Universidade Federal de Minas Gerais

Instituto de Ciências Biológicas

Míriam Maria Silva Costa

Tese de Doutorado

Identificação e Caracterização Imunológica de Antígenos de *Leishmania (Leishmania) chagasi* para o Desenvolvimento de Vacinas e Alternativas em Diagnóstico da Leishmaniose Visceral

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2011

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Tese de Doutorado apresentada ao Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como parte dos requisitos para obtenção do grau de Doutor em Bioquímica e Imunologia.

Orientadores: Prof. Dr. Ricardo Tostes Gazzinelli

Prof. Dra. Hélida Monteiro de Andrade

Instituto de Ciências Biológicas

Universidade Federal de Minas Gerais

2011

Universidade Federal de Minas Gerais -
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ATA DA DEFESA DA TESE DE DOUTORADO DE MIRIAM MARIA SILVA COSTA. Aos quatorze dias do mês de setembro de 2011, às 14:00 horas reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "Identificação e caracterização imunológica de antígenos de *Leishmania Leishmania chagasi* para o desenvolvimento de vacinas e alternativas em diagnóstico da leishmaniose visceral", requisito final para a obtenção do grau de *Doutor em Ciências*: Imunologia. Abrindo a sessão o Presidente da Comissão, Prof. Ricardo Tostes Gazzinelli da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra a candidata para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dra. Clara Lucia Barbieri Mestriner da Universidade Federal de São Paulo, aprovada; Dra. Patricia Cuervo Escobar da Fundação Oswaldo Cruz, aprovada; Dra. Maria de Fátima Martins Horta da Universidade Federal de Minas Gerais, aprovada; Dr. Adriano Monteiro de Castro Pimenta da Universidade Federal de Minas Gerais, aprovada; Dra. Hélida Monteiro de Andrade, co-orientadora, da Universidade Federal de Minas Gerais, aprovada; Dr. Ricardo Tostes Gazzinelli, orientador, da Universidade Federal de Minas Gerais, aprovada. Pelas indicações a candidata foi considerada APROVADA. O resultado final foi comunicado publicamente a candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 14 de setembro de 2011.

Dra. Clara Lúcia Barbieri Mestriner – UNIFESP

Dra. Patricia Cuervo Escobar – FIOCRUZ -RJ

Dra. Maria de Fátima Martins Horta – UFMG

Dr. Adriano Monteiro de Castro Pimenta – UFMG

Dra. Hélida Monteiro de Andrade, co-orientadora - UFMG

Dr. Ricardo Tostes Gazzinelli – orientador - UFMG

Profª Maria Elena de Lima Peres Lopes
Coordenadora do Curso de Pós Graduação
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ICB - UFMG

Dedico este trabalho primeiramente a Deus,

que sempre orientou meus passos e escolhas

e também a meus pais Paulo e Terezinha,

`as minhas irmãs Paula e Camila e ao meu querido marido Thiago

por todo amor, apoio e confiança de sempre!

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LISTA DE ABREVIATURAS

BCG: Bacilo de *Calmette-Guérin*

BSA: Albumina bovina

bp: Pares de bases

DIGE: *Differential Gel Electrophoresis*

DMEM: *Dulbecco's Modified Eagle's Medium*

ELISA: Enzyme linkd immunosorbent assay

FML: Ligantes fucose manose

IFN-γ: Interferon gama

IgG: Imunoglobulina

PSA: Persulfato de amônio

RIFI: Reação de Imunofluorescência Indireta

RT-PCR: *Reverse transcription polymerase chain reaction*

s.c.: Subcutânea

SDS: Dodecil sulfato de sódio

TAP: *Transporter associated with antigen processing*

TE: Tris EDTA

TEMED: *N,N,N',N'-Tetramethyl-ethylenediamine*

TMB: *Tetramethylbenzidine*

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RESUMO

A leishmaniose visceral (LV) é uma doença infecciosa grave causada por protozoários do gênero *Leishmania*. Nas Américas, o cão é o principal reservatório da doença, principalmente nas áreas urbanas, onde encontra-se em grande expansão. A LV é fatal se não for tratada adequadamente e/ou diagnosticada precocemente, porém, a eficácia dos métodos diagnósticos e vacinas disponíveis permanece parcial, sendo necessário o desenvolvimento de novas estratégias. Vários estudos demonstraram o potencial da proteína A2, específica do estágio amastigota de *Leishmania*, como antígeno vacinal e diagnóstico. No presente trabalho, inicialmente, testamos este antígeno, administrado na forma de vacina de DNA e observamos proteção em camundongos contra a infecção por *L. (L.) chagasi* e *L. (L.) amazonensis*. A seguir, descrevemos a proteção, em estudos de fase II, com cães beagles vacinados com a proteína A2 associada ao adjuvante saponina (constituindo a vacina Leish-tec®) contra a infecção por *L. (L.) chagasi*. Com relação ao diagnóstico utilizando este antígeno, observamos que peptídeos provenientes de análises *in silico* da proteína A2, associados a peptídeos de outras proteínas como NH, LACK e K39 apresentaram elevada sensibilidade e especificidade em testes com soros humanos e caninos. No entanto, a busca por antígenos vacinais e diagnóstico na LV é incessante, e o atual estágio de desenvolvimento tecnológico com a possibilidade de utilizar abordagem proteômica para identificação de centenas de antígenos imunogênicos simultaneamente é atraente. Dessa forma, optamos por uma abordagem proteômica associada a análises *in silico* para identificar novos antígenos de *L. (L.) chagasi*. Nossa análise proteômica foi a primeira a utilizar amastigotas purificadas de baço de hamster e, além disso, é o primeiro estudo a mapear a antigenicidade da infecção por *L. (L.) chagasi* utilizando soros de cães de fase aguda e crônica. Todas as proteínas selecionadas por western blot-2DE (WB-2DE) foram mapeadas para epítopos de células B pelo software BepiPred e submetidas `a imuno-ensaios em membranas de celulose resultando na seleção de 25 peptídeos com elevado potencial para o diagnóstico sorológico da LV. Paralelamente, foi utilizada a técnica de DIGE para comparar a expressão diferencial de proteínas entre as formas amastigota e promastigota de *L. (L.) chagasi* e 40 proteínas foram identificadas, sendo 25 aumentadas em promastigotas, 5 aumentadas em amastigotas e 10 com expressão similar em ambas as formas. Além disso, todas as

proteínas identificadas no trabalho, tanto por DIGE como por WB-2DE, foram mapeadas para epítopos de células T utilizando o software NetCTL que permitiu a seleção de potenciais candidatos para o desenvolvimento de vacinas. Por fim, para complementar a análise de predição para células B, as mesmas proteínas imunogênicas identificadas por WB-2DE forma mapeadas utilizando outros dois programas de predição de epítopos (ABCPred e BCPred) e após análise adicional de imuno-ensaios mais 23 peptídeos foram identificados como potenciais antígenos diagnóstico na LVC. Esses novos peptídeos, juntamente com os 25 previamente identificados, foram submetidos a ensaios imuno-enzimáticos que resultaram em 10 antígenos com elevada sensibilidade e especificidade, inclusive maior do que a encontrada pelo kit EIE-LVC, que é o recomendado pelo Ministério da Saúde, que também falhou em detectar cães assintomáticos. Portanto, uma análise em grande escala de *L. (L.) chagasi* através do proteoma foi realizada para identificar epítopos de células B e T com potencial para utilização no desenvolvimento de testes diagnósticos e vacinas.

Palavras-chave: *L. (L.) chagasi*, proteoma, diagnóstico, vacina.

ABSTRACT

Visceral leishmaniasis (VL) is a severe infectious disease caused by a protozoan of the genus *Leishmania*. In the Americas, the dog is the main reservoir of disease, mainly in urban areas where the disease is increasing. VL is fatal if it is not adequately treated and/or if it is not diagnosed early, however the efficacy of diagnostic methods and vaccines available remain partial, therefore it is necessary to develop new strategies. Several studies have demonstrated the potential of A2 protein, an amastigote stage-specific protein family in *Leishmania*, as vaccine and diagnosis. In our studies, using this antigen administered as DNA vaccine, we observed protection in mice against infection by *L. (L.) chagasi* and *L. (L.) amazonensis*. A2 antigen also induces protection in phase II studies with beagle dogs vaccinated with A2 protein associated with the saponin adjuvant (constituting the vaccine Leish-tec®) against infection by *L. (L.) chagasi*. The partial protection induced by A2 antigen in vaccine studies led us to search for new proteins for further vaccine tests and future associations with the A2 antigen. Regarding the diagnosis, we found that the peptides derived from *in silico* analysis of the A2 protein, associated with peptides from other proteins such as NH, LACK and K39 showed high sensitivity and specificity in tests with human and canine sera. However, the search for vaccine and diagnosis antigens of VL is incessant, and the current stage of technological development with the possibility of using proteomic approach to identify hundreds of immunogenic antigens simultaneously is attractive. Thus, we chose a proteomic approach combined with *in silico* analyzes to identify new antigens of *L. (L.) chagasi*. Our proteomic analysis were the first to use amastigotes purified from hamster spleen and, furthermore, is the first study to map the antigenicity of infection by *L. (L.) chagasi* using sera from dogs of acute and chronic phase. All the selected protein by western blot-2DE (WB-2DE) were mapped to B cell epitopes by the BepiPred software and subjected to immunoassay through cellulose membranes resulting in the selection of 25 peptide with high potential for serological diagnosis of VL. In parallel, we used the technique of DIGE to compare the differential expression of proteins between the amastigote and promastigote forms of *L. (L.) chagasi* and 40 proteins were identified, 25 increased in promastigotes, 5 increased in amastigotes and 10 with similar expression in both forms. Moreover, all the proteins identified in the work by both DIGE as by WB-

2DE have been mapped to T cell epitopes using the software NetCTL allowing the selection of potential candidates for vaccine development. Finally, to complete the analysis prediction for B cells, the same immunogenic proteins identified by WB-2DE were mapped using others two programs to predict epitopes (ABCPred and BCPred) and after additional analysis of immunoassays, more 23 peptides were identified as potential diagnostic antigens in the LVC. These new peptides, together with 25 previously identified, were subjected to immuno-enzymatic assays that resulted in 10 antigens with high sensitivity and specificity, even greater than that found by the EIE-LVC kit, which is recommended by the Ministry of Health, which also failed to detect asymptomatic dogs. Therefore, a large-scale analysis of *L. (L.) chagasi* through proteome was performed to identify B and T cell epitopes potentially useful in developing diagnostic tests and vaccines.

Key-words: *L. (L.) chagasi*, proteome, diagnostic, vaccine.

