10. After infection, these animals, when compared to the controls, including the wild-type and non-relevant clones; displayed significant reductions in the parasite burden in the infected footpad, liver, spleen, BM and dLN, which was correlated with a higher production of *Leishmania*-specific IFN-γ by spleen cells. Additionally, spleen cells also produced higher levels of IL-12 and GM-CSF when compared to the control groups, and GM-CSF is the last cytokine related to macrophage activation and resistance in murine models against infection with some *Leishmania* species, such as *L. infantum* (Chávez-

Fumagalli *et al*. 2010; Costa *et al.* 2014), *L. major* (Dumas *et al.* 2003), and *L. donovani* (Murray *et al*. 1995). The NO production was also evaluated in the spleen cell cultures, and showed that the protected animals produced higher levels of NO when compared to the control groups, demonstrating the possible activation of these cells by a NO-dependent mechanism.

Evaluating the profile of T cells involved on the IFN-γ production in the protected animals, the $CD8^+$ T cells proved to be important source of IFN-□ in the vaccinated and infected animals. Depletion of these cells in cultures of spleen cells stimulated with SLA has significantly reduced this production. Although previous reports have shown that the activation of both $CD4^+$ and $CD8^+$ T cells subsets may be important for the killing of parasites in mice vaccinated with different parasite recombinant antigens (Pitcovsky *et al.* 2001; Cunha-Júnior *et al.* 2010; Martins *et al.* 2013), the present study's data suggest that $CD4^+$ T cells may contribute in a less extension to the induction of IFN-γ mediated response elicited by vaccination with B10 and C01 phage clones. The immunization using the isolated B10 and C01 phages induced protection against infection, but the association between clones was more effective in the cross-protection, which was based on a more polarized Th1 response, followed by significant reductions in the parasite load in the infected animals.

This study also demonstrates that the protection of mice against *L. amazonensis* was associated with a significant reduction of IL-4 and IL-10 levels. Very low levels of IL-4 and IL-10 were detected after the stimulation of spleen cells derived from vaccinated and infected mice. On the other hand, spleen cells from control mice showed a significantly higher production of these cytokines. In the evaluation of the humoral response, usually, in *L. amazonensis*-infected BALB/c mice, the IL-4-dependent production of IgG1 antibodies is associated with the progression and severity of disease; whereas IFN-γ is related to the production of IgG2a isotype, and with protection against infection (Coelho *et al*., 2003; Chávez-Fumagalli *et al*. 2010). Here, immunized mice with phage clones that were protected against infection presented higher levels of SLA-specific IgG2a antibodies, as compared to IgG1 levels; correlating with the development of a Th1 immune response observed in these animals. On the other hand, animals of the control groups; which were not protect against infection, showed high levels of *L. amazonensis*-specific IgG1 antibodies.

In conclusion, we have shown that a simple vaccine formulation based on phage-fused peptides can be used to protect mice against *Leishmania sp.* infection. We have successfully cross-protected against TL using two mimotopes derived from *L. infantum*.

The phage clones expressing the target mimotopes protected BALB/c mice against *L. amazonensis,* and this protection was correlated mainly by a $CD8^+$ T cells response primed by high levels of IFN-γ, IL-□□□ GM-CSF, and NO; as well as by low levels of IL-4, IL-10, and antileishmanial IgG1-isotype antibodies. The combination of phage clones was able to improve the cross-protective efficacy against *L. amazonensis*, and this result has led us to the conclusion that these antigens may well be used as vaccine candidates to control other *Leishmania sp*. infections. Studies are under way in order to identify the native proteins that express these target mimotopes.

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New serological tools for improved diagnosis of human tegumentary leishmaniasis

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6. MANUSCRITO 3

6.1. Full title

New serological tools for improved diagnosis of human tegumentary leishmaniasis

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6.5. Abstract

Human tegumentary leishmaniasis (HTL), characterized by skin ulcers that may spread and cause dreadful and massive tissue destruction of the nose and mouth, is considered a neglected tropical disease, and it is a serious threat to global health due to its continuous expansion, favored by the lifecycle of its causative organism that is maintained in domestic animal reservoirs and anthropophilic sand fly species. Serodiagnosis of HTL is a great challenge due to many biological factors, including hampered specificity and/or sensitivity. This investigation addresses the unmet need for new diagnostic markers of HTL, and describes a simple platform to improve the serodiagnosis. A constrained conformational phage display random peptide library combined with a magnetic microsphere-based subtraction strategy was used to identify ligands with potential diagnostic applications. Six clones were selected against IgG antibodies from HTL patients, characterized by sequencing and confirmed by a phage-ELISA using sera from patients developing visceral leishmaniasis (n=20), Chagas disease (n=10), mucosal (n=30) and cutaneous (n=20) leishmaniasis; as well as from healthy subjects living in endemic (n=20) and non-endemic (n=30) areas of leishmaniasis. A wild-type M13-phage clone and a soluble *Leishmania* antigenic extract were used as negative and positive controls, respectively. Three clones reached 100% sensitivity and specificity, without any cross-reactivity with sera from patients with leishmaniasis-related diseases. Briefly, we describe for the first time a set of serological

markers based on three immunodominant mimotopes that showed 100% accuracy, and that could be used in a phage-ELISA assay for the HTL serodiagnosis.

6.6. Introduction

The human tegumentary leishmaniasis (HTL), caused mostly by *Leishmania (Viannia) braziliensis* and *L. amazonensis* species, leads to a broad spectrum of clinical manifestations attributed to intraspecific variability of the parasites . The expansion of metropolitan areas associated with the parasite's lifecycle, maintained in domestic animal reservoirs and anthropophilic sand fly species , presents a significant threat to global health due to its expanding dissemination worldwide. Nearly 1.5 million cases of HTL have been registered annually , and the majority present single or few skin ulcers, but the extreme scarceness of parasites within lesions does not correlate with disease severity and resistance to antimonial therapy . Clinical manifestations of disease are classified as cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL) and mucosal leishmaniasis (ML) , but only 3 to 5% of infected patients will develop a severe disease (DCL or ML), which produces a destructive secondary mucosal lesion in the nose and mouth with extensive disfiguration and pain . Severe manifestation of CL is associated with a polarized Th2, when compared to a Th1 response found in mild manifestations of the disease . Therefore, the T-cell-mediated immune response plays a pivotal role either for cure or aggravation of the disease .

HTL diagnosis is currently performed by a combination of methods and clinical symptoms; therefore, there is an urgent need for more precise, simple and fast diagnosis approach . Currently, diagnosis relies on visualizing parasites in tissue or by culture, serology or detection of parasite DNA . The standard method for laboratory diagnosis of CL and ML is microscopy of Giemsa-stained or hematoxylin-eosin-stained scrapings, aspirate samples, or biopsy samples of skin ulcers or mucosal lesions , but it presents low sensitivity due to the scarceness of parasites within lesions . Another method used is the Montenegro skin test (MST), which measures a delayed-type hypersensitivity (DTH) reaction to an intradermal injection of leishmanial antigens ; however, it requires standardization, trained technician to assure consistent interpretation of results, quality and concentration of antigens injected, as well as it depends of the physiological status of the patients .

Besides those complicating factors, the MST still cannot distinguish between patients with acute symptoms from those cured or under treatment . DNA detection by PCR technique has been described for CL diagnosis, but is not available for patients' diagnostic testing and still requires extensive standardization . Serological tests are employed for HTL diagnosis, and are the most advantageous for being minimally invasive and easy to perform, but lack specificity since HTL-infected patients may present low antibody titers, and yet non-infected individuals living in endemic areas of disease can present false-positive results . Additionally, cross-reactions are also registered in patients infected by other Trypanosomatidae, such as *Trypanosoma cruzi* or *L. infantum* . The lack of accurate diagnosis is one of the most important factors that have led to the expansion of HTL infection, not only affecting global health, but also worsening poverty in low-income countries due to the greater morbidity. Therefore, improved HTL serodiagnosis is mandatory, and the identification of new antigens must aim the development of more sensitive and specific serological assays.

Currently, advanced proteomic strategies to obtain novel biomarkers have relied on subtractive selection cycles within recombinant libraries containing a very large repertoire of random peptides expressed in the capsid surface of filamentous bacteriophage, also called phage display. Peptide ligands are usually identified and affinity-enriched by cycles of bio-selection against a specific target, and selected peptide sequences that mimic epitopes (mimotopes) of native antigens are further characterized by DNA sequencing and validated by immunoassays . Phage display has successfully developed antigens for improved diagnosis of malaria , toxoplasmosis , hepatitis [28], neurocysticercosis [29], strongyloidiasis , Chagas' disease and canine VL [32].

Recently, our group have focused on the development of robust diagnostic platforms using highly specific and sensitive markers for visceral leishmaniasis (VL) selected by phage display [32,33]; but HTL serodiagnosis is still a significant challenge. In the present study, we have performed a stringent subtractive selection in a phage display library expressing constrained (cyclic) peptides, and the selection strategy was further improved by using a magnetic microsphere support and serum samples from CL and ML patients, which led us to the identification of six new mimotopes that were explored as novel diagnostic antigens for the HTL serodiagnosis using a very phage-ELISA immunoassay.

6.7. Materials and Methods

6.7.1. Sera samples

This study was conducted according to the Declaration of Helsinki principles, and it was approved by Ethics Committee from Federal University of Minas Gerais (protocol number CAAE–323431 14.9.0000.5149), Belo Horizonte, MG, Brazil. All patients received an individual copy of the study policy, which was reviewed by an independent person, and all participants gave their consent form in Portuguese, before collection of their blood sample. Serum samples of patients with confirmed diagnosis of HTL were classified as CL (n=20; including 14 males and 6 females, with ages ranging from 30 to 56 years) or ML (n=30; including 19 males and 11 females, with ages ranging from 22 to 63 years) and, in both cases, they were collected in an endemic area of leishmaniasis (Belo Horizonte, Minas Gerais, Brazil). The diagnosis was confirmed by clinical evaluation of lesions, which were compatible with either CL or ML, as well as by direct demonstration of the parasites in Giemsa-stained smears of biopsies of skin (CL) or mucosal fragments (ML) of patients. Analyses from two biopsy samples collected from each patient were submitted to a conventional PCR technique, and they presented positive results for *L. braziliensis* kDNA. None of the patients had been previously treated with anti-leishmanial drugs, before samples collection. Serum samples were also obtained from healthy individuals living in an endemic (n=20, including 12 males and 8 females, with ages ranging from 17 to 42 years, Belo Horizonte, MG, Brazil) or non-endemic (n=30, including 21 males and 9 females, with ages ranging from 15 to 48 years; Poços de Caldas, MG, Brazil) area of leishmaniasis, and they were used as negative controls. Healthy subjects were clinically evaluated and did not present any clinical signs or suspect of leishmaniasis. Serum samples from Chagas disease patients (n=10, including 7 males and 3 females, with ages ranging from 24 to 58 years) and from patients developing VL (n=20, including 13 males and 7 females, with ages ranging from 18 to 62 years) were used to test cross-reactivity. All sera were collected by venipuncture of medial vein in tubes without anticoagulant, and were kept at 37°C by 15 min, when they were centrifuged at 4,000 × *g* for 15 min, and samples were separated and kept at −80°C, until use.

6.7.2. Soluble antigenic extract

L. braziliensis (MHOM/BR/1975/M2904) strain was used. Stationary-phase promastigotes of *L. braziliensis* were grown at 24ºC in Schneider's medium (Sigma, St.

Louis, MO, USA), which was supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble antigenic extract (SLA) of the parasites was prepared as described .

6.7.3. Phage display selection strategy against IgG antibodies from patients and controls

To perform the affinity selection of phage libraries, IgG antibodies were purified from sera of healthy subjects, as well as from patients developing Chagas disease or HTL (CL and ML), and molecules were coupled to magnetic microspheres (beads) conjugated to protein G (Dynabeads, Invitrogen), as previously described . Briefly, 1 x 10^{10} microspheres were washed 3 times in 1 mL of 0.1 M MES buffer pH 5.0, and pools of sera were added to them and prepared individually (270 µL of a pool of sera of healthy subjects, 240 µL of a pool of sera of Chagas disease patients, 300 µL of a pool of sera of ML patients and 300 µL of a pool of sera of CL patients). Preparations of conjugates were brought to a final volume of 600 µL, submitted to an incubation (40 min) under constant agitation, at room temperature, and IgG/microspheres complexes were washed 3 times with 1 mL of 0.1 M MES buffer pH 5.0, to remove the non-conjugated antibodies. Next, the system was washed twice with 1 mL of 0.2 M triethanolamine buffer pH 8.2, and resuspended in 1 mL of covalent coupling buffer (20 mM dimethy pimelimidate/HCl diluted in triethanolamine buffer) for 30 min, under constant agitation at room temperature. The neutralization of unbound sites was made by incubating 1 mL of 50 mM Tris-base pH 7.5, for 15 min and at room temperature. The microspheres were washed 3 times with 1 mL TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20), blocked by addition of 2 mL of blocking solution (5% BSA diluted in TBS-T) for 1 h at 37°C, and resuspended in 200 µL of TBS (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) buffer. To carry out the negative selection, 1 x 10^{11} viral particles from a phage library containing random seven-peptides fused to a minor coat protein of M13 filamentous phages (Ph.D.®-C7C library, New England BioLabs, USA) were diluted in 210 µL of 0.1% TBS-T buffer. The mixture was incubated for 30 min at room temperature with the microspheres coupled to IgG antibodies that had been purified from healthy subjects, and then precipitated by magnetic capture through a Dynal Biotech support (12020, Invitrogen). The supernatant containing the clones that were not adhered to these antibodies was recovered and

transferred to a new tube, and the process was repeated twice. After, the supernatant was recovered and transferred to a new tube containing the microspheres coupled to IgGs from Chagas disease patients, and the procedure was repeated twice. Then, the supernatant containing the phage clones that did not bind to IgG antibodies were recovered and transferred to a new tube containing the microspheres coupled to purified antibodies from CL patients. A new incubation (30 min) was performed at room temperature, and the supernatant was removed. The remained bound phages to the IgG antibodies were washed 10 times with 1 mL of TBS-T and eluted in 500 µL of 0.2 M glycine buffer, pH 2.0. Next, 75 µL of 1 M Tris-base pH 9.0 was added to neutralize the acid pH. The recovered clones were transferred to a new tube and the process was repeated twice, when they were recovered and incubated with the microspheres adsorbed to antibodies from ML patients. The selected phages by these antibodies were recovered; titrated and 96 colonies were isolated. The pellet containing the DNA of each clone was washed in 70% ethanol, centrifuged, and diluted in 20 µL of ultra-pure water to be used in a 1% agarose gel, in order to verify the DNA quality.

6.7.4. DNA sequencing

The DNA from selected phage clones was used for sequencing and identification of target peptides. Sequencing was performed by capillary electrophoresis on ABI 3130 equipment, using BigDye v 3.1 and POP7 polymer (Myleus Biotechnology®, Belo Horizonte, Minas Gerais, Brazil). For the analysis of the generated AB1 files, the sequence scanner software (Applied Biosystems) was employed. The peptide sequences were deduced using the ExPASy server (www.expasy.org), and they were analyzed by BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi) against proteins and motifs of the GenBank database. In order to identify possible false-positive sequences, the Pepbank, MimoDB and SAROTUP programs were used. Further analyses of multiple sequence alignments were performed using the Clustal W2 server (www.ebi.ac.uk).

6.7.5. Validation of selected clones by phage-ELISA

To evaluate the antigenicity of selected phage clones, their immunoreactivity was tested in a phage-ELISA immunoassay. *L. braziliensis* SLA was used as a positive control, whereas the wild-type phage clone was used as a negative control. Flexible microtiter plates (Jet Biofil®, Belo Horizonte, Minas Gerais, Brasil) were coated with individual clones (1 x 10^8 phages per well), WTP clone (1 x 10^8 per well) or *L. braziliensis* SLA (2.0 µg per well), which were diluted in a 100 µL coating buffer (50

mM carbonate buffer) pH 9.6, for 16 h at 4°C. After, free binding sites were blocked using 300 µL of a blocking solution (consisting of TBS plus 0.05% Tween 20 and 5% non-fat dry milk solution), for 1 h at 37°C. Plates were washed three times with TBS-T, and incubated with 100 µL of each serum samples derived from healthy subjects, or those from patients developing TL, VL or Chagas disease (all 1:200 diluted), for 1 h at 37°C. Plates were washed five times with TBS-T and incubated with a 1:10,000 anti-human IgG peroxidase conjugated antibody (SAB3701282 catalog, Sigma-Aldrich, USA), for 1 h at 37ºC. After washing five times with TBS-T, the reaction was developed through incubation with H_2O_2, orto-phenylenediamine, and citrate-phosphate buffer pH 5.0, for 20 min in the dark. The reaction was stopped by adding 25 µL H_2SO_4 2 N, and optical density was measured in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm. Serum samples were evaluated in triplicate in the phage-ELISA immunoassays, and results of each sample were calculated from mean of each triplicate.

6.7.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.0 for Windows). The cut-off values for each antigen were determined by using a receiver operating characteristic (ROC) analysis, and they were chosen to obtain 100% of specificity. ROC curves were calculated and plotted with optical density (DO) values obtained from healthy subjects living in endemic or non-endemic areas of leishmaniasis versus those obtained either for TL, Chagas disease or VL patients. Then, to compare the diagnostic performance of each antigen, the sensitivity (CI 95%) and specificity (CI 95%) were analyzed. The accuracy was evaluated according to the area under the curve (AUC) relative to the ROC curves (CI 95%) and by the Youden index (J). *P* values < 0.05 were considered statistically significant.

6.8. Results

6.8.1. Identification of highly reactive mimotopes against HTL

In the present study, we have used a highly stringent subtractive phage display selection to identify short peptide motifs that would mimic specific epitope sequences of native antigens to diagnosis HTL. For the initial negative selection process, purified IgG antibodies of a pool of healthy subjects followed by IgG from Chagas disease patients were used to exclude the cross-reactive antigens. Then, a positive selection was

sequentially advanced with antibodies derived from CL and ML patients, in order to select common mimotopes that could recognize both disease forms. Ninety-six clones were isolated and individually evaluated by phage-ELISA using pools of sera representing healthy subjects from endemic and non-endemic areas of leishmaniasis (negative), and from HTL patients (positive). The ratio of the optical density (OD) values obtained between positive and negative pools compared with the positive control (*L. braziliensis* SLA) was used to select the most reactive clones, and those clones with ratios above 1.6 observed for SLA were sequenced. Six valid sequences were obtained and these clones were individually validated by a phage-ELISA using individual serum samples. The peptide sequences of the six selected clones are showed in Table 1. An alignment analysis among them showed no consensus motifs (data not shown).

6.8.2. Performance of antigenic markers for HTL serodiagnosis

Diagnostic performance for each selected phage clone was determined, in which we have included the serological panel of HTL patients, cross-reactive sera from Chagas disease and VL patients, as well as control sera of healthy individuals living in endemic or non-endemic areas of leishmaniasis. The reactivity of phages was analyzed in a simple and straightforward phage-ELISA immunoassays. The A10, B7, B10, C12 and H7 clones were specifically recognized by specific antibodies in sera of HTL patients, and no reactivity was observed with sera of healthy individuals living in a non-endemic or endemic area of leishmaniasis, leading to 100% sensitivity and specificity; whereas the C11 clone showed sensitivity and specificity values of 98% and 100%, respectively, when taken into account the endemic healthy controls. Additionally, highly accurate ROC curves (AUC=1.0), Youden index (1.0) and statistically significant values ($P < 0.0001$) were obtained for these phage clones. On the other hand, the WTP clone showed sensitivity values of 52% and 44% to be identified by sera derived from healthy subjects living in endemic or non-endemic areas of leishmaniasis, respectively.

In the next step, we have also analyzed the cross-reactivity of antigenic markers with serum samples of patients developing Chagas disease or VL. For cross-reactivity evaluation with sera from Chagas disease patients, the A10, B7, C12 and H7 clones showed sensitivity values of 100%, whereas B10 and C11 clones presented sensitivity values of 66% and 60%, respectively. The WTP and SLA showed sensitivity values of 44% and 60%, respectively (Table 2). Cross-reactivity with sera from VL patients did not occur for A10, C12 and H7 clones, which presented sensitivity values of 100%, whereas B7, B10 and C11 clones showed sensitivity values of 66%, 76% and 68%,

respectively. The WTP clone and *L. braziliensis* SLA presented sensitivity values of 22% and 46%, respectively (Table 2). In addition, the individual OD values for each serum sample are presented in reaction to each one of the six evaluated phage clones (Figure 1).

6.9. Discussion

The HTL, although still neglected, is becoming an emerging global disease due to its zoonotic parasite, which is maintained in domestic animals reservoirs and transmitted by anthropophilic sand fly vectors, and further expanded by co-infections with HIV. However, to prevent disease dissemination and evolution to severe forms, accurate HTL diagnosis is mandatory. But this has become one of the greatest challenges nowadays, due to the different pathogenetic backgrounds, complex interactions with vectors, cross-reactions with leishmaniasis-related diseases, and others. Therefore, in order to overcome such a quest, we have identified *Leishmania*-specific epitope-based antigens that can distinguish sera from CL and ML patients from other related diseases, as well as from healthy subjects, by using a simple phage-ELISA immunoassay.

Current HTL diagnosis has been based on clinical criteria and laboratorial procedures , but all of them present problems related to their sensitivity and/or specificity, requiring more than one procedure for confirmation. Even parasite DNA detection within lesions by the very specific PCR requires extensive standardization protocols, due to its variable sensitivity , especially considering the low number of parasites in lesions that leads to false-negative results . Immunological methods based on the identification of antileishmanial antibodies in patients´ sera present the greatest advantages among current tests for HTL diagnosis, mainly for being minimally invasive and easy to perform. However, problems with false-negative and false-positive results may occur as a result of the variable immune response to HTL, which is characterized by T-cell response with low antibody titers in circulation . Besides the low humoral response, it also presents cross-reactivity with sera from healthy subjects living in endemic areas of disease, as well as with antibodies from HTL-related diseases, such as Chagas´ disease, VL, malaria, and others .