SUMÁRIO

1. INTRODUÇAO

A leishmaniose visceral (LV) nas Américas é uma zoonose causada pelo protozoário *L. (L.) infantum* [= *L. (L.) chagasi*] que representa grande importância dentre as doenças negligenciadas (Ashford 2000; Desjeux, 2004). Ela encontra-se em grande expansão nas áreas urbanas e oferece muito prejuízo a saúde publica. O cão é o principal reservatório da doença e, portanto tem um importante papel na disseminação do parasita e conseqüente ampliação dos focos endêmicos (Ashford 2000; Chappuis *et al.,* 2007 e Rouf *et al.,* 2009). No Brasil, testes sorológicos como RIFI e ELISA são empregados na rotina de inquéritos epidemiológicos e controle da leishmaniose visceral canina, entretanto, estes testes são questionáveis em relação a sensibilidade e especificidade, o que, conseqüentemente, subestima a taxa real da infecção e garante a manutenção de animais infectados (Sundar *et al.,* 2002; Porrozzi *et al.,* 2007; Rouf *et al.,* 2009). Adicionalmente, uma da metas da Organização Mundial de Saúde é o desenvolvimento de uma vacina protetora para os cães, fator que teria grande impacto no ciclo de transmissão do parasita (WHO, 2003), uma vez que as vacinas presentes no mercado, até o presente momento, oferecem uma proteção parcial dos animais (Fernandes *et al.,* 2008; Palatnik-de-Sousa *et al.,* 2009). Neste contexto, um novo desafio se estabelece com relação a identificação de novos antígenos para serem testados em métodos diagnósticos e vacinas.

Nos últimos anos, o nosso grupo de pesquisa se empenhou em identificar, caracterizar e avaliar antígenos e protocolos de imunização para o desenvolvimento de vacinas e testes diagnóstico para leishmaniose.

Um grande número de antígenos isolados do parasita têm sido avaliados em seu potencial para desencadear uma resposta protetora contra a infecção com *Leishmania.* Inicialmente, em nosso grupo de pesquisa, obtivemos êxito com o antígeno A2 que é uma proteína específica do estágio amastigota de várias espécies de *Leishmania* e foi o primeiro fator de virulência amastigota-específico identificado em *L. donovani* (Zhang & Matlashewski 1997). Iniciamos os testes com camundongos utilizando a proteína recombinante A2 (rA2) associada à rIL-12 como adjuvante, o que conferiu proteção em

camundongos BALB/c desafiados com *L. (L.) amazonensis*. Foi observado redução significativa das lesões na pata e da carga parasitária nos animais imunizados e desafiados com A2, associados `a elevada produção de IFN-γ e de anticorpos IgG2a específicos à proteína e ao parasita (Coelho *et al.,* 2003). Imunização com o antígeno A2 na forma de DNA também induziu proteção contra a infecção com as formas promastigotas de *L. (L.) chagasi* e *L. (L.) amazonensis* em camundongos BALB/c, onde foi observado o desenvolvimento de uma resposta protetora T_H1 (Zanin *et al.,* 2007).

Em função dos resultados promissores obtidos com o antígeno A2 em camundongos, estendemos a nossa análise no modelo canino, uma vez que eles representam o principal reservatório da doença e o principal foco para busca de vacinas (Fernandes *et al.,* 2008). Neste trabalho, foram avaliadas as respostas imune celular, humoral, parasitológica e clínica de cães imunizados com a proteína recombinante A2, associado ao adjuvante saponina, compondo a vacina Leish-tec®. Animais imunizados com Leish-tec® apresentaram aumento significativo da produção de IFN-γ após as imunizações, que se manteve elevado sete meses após o desafio, sob estímulo de rA2 e LcPA (extrato solúvel do parasita). Após o desafio, houve aumento significativo de IL-10 no grupo de animais não vacinados e infectados, quando comparado ao grupo de cães vacinados e infectados. No grupo de cães não vacinados e infectados, apenas 28.5% dos cães permaneceram assintomáticos, sendo que os sintomas clínicos encontrados nos demais foram perda de peso intensa, diarréia profusa e sanguinolenta, caquexia, hepatoesplenomegalia, onicogrifose, lesões de pele e secreção ocular purulenta. Estes sintomas apareceram mais precocemente nos cães do grupo controle. A imunização com antígeno rA2 permitiu a diferenciação sorológica entre animais vacinados e animais infectados, um requisito importante para uma vacina para leishmaniose visceral canina (Fernandes *et al.,* 2008).

Paralelamente aos testes vacinais com A2, também foram avaliados o potencial de alguns peptídeos em métodos diagnósticos sorológico (ELISA) utilizando, além de peptídeos de A2, antígenos de algumas proteínas bem caracterizadas em *Leishmania* como NH, LACK e K39 e suas associações. O peptídeos, provenientes de análises *in silico* das referidas proteínas apresentaram sensibilidade e especificidade elevados (próximo de 100%) quando testados com soros de cães com títulos baixos, médios e

altos, além dos soros humanos, sendo, portanto, promissores para o desenvolvimento de métodos diagnósticos sorológicos e futuramente cromatográficos (Costa *et al.,* 2012).

No entanto, em função da heterogeneidade de moléculas MHC das populações dos hospedeiros e da facilidade do parasita *Leishmania* em evadir a resposta imunológica consideramos a necessidade de identificação de novos antígenos para compor e/ou complementar as vacinas e aplicação em testes diagnósticos. Portanto optamos pela aplicação da análise proteômica que é resultado da integração de uma grande variedade de técnicas que permite identificar, quantificar e caracterizar proteínas, assim como relacionar essa informação com a Bioinformática.

Os recentes progressos na proteômica, como a introdução da espectrometria de massa para análise das macromoléculas associado a técnicas analíticas como a eletroforese bidimensional possibilitaram o estudo desses processos fisiológicos intricados e dinâmicos (Kumari *et al.,* 2008; Petrak *et al.,* 2008). Esta abordagem nos permitiu identificar proteínas diferencialmente expressas, além de proteínas imunogênicas nas formas amastigotas e promastigotas de *L. (L.) chagasi*. Adicionalmente, associamos as proteínas identificadas com análises *in silico* e imuno-ensaios que nos permitiram selecionar epítopos reconhecidos por células B e T para posteriores testes em vacinas e diagnóstico (Costa *et al.,* 2011). Testes diagnósticos em ELISA com 10 peptídeos derivados das proteínas imunogênicas identificadas por 2DE-Western-blot revelaram alta acurácia na análise cuja sensibilidade e especificidade foram elevadas como 88.7% e 95%, respectivamente (Faria *et al.,* 2011).

1.1. Agente Etiológico

Os protozoários do gênero *Leishmania* (Ross, 1903) pertencem a Família Trypanosomatidae, Ordem Kinetoplastida, Filo Sarcomastigophora e Sub-Reino Protozoa. Estes parasitas são digenéticos e apresentam duas formas morfologicamente distintas, as formas amastigotas, que são formas arredondadas com 3-5 μm, aflageladas e encontradas no hospedeiro vertebrado e as formas promastigotas que são alongadas com 5-15 μm, flageladas e encontradas principalmente no inseto vetor (Lainson & Shaw 1987; Grimaldi & Tesh, 1993; Ashford, 2000).

O modelo taxonômico mais abrangente e aceito foi o sugerido por Lainson & Shaw (1987), utilizando como parâmetro o desenvolvimento do parasito no intestino do inseto vetor. As espécies pertencentes ao subgênero *Leishmania*, desenvolvem-se na porção média e anterior do intestino (seção Suprapylaria) e possuem outras características como facilidade de crescimento em meio de cultura NNN tradicional. Já as espécies do subgênero *Viannia*, desenvolvem-se no intestino posterior, na região do piloro (seção Peripylaria) e apresentam lento crescimento em meio de cultivo e desenvolvimento lento (Lainson & Shaw 1987; Grimaldi & Tesh, 1993).

Atualmente, são descritas trinta espécies de *Leishmania*. No Velho Mundo, são registradas dez espécies, todas pertencentes ao subgênero *Leishmania* e dentre elas, sete espécies são conhecidas como capazes de infectar o homem. No Novo Mundo, são registradas vinte espécies, sendo que onze pertencem ao subgênero *Leishmania* e nove espécies pertencem ao subgênero *Viannia* (Grimaldi & Tesh, 1993; Cupolillo *et al.,* 2000). Dentre as espécies pertencentes ao subgênero *Leishmania*, cinco infectam o homem e são as espécies *L. mexicana, L. amazonensis, L. venezuelensis, L. chagasi (= L. infantum) L. major "like" (= L. major)* e dentre as espécies pertencentes ao subgênero *Viannia,* oito infectam o homem e são as espécies *L. braziliensis, L. peruviana, L. guyanensis, L. panamensis, L. lainsoni, L. naiffi, L. colombiensis e L. shawi* (Grimaldi & Tesh, 1993).

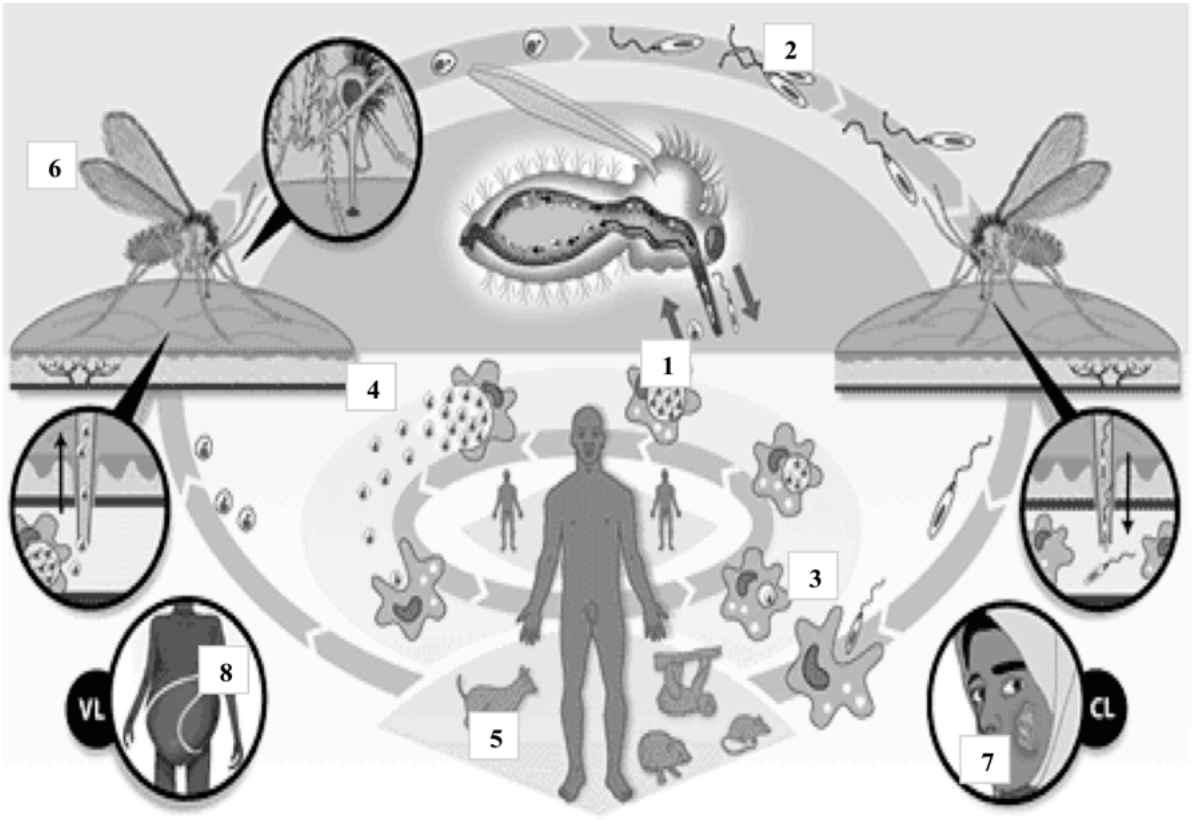
Os protozoários do gênero *Leishmania* necessitam dos hospedeiros vertebrados e invertebrados para completar seu ciclo de vida. Os vetores do parasita são dípteros, hematófagos, com atividade crepuscular e pós-crepuscular que abrigam-se durante o dia em lugares úmidos, sombrios e bem protegidos dos ventos e estão distribuídos principalmente em regiões com climas quentes e temperados. São pertencentes à família Psychodidae, subfamília Phlebotominae, sendo que somente dois gêneros são realmente importantes para a epidemiologia da doença: *Lutzomyia,* encontrados na América Central e América do Sul e *Phlebotomus,* encontrados na África, Ásia e Europa (Young & Ducan, 1994; Ashford, 2000; Bates *et al.,* 2008). Os vetores da *Leishmania* são flebotomíneos fêmeas e aproximadamente 1000 espécies já foram identificadas. Dentre elas 70 são consideradas vetores da doença (Bates *et al.,* 2008).

O ciclo de vida (Figura 1) se inicia quando o vetor fêmea se alimenta em um hospedeiro humano infectado (antroponose) ou em um outro hospedeiro vertebrado infectado (zoonose). O ciclo de vida dentro do intestino dos insetos é complexo e varia entre os subgêneros *Leishmania* e *Viannia* (Gossage *et al.,* 2003; Bates *et al.,* 2008; Wilson *et al.,* 2010). No subgênero *Leishmania*, as formas amastigotas ingeridas são liberadas dos macrófagos no intestino do inseto e se diferenciam em formas proliferativas denominadas promastigotas procíclicas, que 48-72 horas depois, se diferenciam em formas não-proliferativas. Após isso, entre o 7-10 dias após o a ingestão de sangue, elas se diferenciam em promastigotas metacíclicas, que representam as formas infectivas que migram para o aparelho bucal do vetor (Gossage *et al.,* 2003; Bates *et al.,* 2008). A ligação do parassita ao epitélio do intestino é um evento essencial no estabelecimento da infecção, pois impede a eliminação dos protozoários juntamente com as fezes (Bates *et al.,* 2008; Wilson *et al.,* 2010).

As formas metacíclicas são transmitidas para os hospedeiros vertebrados durante a alimentação do vetor, onde se diferenciam em formas amastigotas, e se multiplicam a ponto de lisar os macrófagos, e, conseqüentemente, permitir a infecção de novas células e também do inseto vetor (Matlashewski, 2001; Sacks & Noben-Trauth, 2002).

Considerando a leishmaniose visceral e o ciclo zoonótico rural e urbano, o cão (*Canis familiaris*) tem sido considerado o principal reservatório da doença, devido, principalmente, ao grande número de casos e devido ao grande parasitismo cutâneo destes animais (Abranches *et al.,* 1991). Portanto o cão representa grande importância no ciclo de transmissão da infecção, com ênfase em áreas urbanas. O cão infectado pode ou não desenvolver o quadro clínico da doença cujo sinais são: emagrecimento, alopécia, nódulos ou ulcerações, diarréia, hemorragias intestinais, hepato-esplenomagalia, paralisia de membros posteriores, ceratite com cegueira e caquexia, podendo evoluir para a morte nos casos mais graves. O reconhecimento das manifestações clínicas destes reservatórios é importante para adoção de medidas de controle da doença (Abranches *et al.,* 1991; Ashford *et al.,* 2000; Carvalho *et al.,* 2002; Porrozi *et al.,* 2007). No ambiente silvestre, os reservatórios são as raposas (*Dusicyon vetulus* e *Cerdocyon thous*), os marsupiais (*Didelphis albiventris*) e roedores (*Proechymis oris*). Os principais reservatórios na leishmaniose tegumentar variam de acordo com a espécie. Os principais reservatórios do

parasita *L. amazonensis* são os marsupiais e os roedores (*Proechymis* e *Oryzomys*), para a espécie *L. guyanensis* vários mamíferos foram identificados como hospedeiros naturais, tais como a preguiça (*Choloepus didactylus*), o tamanduá (*Tamandua tetradactyla*), marsupiais e roedores. Já para a espécie *L. braziliensis*, até o momento não se conseguiu identificar definitivamente nenhum animal silvestre como reservatório, no entanto, é freqüente encontrar em espécies domésticas, em grande quantidade, como no cão (Ceará, Bahia, Espírito Santo, Rio de Janeiro e São Paulo), eqüinos e mulas (Ceará, Bahia e Rio de Janeiro) e roedores domésticos ou sinantrópicos (Ceará e Minas Gerais) (Reithinger & Davies, 1999; Cabrera *et al.,* 2003; Garg & Dube, 2006; Campino & Maia, 2010; Ready PD, 2010).



Organização Mundial de Saúde (OMS) 2008 (http://www.who.int/en/)

Figura 1- Ciclo de vida do protozoário *Leishmania.* O ciclo de vida se inicia quando o vetor fêmea se alimenta em um hospedeiro vertebrado infectado (1). As amastigotas ingeridas se diferenciam no intestino do vetor em promastigotas procíclicas e iniciam a multiplicação. Após isso elas se diferenciam em promastigotas metacíclicas, e migram

para o aparelho bucal do vetor (2). As formas metacíclicas são transmitidas para os hospedeiros vertebrados durante a alimentação do vetor. As formas promastigotas metacíclicas são fagocitadas e nos macrófagos (3) se diferenciam em formas amastigotas, e se multiplicam a ponto de lisar os macrófagos e permitir a infecção de novas células (4) ou mesmo a infecção do inseto vetor (6). Em 5, 7 e 8 estão representados os variados hospedeiros vertebrados, um dos sintomas clínicos da leishmaniose tegumentar (lesão ulcerativa) e um dos sintomas clínicos da leishmaniose visceral (hepatoesplenomegalia), respectivamente.

1.2. As Leishmanioses

Espécies do gênero *Leishmania* são agentes etiológicos das diferentes formas clínicas das leishmanioses (visceral, cutânea, mucocutânea e difusa) que, por sua vez, apresentam distribuição geográfica e prevalências distintas (Ashford, 2000; Desjeux, 2004). A maioria dos casos de infecções humans estão relacionadas com hábitos, atividades primárias e/ou secundárias de indivíduos que exploram ou habitam florestas e, recentemente, tem ocorrido um aumento significativo nos casos de leishmaniose em áreas peri-urbanas de grandes cidades, em função de atividades como o desmatamento florestais, entre outras (Ashford, 2000; Desjeux, 2004; Murray *et al.,* 2005).

Dentre os 98 países em que são registradas a ocorrência de leishmanioses, 72 são países em desenvolvimento e 13 estão entre os menos desenvolvidos do mundo (WHO, 2010). Esta doença atinge mais de 12 milhões de pessoas. Cerca de 90% dos casos de leishmaniose visceral (LV) ocorrem no Brasil, Bangladesh, Índia, Nepal e Sudão e 90% dos casos de leishmaniose cutânea (LC) ocorrem no Brasil, Afeganistão, Argélia, Iran, Peru, Arábia Saudita e Síria (Ashford, 2000; Desjeux, 2004; Murray *et al.,* 2005).

Todos os anos, aproximadamente um milhão e meio de pessoas manifestam alguma forma clínica da leishmaniose tegumentar, e cerca de meio milhão de pessoas desenvolvem a forma visceral, sendo a incidência da infecção ainda maior se forem considerados os casos subclínicos (WHO, 2003; Desjeux, 2004).

A leishmaniose tegumentar no Novo Mundo se extende do México a Argentina atingindo países como Martinica, Honduras, Guatemala, Costa Rica, El Salvador, Nicarágua, Panamá, Venezuela, Colômbia, Guiana francesa, Equador, Paraguai, Bolívia, Peru e Brasil, apresentando alta incidência nestes dois últimos países (Ashford, 2000; Desjeux, 2004; Murray *et al.,* 2005). A leishmaniose visceral no Novo Mundo acomete países como Brasil, Paraguai, Argentina, Bolívia, Venezuela, Colômbia, Peru, Guatemala e México (Ashford, 2000).

Em função de seu caráter espectral existem diferentes propostas de classificação para as formas clínica da doença. A Organização Mundial de Saúde (OMS) classifica em leishmaniose cutânea, leishmaniose cutânea difusa, leishmaniose mucocutânea e a leishmaniose visceral (Ashford 2000; WHO, 2003; Desjeux, 2004; Murray *et al.,* 2005).

A leishmaniose cutânea se caracteriza pela formação de úlceras localizadas, podendo ser únicas ou múltiplas. A lesão aparece após um período de latência de 30 dias e permanece restrita ao local da inoculação. O desenvolvimento das lesões variam de acordo com a resposta imunológica dos paciente e a carga parasitária das lesões variam também de acordo com as espécies de *Leishmania* (Desjeux, 2004).

Na leishmaniose cutânea difusa as lesões são disseminadas pelo corpo, mas não são ulcerativas e acomete indivíduos com deficiência na resposta celular. A gravidade desta forma deve-se, principalmente, a dificuldade no tratamento das lesões (Ashford 2000; Murray *et al.,* 2005).