However, in order to overcome such a challenge, we hypothesized that short epitope-based mimotopes would bring greater specificity and sensitivity for HTL serodiagnosis. Therefore, we have proposed a strategy based on phage display, which is

a subtractive proteomic technology that allows selection of short peptides expressed on the surface of filamentous phages against specific targets, resulting in peptides that mimic epitopes of native antigens . The elucidation of protein structures based on short peptide sequences are limited because most of the mimotopes selected against serum antibodies are conformational and present little resemblance to their native antigens , and linear or conformational alignments with a native epitope would be too speculative. In our study, we have not performed linear or 3D structural alignments with protein databases due to the lack of validity of such procedures, and because we have used a constrained peptide library with cyclic heptapeptides, with two cysteine residues at the ends to generate disulfide bridges (PhD-C7C), which already forces a structural conformation. Constrained peptides generally present a higher affinity and specificity and a lower conformational entropy, increasing the probability that they will retain their binding capacity when removed from the phage context .

In the present study, six phage clones were obtained from a highly stringent subtractive phage display selection, which was further improved by a magnetic capturing system using sera from cross-reactive diseases. Clones were individually evaluated against a human serological panel composed by sera from patients with VL and Chagas disease, as well as sera from healthy subjects living in endemic or non-endemic areas of leishmaniasis. A high diagnostic performance was obtained for three clones (A10, C12 and H7), which were able to discriminate TL patients from all controls and related diseases, showing 100% sensitivity and specificity, and remarkably without cross-reactions. The high performance observed was compatible with the diagnostic performance observed in recent studies using other antigens, such as recombinant proteins multiple-epitope chimeric antigens and mimotopes , but with the advantage to be a very simple diagnostic platform with new specific markers for HTL diagnosis without present false-positive results, even when tested against regular cross-reactive conditions, such as *T. cruzi*- or *L. infantum*-infected patients.

The sample size employed in the present study could be considered as a limiting factor, as well as the absence of an independent cohort performed using serum samples of other healthy controls or from patients developing Chagas disease, VL and other HTL-related pathologies. In addition, the absence of a serological follow-up of the treated patients in reaction to the phage clones, which has not been made in the current serological assays, could also be performed. Thus, the present study´ data should be taken as a proof-of-concept of the capacity of these antigens for the serodiagnosis of HTL, and would serve as reference for further serological assays.

Briefly, we have used a stringent phage display selection against IgG antibodies from HTL patients, which resulted in three novel antigens (based on phage clones) that could be produced from bacterial cultures. These three markers were directly applied in a simple serological phage-ELISA immunoassay, and to the best of our knowledge this is the first study describing a new diagnostic tool for the HTL serodiagnosis.

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Leishmania infantum mimotopes and a phage–ELISA assay as tools for a sensitive and specific serodiagnosis of human visceral leishmaniasis

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MANUSCRITO 4

7. MANUSCRITO 4

7.1. Title

Novel mimotopes from *Leishmania infantum* antigens applied for highly accurate serodiagnosis of human visceral Leishmaniasis

7.2. Authors

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7.5. Abstract

Abstract

Visceral leishmaniasis (VL) is a poverty-related zoonotic disease occurring in tropical and subtropical countries in the world. The gold standard diagnostic method of the disease is a parasitological test based on direct detection of amastigote forms in internal aspirates from patients, which is complex, invasive and presents low sensitivity. Serological methods are considered suitable, since they present good sensitivity and specificity, besides of the samples collection is considered less invasive than parasitological methods. Here, we describe a phage display selection strategy by employing sequentially IgG from healthy subjects, and patients developing Chagas disease or VL, aiming to select new bacteriophage-fused epitopes (mimotopes) that could be applied in the serodiagnosis of human VL. In the results, eight phage clones were identified after three bio-selection rounds, and their reactivity was tested by a phage-ELISA, by using sera from VL (n=30), Chagas disease (n=15), mucosal (n=34) and cutaneous (n=10) leishmaniasis patients, as well as sera from healthy subjects living in endemic (n=27) or non-endemic (n=30) areas of leishmaniasis. A wild-type phage clone was used as an antigen control, whereas the recombinant K39 (rK39)-based immunochromatographic test was employed to compare the sensitivity of the evaluated phages. In the results, all clones reached highly accurate diagnosis without significant cross-reactivity. In conclusion, we describe new mimotopes of *Leishmania infantum* antigens that presented 100% sensitivity and specificity, and that could be evaluated as a simple assay for serodiagnosis of human VL.

Keywords: Visceral leishmaniasis; serodiagnosis; mimotopes; phage display; phage-ELISA; specificity.

7.6. Introduction

Introduction

Visceral leishmaniasis (VL) is a parasitic disease occurring in tropical and subtropical countries in the world [1,2]. The outcome of infection varies from an asymptomatic and/or subclinical disease to an acute symptomatic form. Asymptomatic VL is characterized by subjects infected by parasites, but without present apparent impact on their health. On the other hand, in the symptomatic VL, clinical manifestations such as lymphadenopathy, fever, diarrhea, malaise, hepatomegaly and splenomegaly are observed in the patients [3,4]. Chemotherapy to treat VL has been based on the parenteral administration of pentavalent antimonials [3]; however, these products can cause side effects such as anorexia, myalgia, fever, urticaria and arthralgia; besides of toxicity in the liver, spleen and kidneys of the treated patients [5,6].

In this context, an early diagnosis of VL could permits to a faster and effective treatment, with increased possibility of cure of the patients. Unfortunately, the parasitological diagnosis based on direct observation of amastigote forms, besides of its poor sensitivity, requires invasive procedures for samples collection, which limits their use [7,8]. On the other hand, the detection of *Leishmania* DNA by polymerase chain reaction (PCR) technique presents high specificity, but the sensitivity remains also variable [9], probably due to samples processing and the requirement of specialized equipment and labor. In this context, serology could be considered as an alternative diagnostic tool for detection of antileishmanial antibodies in the sera of the patients, since it is simpler and cheaper to be performed, besides of the fact that the samples collection is considered less invasive than the aspirates necessary to the parasitological exams [7].

Recombinant antigens have been evaluated for the serodiagnosis of human VL and the results have been considered satisfactory, when compared to those using crude, soluble or semi-purified extracts of the parasites. Among the most promising antigens, the recombinant K39 (rK39) protein has showed good results for the serodiagnosis of disease [10]. A commercial kit, Kalazar Detect™ Test (InBios International, Inc., Seattle, Wash, USA), was developed and is considered a rapid and non-invasive immunochromatographic strip assay for the qualitative detection of rK39-specific antibodies to members of the *L. donovani* complex in human serum. It is a qualitative and considered to be highly sensitive membrane-based immunoassay employed for the serodiagnosis of human VL; however, it presents problems related to cross-reactivity

with sera from patients with other VL-related diseases, including false-positive results found to healthy subjects living in endemic areas of disease [11-14]. Thus, the search for new antigenic markers could contribute to solve these serological problems.

Efforts to identify novel diagnostic antigens have recently relied on the phage display technology, which is a subtractive proteomic approach that aims to select random peptides (mimotopes) expressed at the outer surface of phage clones [15]. Bacteriophage-fused peptide libraries are submitted to several selection cycles, and selected peptide clones are further characterized and identified by DNA sequencing [16-18]. Phage display generates short peptides that mimic epitopes with antigenic and immunogenic potential for diagnostic and vaccine applications against parasitic diseases, such as malaria [19-21], toxoplasmosis [22,23], canine VL [24-26], Chagas disease [27], neurocysticercosis [28,29], strongyloidiasis [30], and others.

In the present study, a highly stringent subtractive phage display selection was employed aiming to identify new mimotopes that could be applied as antigens for the serodiagnosis of human VL. Besides the unique subtractive selection procedure, the selected mimotopes proved to be highly relevant for serological analysis through a phage-ELISA assay. This study shows novel diagnostic markers applied in a simple assay with high sensitivity and specificity, and are presented as alternative screening tool for the serodiagnosis of human VL.

7.7. Materials and Methods

Material and Methods

Ethics Statement

The present study was approved by Ethics Committee from Federal University of Minas Gerais (COEP/UFMG, protocol CAAE–32343114.9.0000.5149), Belo Horizonte, Minas Gerais, Brazil. In addition, this study was conducted according to the Declaration of Helsinki principles, and all patients received an individual copy of the study policy, which was reviewed by an independent person. Also, a written informed consent was obtained from all patients.

Parasites

Leishmania infantum (MHOM/BR/1970/BH46) strain was used. The stationary-phase promastigotes of the parasites were grown at 24ºC in complete Schneider's medium (Sigma, St. Louis, MO, USA), which was composed by Schneider´s medium

supplemented with 20% 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 *L. infantum* antigenic extract (SLA) was prepared as described [31], and the protein concentration was estimated by the Bradford method [32].

Serum samples

Serum samples from patients with symptomatic VL (n=30, including 17 males and 13 females, with ages ranging from 22 to 58 years) were used. Infection was confirmed by PCR technique targeting *L. infantum* kDNA in aspirates from spleen and/or bone marrow of the patients. None of them have been previously treated with antileishmanial drugs prior to collection of serum samples. All VL patients were selected from an endemic area of disease (Belo Horizonte, Minas Gerais, Brazil). The control group consisted of healthy individuals living in an endemic (Belo Horizonte; n=27, including 15 males and 12 females, with ages ranging from 17 to 42 years) or non-endemic (Poços de Caldas, Minas Gerais, Brazil; n=30, including 18 males and 12 females, with ages ranging from 19 to 48 years) area of VL, and none of them presented clinical signs or suspicious of leishmaniasis. Serum samples from patients with Chagas disease (n=15, including 9 males and 6 females, with ages ranging from 27 to 55 years) were used to test cross-reactivity. The infection was confirmed by hemoculture or by both the Chagatest recombinant ELISA v. 3.0 kit (Wiener Lab, Argentina) and Chagatest hemagglutination inhibition (HAI) assay (Wiener Lab). Sera from tegumentary leishmaniasis (TL) patients developing either mucosal (ML; n=34, including 23 males and 11 females, with ages ranging from 22 to 62 years) or cutaneous (CL; n=10, including 6 males and 4 females, with ages ranging from 18 to 47 years) leishmaniasis were also employed as cross-reactive diseases. The diagnosis was confirmed when parasites were visualized in the Giemsa-stained smears obtained from mucosal fragments or skin biopsies, respectively. Positivity for CL and ML samples was also confirmed by the presence of *L. braziliensis* kDNA through PCR, and by an ELISA assay using *L. braziliensis* SLA as an antigen (data not shown).