A leishmaniose mucocutânea se caracteriza por apresentar uma imunidade celular exacerbada, com granulomas constituídos por linfócitos e macrófagos, com poucos parasitas. Outa característica é a formação de lesões ulcerativas desfigurantes na mucosa e atinge principalmente as cavidades oral e nasal, podendo também acometer os tecidos da laringe, faringe e traquéia (Murray *et al.,* 2005; Desjeux, 2004).

A leishmaniose visceral é a forma mais grave, devido a sua elevada taxa de mortalidade que ocorre principalmente em pacientes não tratados. Ela causa febre, anemia, caquexia, diarréia e hepato-esplenomegalia (Desjeux, 2004).

1.3. Testes Diagnósticos para Leishmaniose Visceral

O uso de métodos diagnósticos sensíveis e específicos, de fácil execução e interpretação, que não necessitem de grande infra-estrutura laboratorial e profissionais especializados, seria de grande importância para um diagnóstico acurado e rápido da LV, principalmente nas localidades onde o acesso a exames laboratoriais mais complexos é limitado (WHO, 2003; Murray *et al.,* 2005; Mettler *et al.,* 2005; Moreira *et al.,* 2007; Porrozzi *et al.,* 2007).

O diagnóstico clínico-epidemiológico da LV é insuficiente para justificar o tratamento, já que a apresentação clínica é comum a diversas outras patologias. Portanto a confirmação por técnicas parasitológicas e ou imuno-enzimaticos é recomendado. Os exames parasitológicos são considerados métodos de referência no diagnóstico da LV, embora envolvam procedimentos invasivos, requeiram laboratoristas experientes, sejam laboriosos e não apresentem sensibilidade ideal. A confirmação de LV pode ser feita pela demonstração direta em esfregaços ou cultivo do parasito obtido de baço, fígado, medula óssea ou linfonodos. A sensibilidade da pesquisa direta em esfregaços em lâmina varia de 95 a 98% para o aspirado de baço (Chulay & Bryceson 1983), 76 a 91% para o de fígado, 52 a 89% para o de medula-óssea e 52 a 69% para o de linfonodos (Zijlstra *et al.,* 1992; Sidding *et al.,* 1988).

Em função dos pontos negativos do diagnóstico parasitológico, avanços foram realizados no diagnóstico sorológico, que é favorecido pela expressiva resposta imune humoral que caracteriza a leishmaniose visceral. A pesquisa de anticorpos, entretanto, deixa a desejar no que concerne à especificidade. Além disso, essas técnicas demandam tempo, equipamentos e laboratoristas treinados. Um dos métodos desenvolvidos foi a reação de fixação do complemento, que se difundiu por apresentar maior sensibilidade e por não ser um método invasivo, dependendo somente da coleta de sangue, além de apresentar maior rapidez na obtenção dos resultados (Hockmeyer *et al.,* 1984).

Logo, foi desenvolvida a reação de imunofluorescência indireta (RIFI) (Duxbury *et al.,* 1964) que apresenta alta sensibilidade de 82 a 95% e especificidade aproximada de 78 a 92%, dependendo da preparação antigênica e da espécie de *Leishmania* utilizada. O RIFI é o teste padrão ouro mundialmente indicado como comprovação final no

diagnóstico da ocorrência ou não de anticorpos de *Leishmania* no homem e é o teste atualmente disponibilizado pelo Sistema Único de Saúde (SUS), com a desvantagem de requerer microscópio de imunofluorescência (Sengupta *et al.,* 1969; Cahil *et al.,* 1970).

Entre os testes imuno-enzimáticos mais difundidos está o ELISA, que é utilizado principalmente para detecção de anticorpos, quando se deseja realizar um levantamento soroepidemiológico prático, menos oneroso e rápido. É sabido que a RIFI apresenta maior grau de especificidade e que o ELISA apresenta maior grau de sensibilidade. No ELISA, os valores de sensibilidade apresentam reprodutibilidade, variando entre 90 e 100%, entretanto, os valores de especificidade são bastante inconsistentes, variando de 71 a 100% (Bray, 1985; Choudhry *et al.,* 1990; Pedras *et al.,* 2008). O ELISA permite diversas variações metodológicas como os métodos indireto, sanduíche, competição e captura, além da utilização de antígenos brutos, solúveis ou recombinantes. Alguns antígenos recombinates como rA2, rK39, rK26, rK39 e rLdccys1 tem se destacado por otimizarem os resultados da sensibilidade e especificidade dos testes em cães, inclusive pela sensibilidade na detecção de animais assintomáticos (Burns *et al.,* 1993; Badaró, *et al.,* 1993; Braga *et al.,* 1998; Bathia *et al.,* 1999; Carvalho *et al.,* 2002; Teodoro-da-Costa *et al.,* 2003; Chappuis *et al.,* 2005; Mettler *et al.,* 2005; Ritmeijer *et al.,* 2005;Porrozzi *et al.,* 2007; Pinheiro *et al.,* 2009; Rouf *et al.,* 2009).

Tentando otimizar não somente a sensibilidade e especificidade, mas também a rapidez na obtenção dos resultados, o custo e a facilidade na coleta do material, houve o desenvolvimento do Direct Agglutination Test (DAT) e do Fast Agglitination Screening Test (FAST) e que tem apresentado bons resultados na LVC (Sundar *et al.,* 1996; Babakhan *et al.,* 2009). Eles apresentam sensibilidade e especificidade semelhantes, entretanto apresentam outras vantagens como a obtenção rápida dos resultados, o processamento de grande número de amostras, não necessita de mão de obra qualificada e somente o uso de amostras do sangue total é suficiente para a análise (Sundar *et al.,* 1996; Schallig *et al.,* 2002; Otramto *et al.,* 2004; Mettles *et al.,* 2005; Babakhan *et al.,* 2009).

Os métodos diagnósticos moleculares que empregam a PCR e diversas amostras biológicas, tais como sangue e aspirado de medula, são os mais sensíveis e específicos

(com sensibilidade acima de 90% e especificidade de 100%), entretanto apresenta elevado custo para o diagnóstico da leishmaniose (Schallig *et al.,* 2002; Reithinger *et al.,* 2002; Gomes *et al.,* 2008).

1.4. Vacinas para a Leishmaniose Visceral Canina

Umas da principais metas da Organização Mundial de Saúde é o desenvolvimento de uma vacina capaz de proteger o cão e conseqüentemente reduzir a taxa de infecção humana para leishmaniose visceral (WHO, 2003).

Várias formulações de vacinas já foram testadas e uma delas é a formulação composta de parasitas mortos ou atenuados (Tabela 1). Neste grupo foi testado *L. braziliensis* associada `a BCG, que apresentou proteção parcial em estudos de fase II, mas não em estudos em campo (Fase III) (Genaro *et al.,* 1996; Mayrink *et al.,* 1996). Foi testado *L. infantum* e/ou *L. major* associado `a BCG que apresentou proteção parcial em estudos de fase I e II (Mohebali *et al.,* 1998) assim como `a associação de *L. major* e BCG e Alum em estudos de fase III (Mohebali *et al.,* 2004). Além disso foi testado *L. infantum* atenuada com gentamicina o que garantiu proteção parcial em estudos de fase II (Daneshvar et al., 2010).

TABELA 1. Vacinas com Parasitas Mortos ou Atenuados

Autor/Data	Composição da Vacina	Proteção
Mayrink et al., 1996 (Fase II)	*L. braziliensis + BCG*	Fase II Proteção Parcial
Genaro et al., 1996 (Fase III)		Fase III – Montes Claros Não houve proteção.
Mohebali et al., 1998	*L. infantum ou L. major + BCG*	Fase I e II Proteção Parcial
Mohebali et al., 2004 (Fase III)	*L. major + BCG + Alum*	Fase III - Iran Proteção Parcial.
Daneshvar et al., 2010	*L. infantum atenuada com gentamicina*	Fase II Proteção Parcial

Também foram testadas Vacinas de DNA, como indicado na Tabela 2. Dentre elas, Ramiro e colaboradores, em 2003, em estudo de fase II obtiveram 60% de proteção em cães imunizados com o antígeno LACK na forma de DNA e uma dose reforço com o vírus vaccinia recombinate, também contendo a região codificadora da proteína LACK e desafiados com *L. infantum* (Ramiro *et al.,* 2003). Proteção parcial também foi observada

em associações semelhantes de LACK com rVV e MVA (Ramos *et al.,* 2008; Ramos *et al.,* 2009). Adicionalmente, foi avaliada a imunização de cães com as cisteínas proteinases do tipo I e II, sob a forma de DNA e de proteína recombinante contra a infecção por *L. infantum*, onde foi observada uma proteção parcial da doença (Rafati *et al.,* 2005).

TABELA 2. Vacinas de DNA (Plasmídeos)

Autor/Data	Composição da Vacina	Proteção
Ramiro et al., 2003	LACK pCIneo LACk + rVV	Fase I e II Proteção parcial.
Ramos et al., 2008	LACK	Fase I e II
Ramos et al., 2009	pORT LACK + rVV pORT LACK + MVA pORT LACK + MVA	Proteção parcial.
Rafati et al., 2005	Cisteína proteinase tipos 1 e 2	Fase I e II Proteção parcial.

Foram testadas Vacinas com frações do parasita, como indicado na Tabela 3. Uma das vacinas foi composta pela fração protéica de 67-94 kDa de L. infantum (LiF2) que inicialmente, em estudos de fase I e II indicaram proteção parcial, mas não em estudos de fase III (Ogunkolade *et al.,* 1988; Dunan *et al.,* 1989). A vacina Leishmune® composta por um complexo glicoprotéico fucose-manose ligante mostrou em um estudo de fase III, proteção de 92% dos cães vacinados, quando expostos em áreas endêmicas da leishmaniose visceral (Palatnik-de-Sousa *et al.,* 1994; Borja-Cabrera *et al.,* 2002; Borja-Cabrera *et al.,* 2010). Além disso, foi testada a fração LiESAP-MDP que garantiu proteção parcial nos estudos de de fase I, II e III (Lemestre *et al.,* 2005; Lemestre *et al.,* 2007).

TABELA 3. Vacinas com Frações do Parasita

Autor/Data	Composição da Vacina	Proteção
Ogunkolade et al., 1988 (Fase II) Dunan et al., 1989 (Fase III)	67-94 kDa *L. infantum* – LiF2	Fase I e II Proteção Parcial. Fase III Não houve proteção.
Palatnik de Souza et al., 1994 Borja-Cabrera et al., 2002 Borja-Cabrera et al., 2010	*L. donovani* - Complexo FML - Leishmune®	Fase I e II Proteção Parcial. Fase III Proteção Parcial.
Lemestre et al., 2005 (Fase II) Lemestre et al., 2007 (Fase III)	*L. infantum* – LiESAp-MDP	Fase I e II Proteção Parcial. Fase III Proteção Parcial.

Também foram testadas Vacinas com antígenos recombinantes, como indicado na Tabela 4. Proteção de 90% foi observada em estudo de fase II no qual cães foram imunizados com uma proteína quimérica composta pelas proteínas ribossomais Lip2a, Lip2b, PO e H2A associadas ao BCG. Os animais imunizados foram desafiados com *L. infantum* (Molano *et al.,* 2003). Fujiwara e colaboradores (2005) avaliaram potencias candidatos para uma vacina composta com a mistura dos antígenos recombinantes TSA, LeIF e LmSTI1 assciado ao adjuvante MPL-SE® em animais infectados com promastigotas de *L. chagasi* e observaram o desenvolvimento de uma resposta Th1. Proteção parcial também foi observada em infecção experimental com promastigotas de *L. infantum* em imunização com as proteínas H1, HASPB1 e MML (Moreno *et al.,* 2007). Face ao potencial do antígeno A2 em estudos de fase I em murinos (Coelho *et al.,* 2003; Zanin *et al.,* 2007), foi iniciado um estudo de fase II com essa proteína recombinante, compondo a vacina Leish-tec®. Os resultados obtidos evidenciaram proteção significativa associado a altos níveis de IFN-γ e baixos níveis de IL-10 nos cães imunizados. Além disso, foi verificado que, após o processo de imunização, os cães permaneceram soronegativos em testes de ELISA e imunofluorescência, corroborando os dados previamente obtidos em camundongos e que indicam que é possível a distinção sorológica entre cães imunizados com essa vacina e cães infectados (Fernandes *et al.,* 2008).

TABELA 4. Vacinas com Antígenos Recombinantes

Autor/Data	Composição da Vacina	Proteção
Molano et al., 2003 Carcelén et al., 2009	Proteína quimérica Q	Fase I e II Proteção Parcial.
Fujiwara et al., 2005 Gradoni et al., 2005	MML + MPL-SE® (rLeIF + rTSA + rLmSTI1)	Fase I e II Proteção Parcial. Fase III Não houve proteção.
Moreno et al., 2007	H1 + HASPB1 + Montanide	Fase I e II Proteção Parcial.
Fernandes et al., 2008	rA2 + Saponina Leish-tec	Fase I e II Proteção Parcial.

1.5. Proteoma de *Leishmania*

Os recentes avanços na proteômica tem possibilitado o estudo de processos biológicos e dinâmicos que associados a progressos paralelamente ocorridos na genômica do parasita *Leishmania*, tem otimizados o entendimento de suas características (Petrak *et al.,* 2008; Kumari *et al.,* 2008; Cuervo *et al.,* 2010). Muitos estudos tem sido realizados utilizando o parasita *Leishmania* afim de entender melhor a sua capacidade de evadir a resposta imune no hospedeiro, seu metabolismo, e também com o intuito de identificar futuros alvos para vacinas e diagnóstico, (Kumari *et al.,* 2008; Cuervo *et al.,* 2010).

Há muitos estudos comparando proteínas diferencialmente expressas entre as formas amastigotas (utilizando as amastigotas axênicas) e promastigotas de *L. infantum,* buscando identificar os fatores de virulência no parasita e que, consequentemente, auxiliam em sua adaptação no hospedeiro vertebrado (El Fakhry *et al.,* 2002; McNicoll *et al.,* 2006; Brotherton *et al.,* 2010). Adicionalmente, estudos com *L. major* comparando o perfil de proteínas entre as formas amastigotas provenientes de lesões e promastigotas também foram realizados e confrontados com a expressão gênica e com a expressão em *L. infantum* utilizando amastigotas axênicas (Leifso *et al.,* 2007), além de outros estudos com a forma cutânea comparando as formas promastigotas e amastigotas axênicas de *L. panamensis* (Walker *et al.,* 2006) e outro estudo coma a mesma abordagem foi realizado em *L. mexicana* (Nugente *et al.,* 2004). Outros trabalhos, com fosfoproteoma, de

comparação entre as formas amastigotas axênicas e promastigotas, utilizando a espécie *L. donovani* também foram descritos. (Morales *et al.,* 2008; Morales *et al.,* 2010).

Mapas proteômicos utilizando somente uma das formas do parasita, tem sido gerados tanto utilizando espécies causadoras de leishmaniose cutânea, como *L. major* (Drummelsmith *et al.,* 2003; Mojtahedi *et al.,* 2008)*, L. amazonensis* (Brobey *et al.,* 2006), *L. braziliensis* (Cuervo *et al.,* 2007) e *L. mexicana* (Paape *et al.,* 2008; Paape *et al.,* 2010), quanto espécies causadoras da leishmaniose visceral (Alcolea *et al.,* 2011; Gupta *et al.,* 2007). Outros trabalhos, avaliaram também a presença de proteínas imunogênicas nas formas amastigotas axênicas e promastigotas de *L. donovani* por western blot 2-D utilizando soro de pacientes humanos infectados (Forgber *et al.,* 2006). Outro estudo, com *L. infantum* avaliou a antigenicidade com soro hiper-imune de coelho (Dea-Ayuela *et al.,* 2006). Proteínas secretadas e/ou liberadas pelo protozoário *Leishmania* tem sido alvo de estudos na busca por importantes mediadores na interação parasita-hospedeiro (Cuervo *et al.,* 2009). Proteoma comparativo entre as formas amastigotas axênicas e amastigotas provenientes de baço de hamster também foi realizado afim de identificar os fatores de virulência necessários para o estabelecimento da infecção no hospedeiro (Pescher *et al.,* 2011).

Neste trabalho utilizamos uma abordagem proteômica associada a análises *in silico* para identificar potenciais antígenos de *L. (L.) chagasi*. Este estudo representa a primeira análise proteômica que utiliza amastigotas purificadas de baço de hamster e, também é o primeiro estudo a mapear a antigenicidade da infecção por *L. (L.) chagasi* utilizando soros de cães de fase aguda e crônica. Foi utilizado a técnica de DIGE para comparar a expressão diferencial de proteínas entre as formas amastigotas e promastigotas de *L. (L.) chagasi*. Além disso, todas as proteínas identificadas no trabalho foram mapeadas para epítopos de células T usando o software NetCTL, que permitiu a seleção de potenciais candidatos para o desenvolvimento de vacinas. Portanto, uma análise em grande escala de *L. (L.) chagasi* através do proteoma foi realizada para identificar epítopos de células B e T com potencial para utilização no desenvolvimento de testes diagnósticos e vacinas.

2. OBJETIVOS

2.1. Objetivo Geral

Identificar e caracterizar antígenos de *Leishmania (Leishmania) chagasi* para o desenvolvimento de vacinas e alternativas em diagnóstico.

2.2. Objetivos Específicos

1. Avaliar a resposta imune e de proteção induzida pelas vacinas de DNA de A2 e NH (Nucleosídeo Hidrolase) contra a infecção por *Leishmania chagasi* e *Leishmania amazonensis* em camundongos;
2. Analisar a proteção imunológica em cães beagles vacinados com a proteína recombinante A2 contra a infecção desafio com *Leishmania chagasi* ;
3. Testar combinação de peptídeos sintéticos provenientes das proteínas A2, NH, LACK e K39 no imuno-diagnóstico da leishmaniose visceral canina e humana;
4. Identificar novos antígenos para diagnóstico e vacina na Leishmaniose Visceral Canina utilizando aboradagem imunoproteômica e de bioinformática;
5. Analisar o potencial imuno-diagnóstico de peptídeos sintéticos provenientes de proteínas identificadas por western blot-2D associadas a analises *in silico.*

3. METODOLOGIA E RESULTADOS: ARTIGOS PUBLICADOS

3.1. Artigo 1. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections.

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Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections

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Abstract

Several antigens have been tested as vaccine candidates against *Leishmania* infections but controversial results have been reported when different antigens are co-administered in combined vaccination protocols. Immunization with A2 or nucleoside hydrolase (NH) antigens was previously shown to induce Th1 immune responses and protection in BALB/c mice against *Leishmania donovani* and *L. amazonensis* (A2) or *L. donovani* and *L. mexicana* (NH) infections. In this work, we investigated the protective efficacy of A2 and NH DNA vaccines, in BALB/c mice, against *L. amazonensis* or *L. chagasi* challenge infection. Immunization with either A2 (A2-pCDNA3) or NH (NH-VR1012) DNA induced an elevated IFN-γ production before infection; however, only A2 DNA immunized mice were protected against both *Leishmania* species and displayed a sustained IFN-γ production and very low IL-4 and IL-10 levels, after challenge. Mice immunized with NH/A2 DNA produced higher levels of IFN-γ in response to both specific recombinant proteins (rNH or rA2), but displayed higher IL-4 and IL-10 levels and increased edema and parasite loads after *L. amazonensis* infection, as compared to A2 DNA immunized animals. These data extend the characterization of the immune responses induced by NH and A2 antigens as potential candidates to compose a defined vaccine and indicate that a highly polarized type 1 immune response is required for improvement of protective levels of combined vaccines against both *L. amazonensis* and *L. chagasi* infections.

Keywords: DNA vaccine; A2 antigen; *Leishmania chagasi*; *Leishmania amazonensis*

1. Introduction

Infection by parasites of the genus *Leishmania* induces a wide spectrum of diseases. In Americas, at least eight different species of *Leishmania* are major ethiologic agents of leishmaniasis, including *Leishmania chagasi* and *L. amazonensis*, which have wide and overlapping areas of transmission and

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are associated with visceral (VL) and tegumentary (TL) leishmaniasis, respectively [1,2].

Due to the high antigenic diversity of the *Leishmania* parasites, a multistage vaccine may be necessary for optimal protection against infections. Although several defined antigenic preparations have been tested as vaccine candidates against *Leishmania* infections, few were shown to induce protection against more than one *Leishmania* species. In addition, distinct results have been reported when different antigens are co-administered in the same vaccination protocol [3–6].

The A2 protein was first identified in *L. donovani* as part of an amastigote stage-specific protein family [7]. Karyotype analysis revealed that A2 genes are conserved in *L. donovani*, *L. chagasi*, *L. amazonensis* and *L. mexicana* species [8]. Immunization with recombinant A2 (rA2) protein or A2 DNA was shown to protect BALB/c mice against *L. amazonensis* [5] or *L. donovani* infection [9,10].