Purification of the IgG antibodies

Purification of IgG antibodies was performed by coupling them onto magnetic microspheres (magnetic beads) conjugated to protein G (Dynabeads, Invitrogen), as previously described [24], with some modifications. Briefly, 2 x 10^{10} particles of microspheres were washed three times in 1 mL of 0.1 M MES buffer [2-(*N*-

morpholino)ethanesulfonic acid], at pH 5.0; and 350 µL of pools of sera from either healthy subjects, patients developing Chagas disease or VL were added and submitted to a 40-min incubation at room temperature under constant agitation. IgG-coupled microspheres were washed four times in 1 mL of 0.1 M MES buffer to remove non-adhered antibodies. The beads-antibody complex was washed three times in 1 mL of 0.2 M triethanolamine buffer pH 8.0, and resuspended in 1 mL of covalent coupling buffer (20 mM dimethy pimelimidate/HCl diluted in triethanolamine buffer) for 30 min at room temperature under constant agitation. The neutralization of unbound reactive sites was performed with 1 mL of 50 mM Tris-base pH 7.5 for 15 min at room temperature. Microspheres were washed twice in 1 mL of TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) buffer, blocked with a 2-mL blocking solution (5% BSA diluted in TBS-T) for 1 h at 37°C, and resuspended in 200 µL of TBS (50 mM Tris-HCl pH 7.5 and 150 mM NaCl) buffer. To verify the coupling process, 5 µL of IgG-coupled beads coupled were incubated with an anti-human IgG peroxidase antibody for 1 h at 37°C (1:10,000 dilution), washed twice with 1 mL of TBS-T, and developed with the TMB substrate. After 30 min, the reaction was stopped by adding 25 µL H_2SO_4 2 N and the optical density was obtained in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 450 nm.

Phage display selection through magnetic capture

To carry out the subtractive selection process of phage display technology, 2 x 10^{11} viral particles from a phage display library containing randomly constrained peptides fused to the pIII coat protein of M13 filamentous phages (Ph.D.®-C7C library, New England BioLabs, USA) were diluted in 190 µL of TBS-T buffer. The mixture was incubated for 30 min at room temperature with the microspheres coupled to the IgG antibodies from healthy subjects, which were magnetically captured (Dynal Biotech support, 12020). The clones-containing supernatant was recovered and transferred to a new tube, and this procedure was repeated twice for subtraction purposes. A second negative selection was performed using IgG-coupled magnetic beads from Chagas disease patients, and the unbound clones present in the supernatant were recovered and transferred to a new tube, when the procedure was repeated twice. For the positive selection, the supernatant-recovered phages were submitted to a selection using IgG-coupled microspheres derived from VL patients, which were incubated for 30 min at room temperature. The supernatant was removed and the bound phages were washed five times in 1 mL of

TBS-T buffer and eluted in 500 µL of 0.2 M glycine buffer, pH 2.0. Next, 75 µL of 1 M Tris-base pH 9.0 were added to neutralize the acidic pH. Subsequently, the recovered clones were transferred to a new tube and the procedure was repeated twice, when phages were recovered and titrated. For this, phage clones obtained in the 3rd bio-selection cycle were diluted from 10^{-1} to 10^{-12} in 500 µL of sterile TBS, mixed with the *E. coli* strain ER3728 (New England BioLabs, USA), grown in liquid medium until reaching the OD_{600nm} of 0.5, and plated on LB agar plates containing 1 mL IPTG/X-gal (1.25 g IPTG, 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 25 mL DMSO). Titration was performed by counting the number of colonies in each plate.

DNA extraction and sequencing of selected phage clones

Ninety-six colonies were individually picked and amplified in 250 µL of LB medium in a sterile culture microplate (BD Falcon TM clear, 96-well microtest TM plate), which was sealed and incubated for 6 h at 37ºC under constant agitation. After incubation, the plate was centrifuged for 20 min by 2,500 x *g*, and the supernatant was transferred to a new plate, in which a PEG/NaCl (20% PEG 8,000 and 2.5 M NaCl) solution was added (1/6 of the total volume). Then, the plate was incubated for 18 h at 4ºC and centrifuged for 1 h. The supernatant was removed and the pellet was resuspended in 500 µL of TES (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 4 M NaI) buffer. The plate was vigorously shaken for 5 min, and 300 µL of a 70% ethanol solution was added. The reaction was incubated for 10 min, and DNA was precipitated by centrifugation in 2,500 x *g* for 10 min, at 4ºC. After discarding the supernatant, the DNA pellet of each clone was washed in 500 µL of 70% ethanol, dried and diluted in 20 µL of ultra-pure water. DNA purity was evaluated in a 1% agarose gel stained with an ethidium bromide (10 µg/mL). Sequencing was performed by capillary electrophoresis on ABI 3130 equipment, using BigDye v 3.1 and POP7 polymer (Myleus Biotechnology®, Belo Horizonte, Minas Gerais, Brazil). For analyses of AB1 output files, the sequence scanner software (Applied Biosystems) was employed.

Bioinformatics

DNA sequences were analyzed by bioinformatics using online servers. The constrained PhD library used for peptide selection presents conformational peptides with cysteines in the borders, therefore, peptide analyses were performed with the 7-mer peptide sequence and the amino acid sequences (ACxxxxxxxCGGGS) contained in the fusion with the pIII bacteriophage capsid protein, as previously described [33]. Amino acid

sequences were deduced based on the nucleotide sequences through the Expasy (http://web.expasy.org/translate/) program and they were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against proteins and motifs of the GenBank database. The putative protein function was predicted by BLAST search alignment in the UNIPROT server (http://www.uniprot.org/). Physicochemical properties of the peptides, such as molecular weight and isoelectric point, were predicted using the Compute pI/Mw tool at the Expasy Server (http://web.expasy.org/compute_pi/). The hydrophobicity and net charge of the peptides were predicted using the Antimicrobial peptide database Server (http://aps.unmc.edu/AP/prediction/prediction_main.php), as described [34].

Phage-ELISA screening

The 96 selected phage clones isolated from the 3th positive bio-selection cycle were pre-validated by a phage-ELISA assay, and comparisons were performed with *L. infantum* SLA. For the assays, 1 x 10^9 phages or 1 µg per well of SLA were incubated with pools of sera (1:200 diluted in TBS buffer) from VL patients or of healthy subjects living in a non-endemic area of disease. The same pools of sera used for IgG purification were also used for the phage-ELISA assay. An anti-human IgG peroxidase conjugated antibody (1:10,000 diluted in TBS buffer; SAB3701282 catalog, Sigma-Aldrich, USA) was used as a secondary antibody, and the reaction was developed after a 30-min incubation with H_2O_2, orto-phenylenediamine and citrate-phosphate buffer, pH 5.0. The reaction was stopped by adding H_2SO_4 2 N and individual OD values for serum samples were obtained in an ELISA microplate spectrophotometer, at 492 nm.

Phage-ELISA for the serodiagnosis of human VL

The selected phage clones were fully tested for the serodiagnosis of human VL in a new phage-ELISA test using the individual sera. For this, titration curves were previously performed to determine the most appropriate concentration of the antigen and antibody dilutions to be used. Flexible microtiter plates (Jet Biofil) were coated with the individual clones that were pre-validated with sera pools (1 x 10^8 phages per well), which were diluted in a 100 µL coating buffer (50 mM carbonate buffer) pH 9.6, for 16 h at 4°C. A wild-type M13 phage clone (WTP, 1 x 10^9 phages per well) was used as a negative control, whereas *L. infantum* SLA (1.0 µg per well) was used as a positive control. After sensitizing the plates with antigens, free binding sites were blocked using 250 µL of a blocking solution (5% non-fat dry milk diluted in TBS-T), for 2 h at 37°C.

After washing plates five times with TBS-T, they were incubated with 100 µL of human sera (1:200 diluted in TBS), for 1 h at 37°C. Then, plates were washed five times with TBS-T and incubated with an anti-human IgGs peroxidase conjugated antibody (1:10,000 diluted in TBS; Sigma-Aldrich), for 1 h at 37ºC. After washing plates five times with TBS-T, the reaction was developed after a 30-min incubation with H_2O_2, orto-phenylenediamine, and citrate-phosphate buffer pH 5.0, in the dark. The reaction was stopped by adding 25 µL of H_2SO_4 2 N, and the OD was obtained in an ELISA microplate spectrophotometer, at 492 nm.

Kalazar Detect™ Rapid Test

The Kalazar Detect™ Test for human VL is an immunochromatographic method used to detect rK39-specific antibodies in human serum. The tested membrane is pre-coated with rK39 protein on the test line region and chicken anti-protein A on the control line region. During testing, the serum sample reacts with the dye conjugate (protein A-colloidal gold conjugate), which has been pre-coated in the test device. The immune complex migrates upward on the membrane by capillary action and reacts with the rK39 antigen on the membrane generating a red line. The presence of this red line indicates a positive result, while its absence indicates a negative result. The technical protocol was developed following the manufacturer's instructions (InBios International, USA).

Statistical analysis

Serum samples were evaluated in duplicate by phage-ELISA and results were analyzed using GraphPad Prism™ (version 6.0 for Windows). The cut-off values for each antigen were determined by using receiver operating characteristic (ROC) analysis, and maximum sensitivity was calculated by establishing a specificity of 100% for all antigens. Contingency tables and the Fisher's exact test ($P < 0.05$) were used to compare the diagnostic performance of the antigens. Sensitivity and specificity (with 95% confidence interval) were estimated, and the accuracy was evaluated according to the area under the curve (AUC) relative to the ROC curves (CI 95%) and by Youden index (J). Agreement beyond chance was assessed using the kappa (k) coefficient (CI 95%) and interpreted according to the following scale: 0.00 to 0.20 (negligible), 0.21 to 0.40 (weak), 0.41 to 0.60 (moderate), 0.61 to 0.80 (good), and 0.81 to 1.00 (excellent).

7.8. Results

Affinity selection, ELISA screening and protein target identification

A highly stringent phage display selection process was performed using pools of sera from healthy subjects and from patients developing Chagas disease or VL. A negative selection was performed to exclude the phages that were cross-reactive with antibodies in sera derived from healthy individuals or from Chagas disease´ patients, whereas the positive selection selected the phage clones that were reactive with sera from VL patients. After three rounds of positive bio-selection, 96 clones were isolated and evaluated in a phage-ELISA, and only those that were highly reactive with sera from VL patients and with low or no reactivity with sera from healthy subjects, were kept for further evaluation. In addition, the ELISA results were comparable with those obtained using *L. infantum* SLA. Individual OD values for each clone were used to calculate the ratio between the positive (VL patients) and negative (healthy subjects) samples, and results higher than those obtained for SLA (1.9) were selected for additional serological analyses. Forty-two clones were selected, DNA sequenced, and their target peptides were identified. Eight phage clones were precisely identified (A3, A5, A8, A11, B2, B9, H11 and G12) and their sequences are shown (Table 1). An alignment analysis was performed and a consensus motif was determined among selected peptide sequences (Table 2). A BLAST analysis for the selected peptides and consensus motifs matched with putative parasite proteins, such as a hypothetical protein (XP_001469726.1), ABC-thiol transporter (XP_001465706.1) and calpain-like cysteine peptidase (XP_001468417.1); and most of them are structurally related with membrane proteins of the parasite (Table 3). The analysis of individual peptides and the highest homology to targets proteins is also presented in a supplementary table. An additional analysis of peptide sequences was performed and their net charge, hydrophobicity and estimated molecular weight are shown (Table 4). Then, the selected phage clones expressing the antigenic peptides were individually evaluated in new serological analyses for the serodiagnosis of human VL.