The nucleoside hydrolase (NH) antigen was initially identified in *L. donovani* as a major fraction of the fucose-mannose ligand (FML) antigenic complex [11]. This surface glycoprotein complex is expressed in *L. donovani*, *L. chagasi*, *L. amazonensis* and *L. major* [12]. Paraguai-de-Souza et al. [13] demonstrated that immunization with recombinant NH (rNH) protein was able to induce partial protection in BALB/c mice against *L. donovani* infection. Partial protection was also obtained by immunization with NH DNA in BALB/c mice against *L. chagasi* or *L. mexicana* infections [14].

Based on previous evidences that A2 and NH antigens induce Th1 immune responses and partial protection against *Leishmania* species, in this work, we investigated the immune responses and protective efficacy of A2 (A2-pCDNA3) and NH (NH-VR1012) DNA and their association (NH/A2 DNA), in BALB/c mice, against *L. chagasi* or *L. amazonensis* infections. DNA vaccines were chosen as an antigenic delivery system, since they are easily prepared and able to induce type 1 and $CD8^+$ cytotoxic T-lymphocyte immune responses [15,16].

2. Materials and methods

2.1. Parasites

Leishmania chagasi (MHOM/BR/1975/M2682) was grown at 26 °C in Dulbecco Modified Eagle medium (DMEM, Sigma), supplemented with 20% heat-inactivated fetal bovine serum (FSB, Cultilab, Brazil), 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoetanol and 20 μg/ml of gentamicin, at pH 7.0. *L. amazonensis* (IFLA/BR/1967/PH-8) was grown at 23 °C in Schneider's medium (Sigma) supplemented with 20% FSB, 20 mM L-glutamine, 50 μg/ml of gentamicin, 200 U/ml of penicillin and 100 μg/ml of streptomycin, at pH 7.4.

2.2. Preparation of antigens

Soluble *L. amazonensis* Antigenic extract (SLA) was prepared from stationary phase promastigotes, as described previously [5]. Briefly, 2×10^8 promastigotes/ml were collected and resuspended in 10 ml of sterile phosphate buffer saline (PBS). After five freezing-thawing cycles, the suspension was centrifuged at 8000 × *g* for 30 min at 4 °C. The supernatant was collected, quantified by the Bradford method [17] and stored at −86 °C.

L. chagasi promastigotes antigenic extract (LcPA) was prepared as described [18]. Briefly, 2×10^8 promastigotes/ml of *L. chagasi* were harvested from late log phase cultures by centrifugation, washed three times in sterile PBS, disrupted by three rounds of freezing-thawing and five cycles (30 s each one) of sonication. The suspension were centrifuged at 8000 × *g* for 30 min at 4 °C and the supernatant was collected, quantified by Bradford method [17] and stored at −86 °C.

2.3. Purification of plasmids

pCDNA3-A2 (A2 DNA) and pET16b-A2 plasmids were kindly provided by Dr. Greg Matlashewski (Microbiology Immunology Department, McGill University, Montreal, Quebec, Canada) and VR1012-NH (NH DNA) and pMALc2-NH by Dr. Clarisa Palatnik de Sousa (Departamento de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). *Escherichia coli* DH5α cells transformed with A2 DNA, NH DNA or with empty plasmids (pCDNA3 or VR1012) were cultured in Luria Bertani (LB; Gibco) liquid medium containing ampicilin (100 μg/ml). Endotoxin-free plasmids DNA were isolated using maxipreps DNA purification kits (Qiagen). Plasmids concentrations were determined by spectrophotometry (260:280 nm), using the Gene Quant II apparatus (Pharmacia Biotech).

2.4. Purification of recombinant proteins

pET16b-A2 plasmid was utilized for expression and purification of recombinant A2 (rA2; ~53 kDa) protein, as previously described [19]. The recombinant NH (rNH) protein expression was obtained after excision of its encoding sequence from pMALc2-NH plasmid, using EcoRI and XbaI endonucleases and ligation into pPROEX-b vector. Then, *E. coli* DH5α cells were transformed with pPROEX b-NH and grown in the presence of 1 mM of IPTG for 3 h (Promega, Montreal, Canada). Cells were disrupted by five cycles of freezing-thawing, followed by mild ultrasonic treatment (five cycles of 30 s each one with an ultrasonic processor) and then were centrifuged at 13,000 × *g* for 30 min at 4 °C. After protein expression, recombinant NH (NH-HIS ~36 kDa) protein was purified by nickel affinity chromatography, according to the manufacturer's instructions (Qiagen).

2.5. Mice and immunization protocol

Groups of female BALB/c mice ($n = 8$), 4–6 weeks old, were immunized intramuscularly in their left hind with 100 μg of A2 DNA, NH DNA or with NH/A2 DNA (100 μg of each plasmid). Two plasmid doses were administered in PBS plus sucrose 25%, at 3-weeks intervals. Control mice

received 100 μg of either pCDNA3 or VR1012 vectors alone or in combination (100 μg each), in PBS plus sucrose 25% or only PBS plus sucrose 25%.

2.6. Challenge infection

Four weeks after the last vaccine dose, BALB/c mice were infected in the tail vein with 1×10^7 late log phase *L. chagasi* promastigotes. Mice were sacrificed 35 days after challenge, when liver and spleen were harvested for determination of parasite burdens and cytokine responses.

For *L. amazonensis* infection, mice were challenged with 1×10^6 late stationary phase promastigotes into their right hind footpad. In these animals, the course of disease was monitored at weekly intervals, by measuring footpad thickness with a metric caliper. Results were expressed as the increase in the thickness of the infected hind foot compared to the uninfected left foot. After lesion onset, the course of infection was monitored during 9 weeks, when tissue skin fragments were harvested for parasite burden determinations and spleen cells were collected for evaluation of cellular immune responses.

2.7. Parasite quantification

Liver and spleen were collected to determine the parasite burdens in mice challenged with *L. chagasi*, as previously described [18]. Briefly, organs were weighted, fragmented and a tissue homogenate was obtained in 1 ml of DMEM plus 20% FBS, at pH 7.0. Five-fold serial dilutions were performed in 96-well flat-bottom microtiter plates (Nunc, Nunclon), in duplicate, and plates were incubated at 23 °C. Plates were evaluated for parasite growth using a microscope (Axiovert 25, Zeiss). The number of viable parasites was determined from the highest dilution at which promastigotes had grown, after 12 days of incubation

The number of viable parasites at the site of *L. amazonensis* infection was determined by a limiting dilution assay, as described previously [5]. Briefly, skin fragments were excised and homogenized in Schneider's medium supplemented with 20% FBS, at pH 7.4. Each tissue homogenate was serially diluted in 96-well plates (Nunc, Nunclon), in duplicate, and incubated at 23 °C. The wells containing motile promastigotes were identified and the number of viable parasites was determined from the highest dilution at which promastigotes had grown after 7 days of incubation.

2.8. Cytokine production assays

Splenocyte cultures were performed as described previously [5]. Briefly, single-cell preparations (5×10^6 cells/ml) obtained from spleen were plated, in duplicate, in 24-well plates (Nunc, Nunclon). Cells were incubated in DMEM (background control) or separately stimulated with concanavalin A (positive control; 5 μg/ml), rA2 or rNH proteins (10 μg/ml), SLA or LcPA (50 μg/ml) in 5% CO_2, for 48 h, at 37 °C. Levels of IFN-γ, IL-4 and IL-10 were assessed in supernatants by ELISA, as described elsewhere [5,20].

2.9. Statistical analysis

All data comparisons were tested for significance by using unpaired Student's *t*-test or Kruskal–Wallis test. Differences were considered statistically significant when P values were <0.05.

3. Results

3.1. Immunization with A2 DNA, but not NH DNA, confers protection in BALB/c mice against L. chagasi or L. amazonensis

The protective effect of immunization of BALB/c mice with NH, A2 or NH/A2 DNA vaccines against *L. chagasi* infection was evaluated by measuring parasite loads in liver and spleen (Fig. 1). Non-significant reductions in parasite numbers in liver or spleen were observed in mice immunized with NH DNA, when compared to saline or VR1012 immunized animals. In contrast, mice immunized with A2 DNA or NH/A2DNA displayed significant reductions in both hepatic and splenic parasite loads, as compared to saline, pCDNA3, VR1012/pCDNA3 and NH groups. Non-significant differences were observed between A2 and A2/NH groups.

After *L. amazonensis* challenge infection, the protective efficacy of immunization was evaluated by measuring lesion development (Fig. 2A) and parasite loads in the infected footpads (Fig. 2B). No significant reduction in footpad swelling or parasite loads was observed in animals immunized with NH DNA, as compared to saline or VR1012 immunized animals. In contrast, mice immunized with A2 DNA showed decreased edema of the infected footpads as compared to saline or pCDNA3 groups (Fig. 2A). The reduction in lesion development observed in A2 DNA group correlated with a 10^6 decrease in parasite burden in the infected footpads as compared to saline or pCDNA3 (Fig. 2B). Mice immunized with NH/A2 DNA also showed significant reductions in footpad

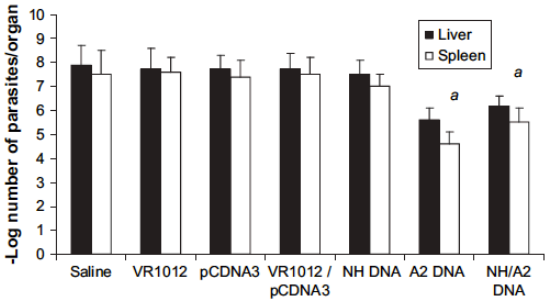

Fig. 1. *Leishmania chagasi* protection assays. Mice were vaccinated as described in Section 2 and challenged i.v. with 1×10^7 *L. chagasi* promastigotes. Parasite burden determinations were performed by limiting dilution assays, 35 days after infection. Experiments were performed in triplicate. Each bar represents mean ± standard deviation of results obtained from four mice per group. The letter in italic (*a*) indicates significant differences as compared to saline and control DNA groups (pCDNA3, VR1012 or VR1012/pCDNA3).

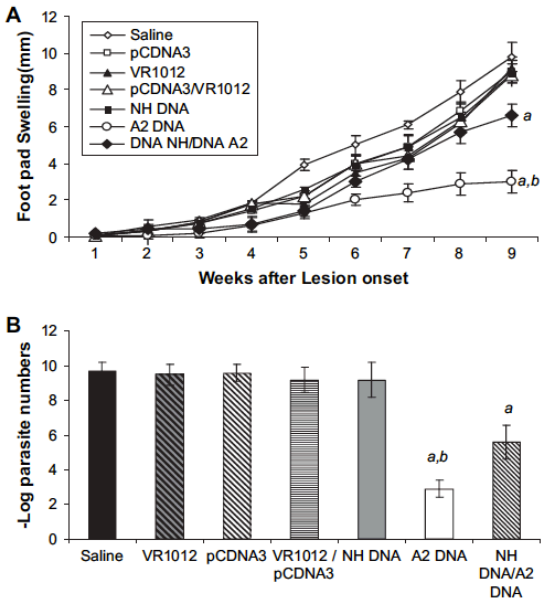

Fig. 2. *Leishmania amazonensis* protection assays. Mice were vaccinated, as described in Section 2.5 and challenged with 1×10^6 late stationary phase *L. amazonensis* promastigotes. Panel (A) shows lesion development in immunized groups, monitored weekly with a caliper. Panel (B) shows parasite loads per mg of tissue fragments, as determined by a limiting dilution assays, 9 weeks post-challenge. Experiments were performed in triplicate. Each point or bar represents the mean ± standard deviation of results obtained from four mice per group. Italic letters indicate significant differences as compared to saline and control DNA groups (pCDNA3, VR1012 or VR1012/pCDNA3) (*a*) or as compared to the NH/A2 DNA group (*b*).

swelling and parasite loads in comparison to saline or VR1012/pCDNA3 groups; however, both lesion size and parasite loads were significantly higher as compared to A2 DNA group.

3.2. Characterization of the cellular (IFN-γ, IL-4 and IL-10) response in immunized animals, before challenge infection

The production of IFN-γ, IL-4 and IL-10 by spleen cells of immunized mice was evaluated prior to (30-days after second vaccine dose) and after challenge infection (35-days after *L. chagasi* infection and 9 weeks after *L. amazonensis* infection).

Prior to challenge infection (Fig. 3A), spleen cells taken from animals immunized with either NH DNA, A2 DNA or NH/A2 DNA produced high levels of IFN-γ in response to each recombinant protein, in comparison to saline or control DNA groups (pCDNA3, VR1012 or VR1012/pCDNA3); however, mice immunized with A2 DNA produced significantly higher levels of this cytokine as compared to NH DNA group, after specific recombinant protein stimulation. Mice immunized with NH DNA showed a significantly higher IL-4 production (Fig. 3B), in comparison to the other groups. IL-10 levels were not significantly different among the different groups, except for NH DNA group which showed an increased production of this cytokine in response to rNH stimulation.

3.3. Characterization of the cellular (IFN-γ, IL-4 and IL-10) response in immunized mice, after challenge infection with L. chagasi *or* L. amazonensis

After *L. chagasi* challenge infection, lower IFN-γ levels (Fig. 4A) and significantly higher IL-4 and IL-10 levels (Fig. 4B) were detected in response to LcPA stimulation, in mice that received saline or control DNA, as compared to the levels produced before challenge. In contrast, immunization with A2 DNA induced a higher IFN-γ production and lower IL-4 and IL-10 levels in response to both rA2 and LcPA as compared to saline, control DNA groups or NH DNA (after stimulation with NH or LcPA). Although splenocytes from NH/A2 DNA immunized animals produced increased IFN-γ levels (Fig. 4A) in response to rNH, rA2 or LcPA stimulation, these animals also produced significantly higher IL-4 and IL-10 levels (Fig. 4B), as compared with A2 DNA immunized mice. These data indicate that, after *L. chagasi* infection the IFN-γ response was boosted in NH/A2 DNA immunized animals but this was not sufficient to significantly improve the control of parasite replication, when compared to the levels observed in mice immunized with A2.

After *L. amazonensis* challenge infection, mice that received saline or control DNA produced low levels of IFN-γ (Fig. 4C) and higher IL-4 and IL-10 levels (Fig. 4D) in response to SLA, as compared to those observed before challenge. Splenocytes of mice immunized with NH DNA showed a similar cytokine profile. Moreover, IFN-γ production in response to rNH or SLA was not significantly boosted after infection in the NH DNA immunized mice. Mice immunized with A2 DNA, in contrast, showed a significant and sustained IFN-γ production and very low IL-4 and IL-10 levels as compared to the levels obtained prior to infection. Significantly higher IFN-γ levels and lower IL-4 and IL-10 in response to rA2 or SLA were also observed in these mice as compared to saline and pCDNA3 groups. A similar profile was observed in NH/A2 mice immunized; however these mice produced lower IFN-γ and higher IL-4 and IL10 levels in response to rNH, rA2 or SLA stimulation, as compared to A2 immunized animals.

4. Discussion

Protective immunity against *L. chagasi* and *L. amazonensis* is dependent of IFN-γ production, a marker of type 1 immune response [3,20–23]. In agreement, the significant reduction of parasite loads observed in mice immunized with A2 DNA in skin (*L. amazonensis* challenge) or in liver and spleen (*L. chagasi* challenge) was associated with high IFN-γ and low IL-4 and IL-10 production, before and after challenge infection. Similar findings were previously reported indicating that immunization with A2 antigen is protective against *L. donovani* and *L. amazonensis* [5,9,10].

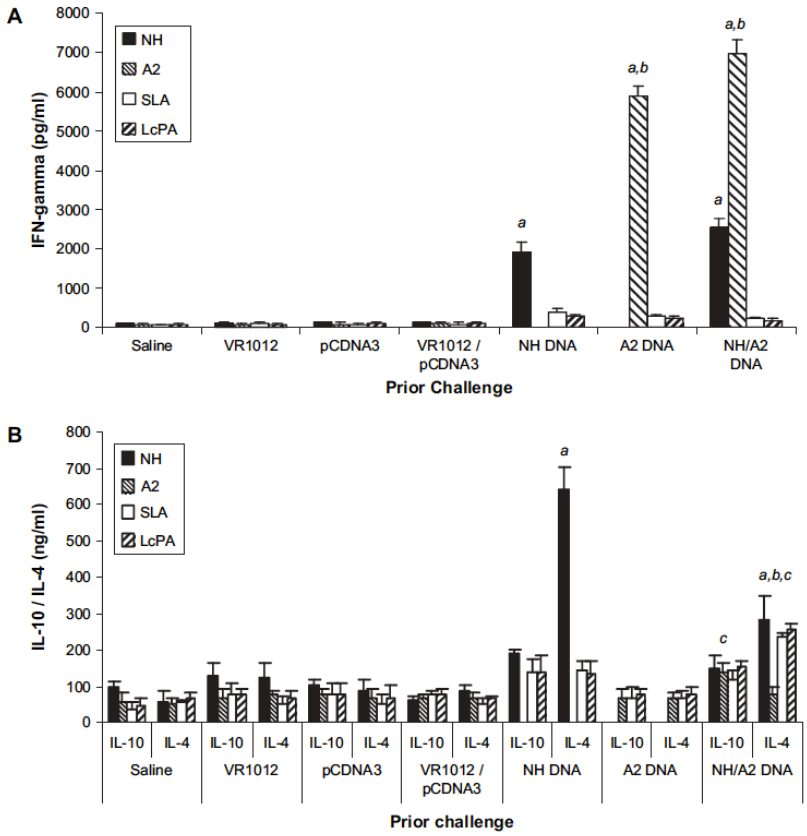

Fig. 3. Production of IFN-γ, IL-4 and IL-10 by spleen cells prior challenge infections. Panel A shows IFN-γ levels and panel B IL-4 and IL-10 levels, as determined by ELISA in culture supernatants of spleen cells stimulated with rNH or rA2 proteins (10 μg), SLA or LcPA (50 μg). Each bar represents mean ± standard deviation of results obtained from four mice per group. Letters in italic indicate statistical significant differences as compared to saline and control DNA groups (pCDNA3, VR1012 or VR1012/pCDNA3) (*a*), as compared to NH DNA group (*b*) or as compared to A2 DNA immunized group (*c*).

Mice immunized with NH DNA were not protected against *L. chagasi*, since significant reduction on parasite burden in liver and spleen were not detected. Partial protection in BALB/c mice immunized with rNH protein or NH DNA and challenged with *L. donovani* or *L. chagasi* was previously reported by Paraguai-de-Souza et al. [13] and Aguilar-Be et al. [14], respectively. Differences in immunization protocols and virulence of parasites might explain these different results.

Significant reductions of parasite numbers at both spleen and liver were observed in A2 immunized mice. The evaluation of parasite loads in the spleen, although frequently neglected in vaccine studies, might be an important marker of vaccine efficacy against visceral leishmaniasis, since the requirements for effective immune mechanisms may not be adequately induced by vaccination in the specific tissue sites. Moreover, during the early stages of visceral infection in BALB/c mice, parasites multiply in large numbers in the liver. Lately, the hepatic parasite load tends to decrease while the splenic parasitism tends to increase [21,24,25]. Thus, it seems that liver serves as an indicator of the initial multiplication of parasites and spleen as a reservoir for them [25].

After *L. amazonensis* infection, the IFN-γ production primed by NH DNA immunization was not boosted. The lack of a boost effect may not be attributed, however, to the lack of NH expression by *L. amazonensis*, since anti-NH antibodies present in sera of NH DNA immunized mice reacted with NH native protein in antigenic preparations of *L. amazonensis* or *L. chagasi* (data not shown). However, before challenge, NH immunized animals displayed increased levels of IL-4 in response to NH stimulation, as compared to saline controls. After challenge, an increased production of both IL-4 and IL-10 in response to NH or SLA was observed in these animals, when compared to A2 immunized mice. Thus, the failure of NH DNA immunized mice to control *L. amazonensis* infection might be attributed to the higher IL-4 and IL-10 levels observed in these animals, when compared to the levels observed in A2 immunized mice.