Diagnostic performance of selected clones

The individual diagnostic performance of the eight selected phage clones was investigated by testing their reactivity in a simple phage-ELISA assay using a panel of human serum samples. Additionally, a wild-type phage clone without fused peptides was used as negative control, whereas the *L. infantum* SLA was used as a positive control. The purpose of this analysis was to identify the clones that correctly recognized

sera from VL patients, without presenting cross-reactivity with antibodies from healthy subjects living in an endemic area of leishmaniasis. All eight clones specifically differentiated VL patients from those healthy subjects, thus avoiding false-positive results in this important population (Table 5). A small exception was observed for the B2 clone that recognized one serum outlier among healthy subjects living in an endemic area of VL. Further investigation is required for this specific clone to demonstrate if this was a false-positive result or if the healthy subjected has a sub-clinical infection. Despite this unique outlier, all phage clones showed highly accurate ROC curves (AUC=1.0), kappa index (1.000) and statistically significant values ($P < 0.0001$), when analyzed by Fisher's exact test. ROC curves for all antigens were constructed by fixing the specificity in 100%, and sensitivity values for all clones reached 100%, whereas the WTP clone and *L. infantum* SLA presented values of 22% and 55%, respectively.

When a comparison of clones' efficacy was performed to serologically differentiate VL patients from those with Chagas disease or TL, as well as from those healthy subjects living in a non-endemic area of VL; all eight clones showed 100% sensitivity. The WTP clone presented sensitivity values of 40%, 13.3% and 60%, respectively, whereas *L. infantum* SLA showed sensitivity values of 56%, 52% and 100% for these groups, respectively (Table 6). All individual OD values for each serum sample against all eight phage clones, including controls, are presented in Figure 1. To compare the sensitivity of our new epitope-based antigens with a commercially available kit for the human VL serodiagnosis, sera of VL patients were tested with the Kalazar Detect™ Rapid Test, and positivity was observed only in 24 out of 30 samples (20% false-negatives). Using sera of healthy subjects living in a non-endemic area of VL, the immunochromatographic test presented negative results for all of the 30 samples that were evaluated, while using sera derived from healthy individuals living in an endemic area of VL, 20 out of 27 (about 25% of false-positives) samples presented negative results, reaching only a moderate performance, with significant misdiagnosis of both VL positive and negative samples. The excellent performance reached by the eight clones suggests that our new epitope-based antigens are the most specific and sensitive markers to date.

7.9. Discussion

Immunological methods have been chosen as the main strategy for VL diagnosis, mainly in field screening tests. They present advantages in relation to the classical parasitological methods, as well as to the molecular detection by PCR technique, due to its easier procedure and adequate sensitivity and specificity, besides being less invasive than aspirates [35]. We have used the phage display technology to select new antigens for improvement of serodiagnosis of human VL, which has been successfully applied in the search of new diagnostic markers for other parasitic diseases, such as neurocysticercosis [28,29], transmissible gastroenteritis virus infection [36], leprosy [37], strongyloidiasis [30], and bovine anaplasmosis [38]. The phage display technology has led us to select and characterize eight novel phage clones that were able to distinguish VL patients from healthy subjects living in an endemic area of disease, and also discriminated VL patients from those with Chagas disease. Importantly, cross-reactivity has been reported when other serological tests are used in these populations, as well as in patients from whom serum reactivity was maintained after treatment discharge [11,12]. Although our mimotopes were not serologically investigated in this situation, it is certainly a future perspective to be performed.

For canine VL, Costa et al [24] used a sequential selection of phage-displayed peptides and identified new antigens, which achieved sensitivity and specificity values of 100% for canine VL serodiagnosis, and did not present false-positive results with *Trypanossoma cruzi*-infected or *Ehrlichia canis*-infected dogs´ sera, including healthy animals immunized with Leishmune® or Leish-Tec® vaccines. Our study described a similar phage display strategy to identify antigenic mimotopes for human VL serodiagnosis, which did not present cross-reactivity with antibodies from sera of Chagas disease patients or from healthy subjects. The eight selected phage clones were able to serologically discriminate the *L. infantum*-infected patients, with 100% sensitivity and specificity through a simple phage-ELISA assay.

As previously described [39-41], different classes of recombinant antigens have been evaluated for serodiagnosis of human VL, and most of them present strong humoral response during the active disease. rK39 protein is considered one of the most effective antigenic markers for the VL serodiagnosis, with sensitivity near 98% in India [42]. In Brazil, the sensitivity and specificity values found for this recombinant antigen have ranged between 82 and 100% [43-51], although problems related to cross-reactivity with sera of patients developing other parasitic diseases, as well as with healthy endemic subjects, have led to false-positive results in the serological assays [11-14].

In our study, although all VL patients presented positive PCR for *L. infantum*, six of them were not detected by the rK39 immunochromatographic test. In contrast, all selected phage clones have correctly identified all VL patients. Furthermore, our eight clones showed a high performance using a direct and easy phage-ELISA test, suggesting that these clones together with this simple immunoassay compose the best platform for VL serology. Regarding their sensitivity and specificity, the clones´ performance was compatible with diagnostic parameters described for the serodiagnosis of canine VL elsewhere [24], but was superior to other recombinant antigens, such as rA2 [52-55], rHSP83 [56] and rLiHyp1 [57]; which were applied for VL serodiagnosis, but most of them were not tested or could not distinguish the clinical forms of disease.

Antigenicity is defined as the ability of the peptide or whole antigen to bind to a selective monoclonal antibody or target [58]. Large repertoires of random sequences displayed on the bacteriophage capsid offer the advantage that large numbers of sequences can be screened in a relatively short time. Phage-displayed peptides as diagnostic candidates possess main advantages, such as the fact that they are effectively antigenic [59], the amplification of bacteriophages is simple and inexpensive compared with the conventional route of peptide synthesis and/or production of recombinant proteins, and besides those advantages, the final product is composed by a high number of virions that express mimotopes on their surface, which also improves the reactivity against antibodies of patients´ sera.

In our study, Ff filamentous phages of M13 class were used to select the antigenic clones. These phages belong to a non-lytic class, where the bacteria are not disrupted during the purification process; in consequence, one could speculate that there is only a low presence of contaminants endotoxins, which additionally could be removed by using commercial kits [60,61]. In this context, our molecules could be considered as having a satisfactory purity to be employed as antigens in the phage-ELISA assays. In addition, the diagnostic phages present also advantages such as the fact of them potentially exclude confusing signals from other non-diagnostic determinants, besides of these purified preparations can be stored in optimized buffers for months to years [62]".

It is interesting to note that at least two amino acid residues within the seven-motif peptides were shared among the eight clones, which have led to the consensus motif TFLRSIVPS. This motif is structurally related with membrane proteins of the parasite, with the highest hit in a hypothetical protein (XP_001464507.2) of unknown function, but suggests that our peptides mimic specific surface proteins from species of the VL

complex. This fact also may explain the similar ELISA reactivities found in the sera of VL patients for the eight evaluated phage clones.

As a limiting factor of the present study, phages were screened using a Ph.D.-C7C Phage Display Peptide Library Kit, which displayed peptides fused to their pIII protein. This protein is expressed at 5 copies per molecule, of which all can be incorporated with short peptides without interfering in the phages´ infectivity. On the other hand, major coat pVIII protein is expressed at 2,700 copies per virion, of which about 10% can be reliably fused to peptides. As a result, peptides expressed as pIII fusions are present at lower valency, whereas pVIII fusions are present at higher valency in the phage [63]. Therefore, the diagnostic performance of our phages could be improved by the construction of target epitopes incorporated to the pVIII proteins of the phages. In addition, the incorporation of other antigenic epitopes in the same or in other phages could be also considered as an additional strategy to improve the diagnostic performance of these evaluated antigens, based on phage-displayed multi-epitopes containing *L. infantum* antigens represented by their selected mimotopes. The sample size used in the present study could be also considered a limiting factor, as well as the absence of a serological follow-up of treated patients in reaction to the phage clones, which has not been made.

Taken together, the present study´ data showed a 100% accuracy for the eight phage-fused mimotopes, when a very simple phage-ELISA assay was performed. This strategy has led to a powerful platform for VL serodiagnosis, based on the low cost and easily to reproduce, and could well be used as an alternative serological tool for population screenings in public health programs for VL monitoring.

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MANUSCRITO 5

(Em submissão)

8. MANUSCRITO 5

8.1. Título

"Avaliação da antigenicidade de fagos e seus peptídeos sintéticos no diagnóstico das leishmanioses em humanos e cães"

8.2. Autores

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8.5. Introdução

As leishmanioses são um grupo de doenças infecto-parasitárias causadas por parasitas protozoários do gênero Leishmania. São endêmicas em 98 países no mundo, sendo que mais de 350 milhões de pessoas estão expostas ao risco de infecção. Estima-

se que cerca de 2 milhões de novos casos ocorram por ano, sendo que 1,0 a 1,5 milhões correspondam a casos de leishmaniose tegumentar (LT), e 500.000 casos sejam de leishmaniose visceral (LV), a qual ocasiona cerca de 60.000 óbitos por ano (WHO, 2014). A LT apresenta manifestações clínicas distintas, como leishmaniose cutânea (LC), leishmaniose cutânea difusa (LCD) e leishmaniose mucosa (LM) (Grimaldi e Tesh, 1993).

Já a leishmaniose visceral (VL) é uma doença zoonótica endêmica do Brasil, onde os cães são os principais reservatórios parasitários domésticos da doença, e as percentagens de cães infectados que vivem nessas regiões onde a VL canina (CVL) é endêmica variaram de 10% a 62%.

As Lesihmanioses são um grupo heterogêneo de doenças cujas manifestações clínicas são bem distintas e expressivas tanto no homem quanto no cão. Mesmo com os avanços tecnológicos no campo de diagnóstico ainda assim não existe um teste sorológico de amplo espectro, rápido, sensível, específico e que seja capaz de distinguir as formas diversas, sendo portanto necessário o desenvolvimento de pesquisas na área.

A técnica de *Phage Display* é uma técnica de biologia molecular que consiste na busca de novos antígenos frente a uma molécula alvo de interesse. Para isto utilizam-se de vírus de bactérias geneticamente modificados, fagos, que expressam peptídeos exógenos em seu capsídeo viral (Smith, 1997) e (Barbas, 2001). A exibição de fagos tem sido utilizada para selecionar mimotopos com diversa aplicabilidade, como: vacinas (Manoutcharian et al., 2004), (Gu et al., 2008), (Frenkel et al., 2000); agentes terapêuticos (Manoutcharian et al., 2001); e principalmente com antígenos diagnósticos para doenças infectocontagiosas e parasitárias (Costa et.al., 2014, 2016).

Em estudos anteriores dois mimotopos de *Leishmania infantum* identificados por *Phage Display* (Fago B10 e C01) foram avaliados como candidatos vacinais em camundongos BALB / c contra a infecção desafio por *Leishmania infantum* (Costa, 2014) e *Leishmania amazonensis* (Costa, 2015), confirmando seu potencial imunogênico na proteção específica e heteróloga. Estes resultados demonstraram indício também de um possível potencial antigênico desses dois clones de bacteriófagos e seus respectivos peptídeos sintéticos, o que levou ao delineamento do presente trabalho, a fim de comparar a acurácia e eficácia daqueles antígenos em diagnosticar a Leishmaniose Visceral Canina (LVC), Leishmaniose Visceral e Tegumentar Humana (LVH e LTH, respectivamente) em detrimento a soros negativos e de doenças que geram reações cruzadas.

8.6. Materiais e Métodos

Animais do Estudo

A amostra consistiu de 100 cães (*Canis lupus familiaris*) constituídos por machos e fêmeas de diferentes raças e idades, coletados de uma área de Belo Horizonte, Minas Gerais, Brasil, que é endêmica para LVC. As amostras de soro sanguíneo de animais Com LVC foram selecionados com base em dois testes serológicos (IFAT [IFATLVC Kit Bio-Manguinhos] e ELISA [Kit EIE-LVC Bio-Manguinhos], ambos da Bio-Manguinhos, (Fiocruz, Brasil) para *Leishmania spp*. Cães com um título IFAT de 1:40 ou reatividade no ELISA abaixo do valor de corte indicadas pelo fabricante foram consideradas soronegativas. Animais com um título IFAT de 1:40 e um valor de ELISA sobre o ponto de corte foram soropositivos. Assim, os cães sintomáticos (n: 51) foram aqueles que foram positivos pelo IFAT e ELISA mas que também tiveram resultados positivos no parasitológico e no PCR (PCR-RFLP) em amostras de sangue, e que apresentaram mais de três sintomas clínicos (perda de peso, alopecia, adenopatia, onicogrifose, hepatomegalia, conjuntivite e dermatite espoliativa no nariz, cauda, E pontas da orelha). Cães saudáveis (n: 15) foram selecionados de uma área de Belo Endêmica para LVC, e de área não endêmica (n: 9), ambos apresentaram resultados sorológicos negativos: (IFAT e ELISA), parasitológicos (PCR) e livre de quaisquer sinais clínicos ou sintomas de leishmaniose. Animais saudáveis vacinados com Leish-Tec (n: 7) foram isolados em canis para evitar o contato com vetores transmissores de leishmaniose, e cães que foram experimentalmente infectados com T. cruzi (n: 8) ou E. canis (n: 10), também foram utilizados neste estudo. Estas amostras de soros foram obtidas de projetos anteriores (Bahia et. al., 2002) e (Carneiro, et.al., 2006). As amostras de soro foram Fornecido por Alexandre Barbosa Reis (Departamento de Análises Clínicas, Universidade Federal de Ouro Preto, Ouro Preto, Brasil), Maria Norma Mello (Departamento de Parasitologia, Universidade Federal de Minas Belo Horizonte, Brasil), e Ana Paula Fernandes (Departamento de Análises Clínicas e Toxicológicas, Universidade Federal de Minas Belo Horizonte, Brasil), (vide tabela 1).

Pacientes do Estudo

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa Humana (COEP) da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG (número de protocolo: CAAE-323431 14.9.0000.5149). Antes de coleta de amostras, os

pacientes receberam uma cópia individual da política de estudo, a qual foi revisada por uma pessoa independente, e todos os participantes assinaram um termo de consentimento livre e esclarecido (TCLE) em português antes que suas amostras fossem coletadas. Todos os soros foram colhidos realizando uma venopunctura da veia mediana em tubos sem anticoagulante e mantidos a 37 ° C durante 15 min, depois foram centrifugados a 4000 × g durante 15 min. As amostras foram então separadas e mantidas a -80 ° C até à sua utilização.

Para análise da antigenicidade frente a LVH as amostras de soro foram obtidas de pacientes com Leishmaniose Visceral (n: 39, incluindo 23 homens e 16 mulheres, com idades variando de 25 a 50 anos) cujo diagnóstico foi confirmado por meio de avaliação clínica e acompanhamento ambulatorial, pela demonstração direta dos parasitas em aspirados de medula bem como PCR. As amostras de soro também foram obtidas de indivíduos saudáveis que viviam em áreas endêmicas (n:39, incluindo 20 homens e 19 mulheres, com idades variando de 25 a 50 anos), de indivíduos com Doença de Chagas (n: 14, incluindo 8 homens e 6 mulheres, com idades variando de 24 a 58 anos), totalizando 92 pacientes (Table 1).

Já as amostras de soro obtidas de pacientes com diagnóstico de LT, classificados como LC (n: 14, incluindo 11 homens e 3 mulheres, com idades variando de 30 a 56 anos) ou ML (n: 8, incluindo 6 homens e 2 mulheres , com idade variando de 28 a 57 anos), que em ambos os casos, foram coletados de uma área endêmica de leishmaniose (Belo Horizonte). O diagnóstico foi confirmado por meio de avaliação clínica das lesões, bem como pela demonstração direta dos parasitas em esfregaços manchados com Giemsa de biópsias de pele (CL) e/ou fragmentos mucosos (ML), bem como pela PCR Técnica para identificar o DNA de kinetoplastide *L. braziliensis*. Todos os pacientes também foram submetidos a um teste intradérmico positivo em Montenegro. Além disso, nenhum dos pacientes tinha sido previamente tratados com drogas antileishmaniais antes de suas amostras foram coletadas. Soros de indivíduos saudáveis tambem foram coletados (n: 25, incluindo 14 homens e 11 mulheres, com idades variando de 30 a 56 anos) totalizando 47 pacientes na análise da antigenicidade para LTH (Table 1).

Parasitos

Leishmania (V.) braziliensis (MHOM / BR / 1975 / M2904) foram cultivados a 24 ° C em meio de Schneider (Sigma, St. Louis, MO, EUA) suplementado com 20% de soro fetal bovino inactivado (FBS, Sigma), L-glutamina 20 mM, 200 U/ml de

penicilina e 100 ug/ml de estreptomicina, a pH 7,4. O extrato antigênico de *L. braziliensis* solúvel (SLA) foi preparado a partir de 2 x 10^8 promastigotas de parasita em fase estacionária, conforme descrito em outra parte (Coelho et al., 2003). De *L. Infantum* solúvel (SLA), após poucas passagens em cultura líquida, 2 x 10^8 promastigotas por mL, num volume de 5 mL, foram lavadas 3 vezes em 5 mL de tampão fosfato estéril frio Salina (PBS). Após cinco ciclos de congelação e descongelação, o a suspensão foi centrifugada a 8.000 g durante 20 min a 4ºC, e a o sobrenadante contendo SLA foi recolhido em aliquotas de 500 uL e armazenado a -80ºC, até à sua utilização. A concentração de proteína de ambos extratos foram estimados pelo método de Bradford.

Biosseleção e Amplificação dos Fagos (B10 e C01)

Os procedimentos técnicos utilizados para selecionar os fagos B10 (seq: LSFPFPG) e C01 (seq: FTSFSPY) foram realizados anteriormente e descrito (Costa et al., 2014). A amplificaçao dos dois clones de bacteriófagos foi realizada fazendo-se inicialmente o pré-inóculo em 5ml de meio LB líquido, 5ul de tetraciclina, e 10ul do backup de bactéria congelada e infectada com o respectivo fago. Posteriormente (16 horas após) foi feito o inóculo, o qual foi adicionado 1ml do pré-inóculo a 200ml de LB líquido. Incubou-se durante 5 h com agitação constante a 37 ° C. Após a incubação, foi centrifugado durante 20 min a 2.250 g e o sobrenadante foi transferido para tubos falcon de 50ml, nos quais foi adicionado polietilenoglicol (PEG) -NaCl (20% de PEG 8000 e 2,5 M de NaCl) na proporção de 1/6 do total volume do sobrenadante transferido. Os tubos ficaram incubados durante 16 h a 4 °C. Depois, foi centrifugados durante 1 h, o sobrenadante foi removido, e o sedimento foi ressuspenso em 500 ul de uma solução composta de 10 mM Tris-HCl (pH 8,0). Foi feito alíquotas dos fagos em suspensão, dosados no espectrofotômetro nas absorbâncias (Abs) de [269 – 320], e mantidos a 4ºC até o uso.

Sequenciamento e Síntese Peptídica

Os procedimentos técnicos para realizar o sequenciamento foi descrito previamente, fagos B10 (seq: LSFPFPG) e C01 (seq: FTSFSPY), (Costa et al., 2014). Já a síntese peptídica foi descrita inicialmente por Woolley & Merrifield (1963), em fase sólida (SPPS) consiste na fixação do aminoácido C-terminal do peptídeo sobre um suporte sólido insolúvel e depois aumentar a cadeia peptídica por adições sucessivas de resíduos no sentido C-terminal ao N-terminal. As junções α-aminas dos resíduos

utilizados são protegidas temporariamente numa ligação instável com um grupo Fmoc (9-fluorenilmetiloxicarbonil) que se tornam lábeis em meio básico. As cadeias laterais reativas são bloqueadas por um grupo estável (por exemplo: Trt, Acm) durante todos os ciclos e desprotegidas ao término da síntese do peptídeo, devido a um forte tratamento ácido.

O produto de síntese bruto é submetido à cromatografia líquida de alta pressão (HPLC） de fase reversa. Os componentes presentes são eluídos, utilizando-se um gradiente que varia conforme a sequencia peptídica. O peptídeo purificado é então submetido à espectrometria de massa para a confirmação de sua massa molecular.

Ensaio Imunoenzimático (Fago-ELISA e Peptídeo-ELISA)

As curvas de titulação anteriores foram realizadas para determinar a concentração de fago mais apropriada e a diluição de anticorpo a ser usada. As placas de microtitulação JetBiofilm® foram revestidas, com os fagos B10 e C01 respectivamente. Contendo 1,0 x 10^9 fagos por poço, diluídos em tampão de revestimento 100 ul/poço (tampão carbonato 50 mM) (pH 9,6) durante 16 horas a 4 ° C. Foi utilizado um clone de fago selvagem (naive) como controle do experimento. Já a sensibilização com os peptídeos sintéticos foi realizada em 100 ul/poço (tampão carbonato 50 mM) (pH 9,6) durante 16 horas a 4 ° C, na concentração 2,0 ug/poço de cada peptídeo (peptídeo B10 e C01 respectivamente). Após as sensibilizações, os locais de ligação livres foram bloqueados utilizando 200 ul de uma solução de bloqueio (consistindo em PBS, Tween 20 a 0,05% e 5% de BSA) durante 1 h a 37 ° C. Após lavagem das placas 5 vezes com TBS-T, incubaram-se com 100 ul de soro individuais caninos e humanos (Diluído 1:100 em PBS, Tween 20 a 0,05% e 5% de BSA) durante 1 h a 37°C. As placas foram lavadas 5 vezes utilizando TBS-T e incubadas com uma IgG (1: 5.000 e 1:10.000 respectivamente) IgG anti-dog e anti-humano peroxidase conjugado (Sigma, St. Louis, MO, EUA) durante 1 h a 37°C. Depois as placas foram lavadas 5 vezes com TBS-T, a reação foi desenvolvida através de incubação com H_2O_2, o-fenilenodiamina e citrato-fosfato (PH 5,0) durante 30 min no escuro. A reação foi parada adicionando 25 ul de H_2SO_4 (2 N) e a densidade óptica a 492 nm foi lida em espectrofotômetro de microplaca ELISA (Bio-rad®).

Análise Estatística

Os resultados foram inseridos em planilhas do Microsoft Excel (versão 10.0) e analisados usando GraphPad Prism^{TM} (versão 5.0 para Windows). Os limites inferiores de positividade (cut-off) para os antígenos de diagnóstico foram estabelecidos para determinar a razão de verossimilhança máxima calculada pelas curvas ROC. A capacidade de diagnóstico de cada antígeno foi medida avaliando a sua sensibilidade, especificidade e intervalos de confiança de 95% (IC 95%). O teste t de Student não pareado foi utilizado e foram consideradas diferenças significativas com P <0,05.

8.7. Resultados

Avaliação da antigenicidade dos fagos B10 e C01 e seus respectivos peptídeos sintéticos

No presente estudo, quatro antígenos, os fagos B10 e C01, e seus respectivos peptídeos sintéticos cujas sequencias são: LSFPFPG e FTSFSPY foram avaliados como marcadores diagnósticos para LVC, LVH e LTH. Mais apropriadamente, estes antígenos foram utilizados em ensaios sorológicos por ELISA para detectar cães doentes com LV e pacientes doentes com leishmaniose visceral, e tegumentar (forma cutânea e mucosa). Como controle dos experimentos foi-se utilizado o fago selvagem que não é geneticamente modificado e que, portanto não apresenta o peptídeo exógeno expresso externamente ao seu capsídeo viral, toda absorbância por ele apresentada se dá meramente pela presença das proteínas intrínsecas do vírus. Como forma de comparação entre testes utilizou-se extratos solúveis de leishmania (*SLA L.infantum e SLA L.braziliensis*).

De forma geral ao analisar os resultados obtidos em todo trabalho, verifica-se uma melhor acurácia do teste nos soros testados frente aos fagos em detrimento àqueles frente aos peptídeos sintéticos e por isso o índice Kappa de concordância entre os testes foi realizado apenas nos grupos dos fagos em relação aos extratos solúveis (Table 5).

Na análise da antigenicidade destes 4 antígenos frente a avaliação da leishmaniose visceral canina a qual foi o alvo de biosseleção original destes antígenos, obteve-se bons resultados e ao compararmos os fagos com seus peptídeos não houve grandes diferenças estatísticas como ocorreram nas outras doenças avaliadas (LVH e LTH); 100,00% de sensibilidade e 98,00% de especificidade do peptídeo B10, na avaliação de um total de 100 amostras de cães, enquanto o próprio fago apresentou 100,00% sensibilidade e 98,25% de especificidade. Fago e peptídeo B10 apresentaram (AUC 1,000) e (p< 0.0001*). Em relação ao peptídeo C01 esta avaliação girou em torno de 98,08% sensibilidade e 98,25% especificidade (AUC 0,999) e (p< 0.0001*), o fago

C01 100,00% sensibilidade e 98,00% de especificidade para avaliar as mesmas 100 amostras com(AUC 1,000) e (p< 0.0001*). Já os controles do experimento como o fago selvagem apresentou 78,85% de sensibilidade e 42,86% de especifidade; o *SLA de L. infantum* mostrou sensibilidade de 65,38% e especificidade de 95,59% (AUC 0,926) e (p< 0.0001*).

O índice *Kappa* foi feito avaliando a concordância entre o fago B10 e o *SLA de L. infantum* apresentando índice de 0.718 com intervalo de confiança (IC95% 0.530 – 0.906) que demonstra uma substancial concordância. Esta mesma concordância também foi verificada entre o fago C01 e o *SLA de L. infantum.*

Na avaliação da leishmaniose visceral humana (LVH), o fago B10 apresentou 100,00% de sensibilidade e 98,11% de especificidade (AUC 1,000) e (p< 0.0001*), enquanto seu peptídeo sintético apresentou 87,18% de sensibilidade e 84,91% de especificidade (AUC 0,930) e (p< 0.0001*). Já o fago C01 apresentou 92,31% de sensibilidade e 98,11% de especificidade (AUC 0,994) e (p< 0.0001*), enquanto seu respectivo peptídeo sintético demonstrou 87,18% de sensibilidade e 79,25% de especificidade (AUC 0,920) e (p< 0.0001*). O fago selvagem apresentou 76,92% sensibilidade e 71,70% de especificidade (AUC 0,777) e (p< 0.0001*),, enquanto o SLA *de L. infantum* apresentou 97,44% sensibilidade e 83,02% especificidade (AUC 0,897) e (p< 0.0001*).

O índice *Kappa* foi feito avaliando a concordância entre o fago B10 e o *SLA de L. infantum* apresentando índice de 0.783 com intervalo de confiança (IC95% 0.582 – 0.985) que demonstra uma substancial concordância. Entre o fago C01 e o *SLA de L. infantum* o índice foi de 0.913 com intervalo de confiança de (IC95% 0.701 – 1.00) demonstrando uma concordância quase perfeita.

Já na avaliação da leishmaniose tegumentar humana (LTH), o fago B10 apresentou 100,00% de sensibilidade e 96,00% de especificidade (AUC 1,000) e (p< 0.0001*), enquanto seu peptídeo sintético apresentou 86,36% de sensibilidade e 72,00% de especificidade. Já o fago C01 apresentou 100,00% de sensibilidade e 98,00% de especificidade (AUC 1,000) e (p< 0.0001*), enquanto seu respectivo peptídeo sintético demonstrou 95,45% de sensibilidade e 24,00% de especificidade. O fago selvagem apresentou 27,27% sensibilidade e 88,00% de especificidade, enquanto o SLA *de L. infantum* apresentou 81,82% sensibilidade e 96,00% especificidade (AUC 0,914) e (p< 0.0001*).

O índice *Kappa* foi feito avaliando a concordância entre o fago B10 e o *SLA de L. infantum* apresentando índice de 0.829 com intervalo de confiança (IC95% 0.547 –

1.00) que demonstra uma concordância quase perfeita. Entre o fago C01 e o *SLA de L. infantum* o índice foi de 0.786 com intervalo de confiança de (IC95% 0.507 – 1.00) demonstrando uma concordância substancial (Table 5).

8.8. Discussão

No *Phage Display* os peptídeos apresentados por fagos filamentosos da classe M13 possuem diversas vantagens das quais a principal se deve ao fato, dos peptídeos serem ao mesmo tempo imunogênicos e antigênicos (Manoutcharian et. al, 1999).

O presente estudo descreve quatro antígenos (Fago B10 e C01) e seus respectivos peptídeos sintéticos (LSFPFPG e FTSFSPY) que são capazes de diferir distintas formas de leishmanioses, seja no cão ou no homem (LVC, LVH e LTH). Os clones de fagos utilizados neste trabalho já foram descritos anteriormente (Costa et.al.,2014 e 2015) em dois artigos que avaliaram apenas o seu potencial imunogênico, mas não antigênico. Os peptídeos sintéticos, por sua vez nunca foram testados e não há nenhum trabalho prévio que os caracterize.

O fago apresenta melhor sensibilidade e especificidade quando comparado ao seu peptídeo específico em ensaios de ELISA, isto é confirmado por diversos estudos de diagnóstico, em que é observada queda da sensibilidade e especificidade quando se utiliza o peptídeo sintético ao invés do fago com o peptídeo conformacional (Gu et. al, 2008; Costa et. al, 2014). Parece existir uma maior estabilidade do peptídeo do fago devido às proteínas estruturais do capsídeo viral, o que não ocorre quando se utiliza o peptídeo sintético sozinho. Estes fagos são rastreados a partir de uma biblioteca conformacional de bacteriófagos (Ph.D.-C7C Phage Display Peptide Library Kit), que exibem peptídeos fundidos à proteína pIII, estrutural do vírus. Cada vírus apresenta cerca de cinco cópias desta proteína de revestimento pIII, que confere certa estabilidade ao peptídeo quando este é testado em ELISA, ou seja, imobilizado-o em estrutura rígida como placa de adsorção(Gu et. al, 2008). Mas neste trabalho o perfil comparativo entre fago e peptídeo foi similar, pelo menos nas doenças: LVC e LVH. Acreditamos que seja devido à otimização do protocolo de ELISA em que foi utilizado solução (PBS-T 0,05% e 5% de BSA) no bloqueio e na diluição dos soros, o que nos encoraja a tentar desenvolver e otimizar futuramente uma plataforma de diagnóstico de sistema eletroquímico utilizando os fagos e seus peptídeos sintéticos (LSFPFPG e FTSFSPY).

Como os fagos têm pouca estabilidade, mantendo viabilidade próximo de 30 dias seria interessante propor plataformas que aperfeiçoem seu uso ou então o uso de seu peptídeo sintético (Santos et.al, 2012). Santos e seus colaboradores trabalharam com

fagos específicos e incorporaram seus peptídeos sintéticos em bioeletrodo, de grafite modificado com ácido poli-3-hidroxifenilacético, para detectar diretamente os soros de animais infectados por *Anaplasma marginale* onde foram demonstrados e confirmados os diagnósticos por bioimpedância. O sistema de sensores eletroquímicos provou ser altamente eficaz na discriminação de soros de animais positivos e negativos (Santos et.al, 2012).

Neste trabalho, os melhores fagos para discriminar soros de cães e de pacientes humanos com leishmanioses não foram os que apresentaram melhores índices de concordância entre testes (Índice *Kappa*) em relação aos extratos solúveis de leishmania (*L. infantum* e *L. braziliensis*). Isso é importante ressaltar já que como não há até o momento um padrão-ouro, 100% sensível e específico para o diagnóstico sorológico das leishmanioses, quando utilizamos o índice *Kappa* podemos perceber que os fagos apresentam melhor curva ROC, acurácia, área sob a curva (AUC) e *p-value* ($p<0.0001$*), sendo, portanto o SLA um parâmetro razoável de comparação, com restrito poder de estudo. Tanto que muitos trabalhos atuais relatam dificuldade de padronização de preparo e variável sensibilidade e especificidade dos extratos (Martins et.al, 2015 e 2016); (Lage et.al, 2016); (Coelho et.al, 2016).

É bem estabelecido que a resposta de células B policlonais antígenos-específicos é um sistema imunológico adaptativo de mamíferos que assegura o reconhecimento de múltiplos epítopos de um antígeno. Antígenos podem ser substâncias grandes e complexas (Kennedy et. al, 1990), conseqüentemente, uma resposta imunológica eficaz envolve freqüentemente a produção de muitos anticorpos diferentes. Isso significa que uma ferramenta de diagnóstico baseado em mais de um epítopo será mais eficaz e apresentará um amplo reconhecimento por anticorpos (Kennedy et. al, 1990) por isso pode-se realizar bioensaios para testar epítopos associados em um sistema eletroquímico a fim de desenvolver um biossensor rápido, sensível, específico de excelente peformace diagnóstica.

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9. CONCLUSÃO GERAL DA TESE

- A tecnologia de phage display utilizada no presente trabalho, por meio de uma abordagem inédita para as leishmanioses, bioseleção negativa e positiva, possibilitou a identificação de novos imunógenos que se mostraram efetivos na proteção contra as leishmanioses e no diagnóstico diferencial sorológico das mesmas.

- Os clones de fagos (B10 e C01) selecionados conferiram proteção em camundongos BALB/c contra a *Leishmania infantum* e *Leishmania amazonensis* cuja proteção cruzada foi eficiente. Esta proteção foi correlacionada principalmente às células T CD8+, caracterizada por alto nivel de IFN-γ, IL-12 e GM-CSF, e baixo de IL-4 e IL-10; bem como por baixos níveis de anticorpos IgG1-isotipo.

- Os clones de fagos (B10 e C01) selecionados também conferiram potencial antigêncico de alta acurácia no diagnóstico sorológico da LVC e da LVH, bem como relativa em relação a LTH, formas mucosa e cutânea.

- Estes achados afirmam que os fagos (B10 e C01) poderiam constituir novos candidatos a vacinas contra LV e LT, bem como novas plataformas de diagnóstico sorológico das leishmanioses.

10. PERSPECTIVAS

- Testar estes novos antígenos (B10 e C01) frente à PBMC's de humano, para avaliar nestas células sua imunogenicidade.

- Avaliar seu potencial para o diagnóstico humano de LVH e LTH, por meio da técnica de ELISA, utilizando um painel sorológico amplo com expressivo (n) amostral.

- Realizar experimentos baseados em imunoproteômica e/ou análise reversa (phage reverso) para identificar as proteínas nativas de tais epitopos presentes em *L. infantum*.

- Clonar as proteínas nativas e avaliar sua eficácia protetiva contra a leishmaniose visceral e tegumentar.

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12. ANEXOS

12.1 Anexo 1

PATENTE 1: Documento comprobatório de depósito de patente nacional.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Federal de Minas Gerais

1.2 Qualificação: Instit. de Ensino e Pesquisa

1.3 CNPJ/CPF: 17217985000104

1.4 Endereço Completo: Av. Antonio Carlos, 6627 - Pampulha, Belo Horizonte - MG, Brasil

1.5 CEP: 31270-901

1.6 Telefone: (31) 3409-4774 1.7 Fax: (31) 3409-6430

1.8 E-mail: patentes@ctit.ufmg.br

☐ continua em folha anexa

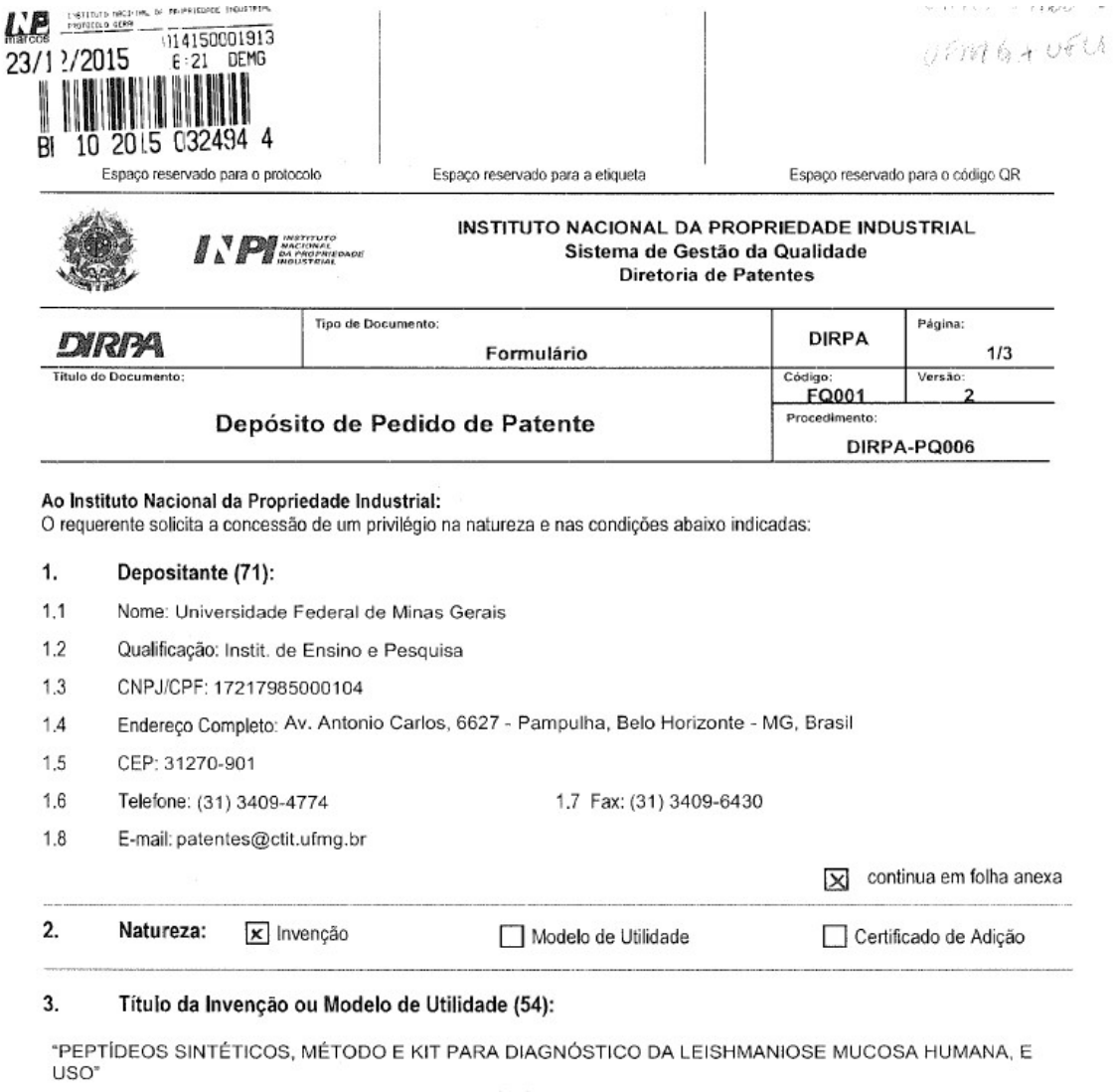
2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSE VISCERAL CANINA, PEPTÍDEOS SINTÉTICOS E USO

12.2. Anexo 2

PATENTE 2: Documento comprobatório de depósito de patente nacional.



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1.6 Telefone: (31) 3409-4774 1.7 Fax: (31) 3409-6430

1.8 E-mail: patentes@ctit.ufmg.br

☒ continua em folha anexa

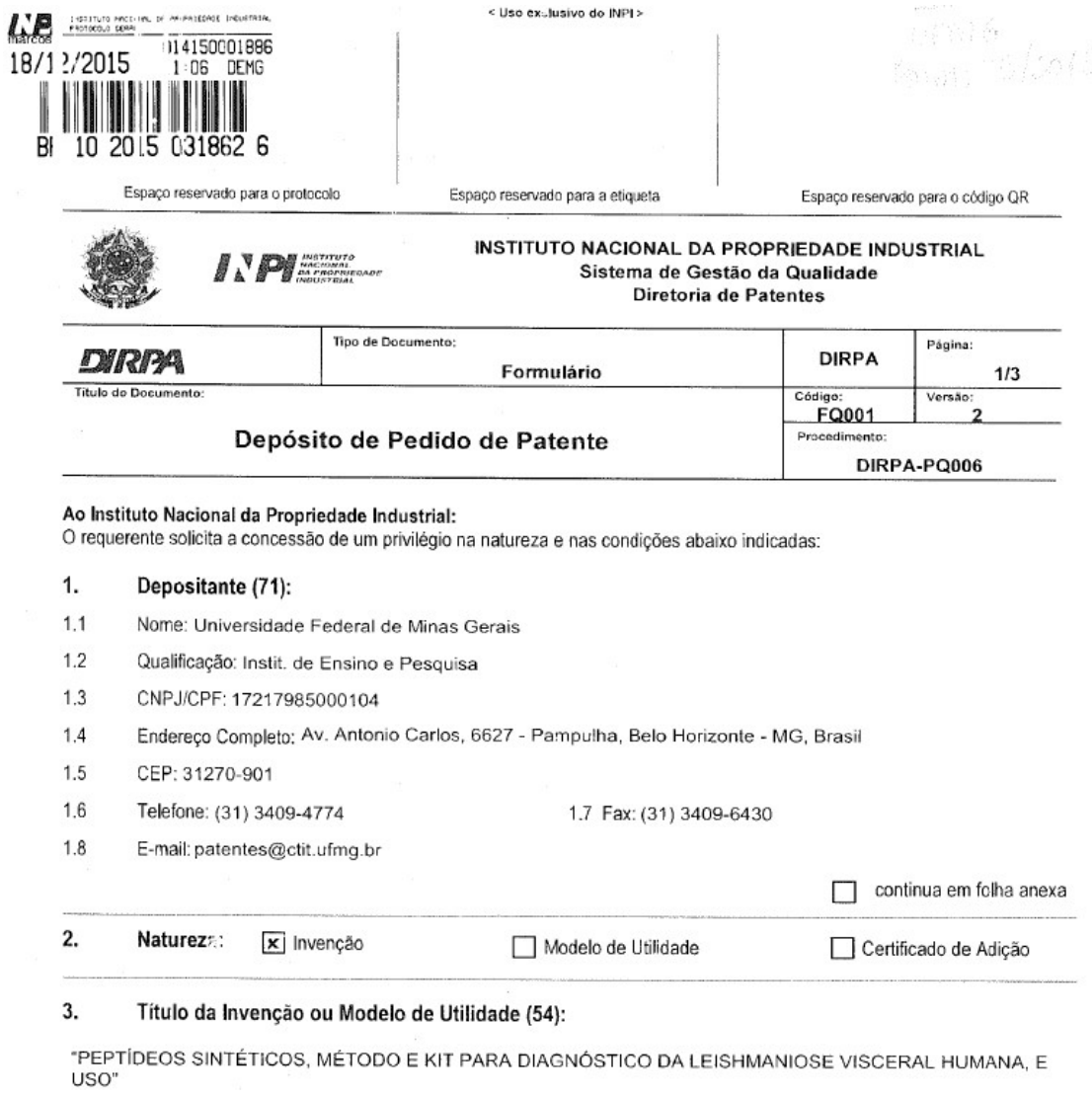
2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

"PEPTÍDEOS SINTÉTICOS, MÉTODO E KIT PARA DIAGNÓSTICO DA LEISHMANIOSE MUCOSA HUMANA, E USO"

12.3. Anexo 3

PATENTE 3: Documento comprobatório de depósito de patente nacional.



< Uso exclusivo do INPI >

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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Federal de Minas Gerais

1.2 Qualificação: Instit. de Ensino e Pesquisa

1.3 CNPJ/CPF: 17217985000104

1.4 Endereço Completo: Av. Antonio Carlos, 6627 - Pampulha, Belo Horizonte - MG, Brasil

1.5 CEP: 31270-901

1.6 Telefone: (31) 3409-4774 1.7 Fax: (31) 3409-6430

1.8 E-mail: patentes@ctit.ufmg.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

"PEPTÍDEOS SINTÉTICOS, MÉTODO E KIT PARA DIAGNÓSTICO DA LEISHMANIOSE VISCERAL HUMANA, E USO"

12.4. Anexo 4

Comprovante de aprovação do projeto pelo CEUA da UFMG.

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o Protocolo nº. 134 / 2013, relativo ao projeto intitulado "Biotecnologia de phage display aplicada no desenvolvimento de vacinas e diagnóstico da leishmaniose visceral canina", 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 11/07/2013. Este certificado espira-se em 11/07/2018.

CERTIFICATE
We hereby certify that the Protocol nº. 134 / 2013, related to the Project entilted "Phage display biotechnology employed in the development of vaccines and in the diagnosis of canine 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 11/07/2013. This certificates expires in 11/07/2018.

FRANCISNETE GRACIANE ARAUJO MARTINS
Coordenador(a) da CEUA/UFMG
Belo Horizonte, 11/07/2013.

Atenciosamente.

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

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