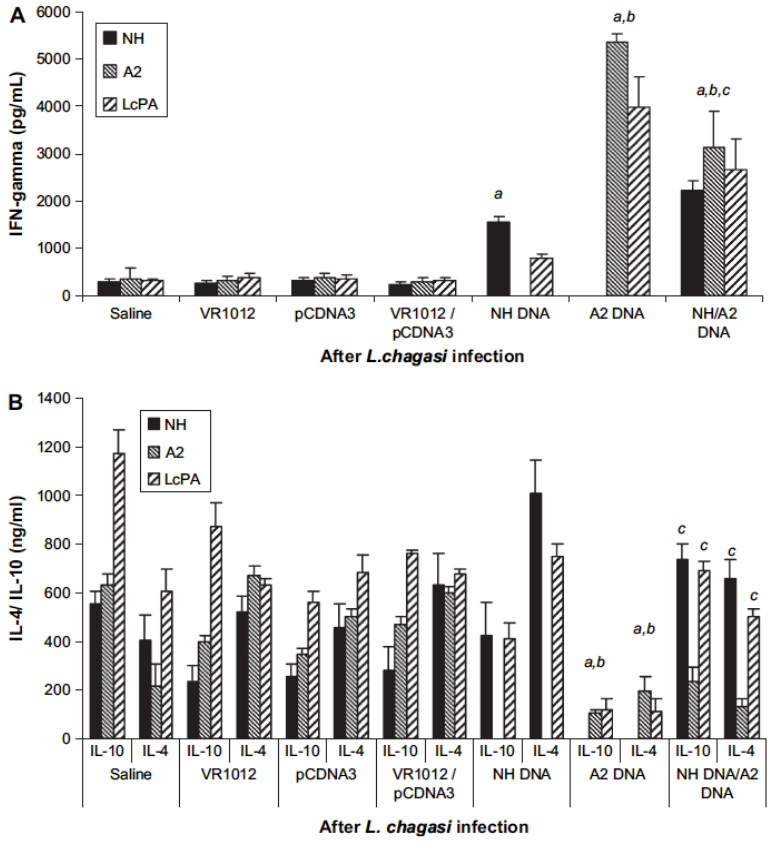

Fig. 4. Production of IFN-γ, IL-4 and IL-10 by spleen cells 35-days after *L. chagasi* or 9 weeks after *L. amazonensis* challenge infections. IFN-γ, IL-4 and IL-10 were determined by ELISA in culture supernatants of spleen cells stimulated with rNH or rA2 proteins (10 μg), SLA or LcPA (50 μg). Each bar represents mean ± standard deviation of results obtained from four mice per group. Panels A and C show IFN-γ levels after *L. chagasi* or *L. amazonensis* infections, respectively. Panels B and D show IL-4 and IL-10 levels after *L. chagasi* or *L. amazonensis* infections, respectively. Italic letters indicate statistical significant differences as compared to saline and control DNA groups (pCDNA3, VR1012 or VR1012/pCDNA3) (*a*), as compared to NH DNA group (*b*) or as compared to A2 DNA group (*c*).

Recent evidence suggest, that in *L. amazonensis* experimental infection, combined effects of low levels of IFN-γ and high levels of parasite-specific antibodies promote the rapid recruitment of immature or insufficiently activated macrophages, that favors the replication of amastigotes and progression of disease [20]. IL-4 and IL-10 contributes partially to these deficient immune responses [23,27]. IL-10 has multiple effects in suppressing microbicidial leishmaniasis activity in the macrophages, IFN-γ production and, consequently, preventing parasite clearance in susceptible mice [21,22,25,28,29].

Although decreased edema and parasite loads were observed in NH/A2 DNA immunized mice as compared to control groups, the association of NH DNA and A2 DNA partially inhibited the A2 DNA protective effect. Similar results were observed in mice immunized with recombinant A2 and LACK proteins [5]. The IFN-γ levels induced by A2 were completed inhibited in A2/LACK immunized animals, suggesting that an antigenic competition mechanism would be involved. The failure to induce protection by immunization with LACK antigen was also accompanied by high levels of IL-4 and IL-10 [5,18]. Since NH/A2 immunized mice were still able to produce significant amounts of IFN-γ in response to rA2, rNH, LcPA or SLA, it seems that the inhibition observed is not due to antigenic competition, but might be attributed to the high IL-4 and IL-10 levels. Stober et al. [29] demonstrated that pre-challenge high IL-10 production was associated with failure of rLACK protein to protect BALB/c mice against *L. major* infection and suggested that the ratio of IFN-γ/IL-10 levels may provide an indicator of vaccine success in this murine model. IL-10 has been shown to participate of parasite persistent in spleen of *L. donovani* infected mice. Mixed immune responses with concomitant production of IFN-γ, IL-4 and IL-10 are also observed in *L. donovani* infections and the progressive development of splenic pathology was associated with high levels of both TNF and interleukin IL-10. IL-10 promotes impaired DC migration into T-cell areas with consequent ineffective T-cell priming [30].

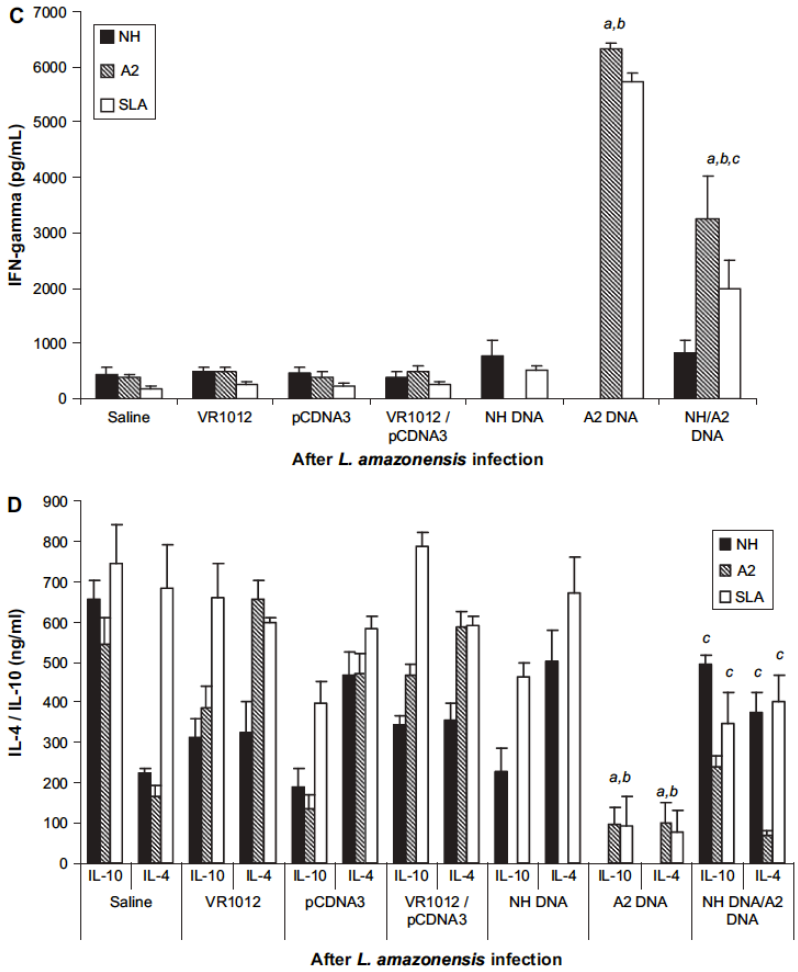

Fig. 4. (*Continued*)

Taken together, our data confirm that high IFN-γ and low IL-4 and IL-10 levels are required for protection in BALB/c mice against *L. chagasi* and *L. amazonensis* infection. The association of well-characterized antigens and potent adjuvant systems, such as viral expression vectors might achieve this polarized type 1 immune response. In this way, results presented herein extended the characterization of the immune responses induced by NH and A2 antigens as potential candidates to compose a vaccine applicable to the complex epidemiological context of leishmaniasis in Americas.

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3.2. Artigo 2. Protective immunity against challeng with *Leishmania (Leishmania) chagasi* in beagles dogs vaccinated with recombinant A2 protein.

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Protective immunity against challenge with *Leishmania* (*Leishmania*) *chagasi* in beagle dogs vaccinated with recombinant A2 protein

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ABSTRACT

In this study, we investigated in dogs the immunogenicity and protective immunity against *Leishmania* (*Leishmania*) *chagasi* infection induced by vaccination with a formulation containing the recombinant A2 protein, an amastigote specific antigen, and saponin. Vaccinated animals produced significantly increased levels of total IgG and IgG2, but not IgG1 anti-A2 antibodies, and remained negative in conventional leishmaniasis serodiagnostic methods. Significantly increased IFN-γ and low IL-10 levels were detected in vaccinated animals before and after challenge, as compared to control animals. Importantly, while the symptoms onset appeared as early as three months after infection in most control dogs, 14 months after challenge, 5 out of 7 vaccinated dogs remained asymptomatic. Therefore, immunization with rA2 antigen was immunogenic and induced partial protection in dogs, and allowed the serological differentiation between vaccinated and infected animals, an important requirement for a canine visceral leishmaniasis (CVL) vaccine.

1. Introduction

Leishmaniasis is an infectious disease with an overall prevalence of 12 million cases and 350 million people at risk of infection [1]. *Leishmania* parasites are transmitted through the bite of an infected sand fly to vertebrate hosts, leading to a spectrum of clinical manifestations, including visceral leishmaniasis [1]. *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *chagasi*, which are now considered synonymous species [2], and *Leishmania* (*Leishmania*) *donovani* are the major etiologic agents of visceral leishmaniasis (VL), a fatal infection if diagnosis and treatment are not promptly established. Dogs are highly susceptible to infection and considered the major reservoir for *L.* (*L.*) *infantum* and *L.* (*L.*) *chagasi*, in different geographical regions of the globe [3]. Canine visceral leishmaniasis (CVL) is assuming increasing importance in countries around the Mediterranean Basin and in the Americas. Research has recently revealed much new information, but advances in treatment and control have been disappointing. Treatment of infected dogs is of limited effectiveness and not recommended in endemic regions, since dogs that respond to treatment may still be a source of parasites [3,4].

The epidemiological control of VL depends of actions against the vectors (sand flies) and reservoirs, mainly the domestic dogs. Therefore, the development of a protective vaccine against CVL is an alternative approach for interrupting the VL domestic cycle [3,5]. Ideally, this vaccine should reduce symptoms, tissue parasitism as well as vector transmission rates. In addition, it is recommended that it allows for the serological differentiation of vaccinated and infected dogs, by means of serological tests using promatigote

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antigens, i.e. indirect immunofluorescence (IFI) or ELISA, since culling of sero-positive dogs is a major VL control measure adopted in endemic areas in Americas.

The A2 antigen has been identified in *L.* (*L.*) *donovani* and represents the first amastigote-specific virulence factor identified in *Leishmania*. A2 genes are present within a multi-gene family that code for A2 proteins ranging from 45 to 110 kDa, which are required for *Leishmania* survival in mammalian hosts [6–8]. A2 antigens, administered as recombinant protein or DNA, are protective against *L.* (*L.*) *donovani*, *Leishmania* (*Leishmania*) *amazonensis* and *L.* (*L.*) *chagasi* infections in mice [9–12]. Anti-A2 antibodies have been detected in sera samples from patients with active VL, confirming that A2 proteins are expressed during active infection [13,14]. Anti-A2 antibodies are also detected in sera of infected dogs [14], including symptomatic and asymptomatic animals, though higher numbers of asymptomatic animals are reactive as compared with the symptomatic dogs [15]. Moreover, A2 antigen was one of the antigens identified through a *L.* (*L.*) *chagasi* T-cell antigens screening [16], indicating the presence in A2 antigen of T cell epitopes, an important requirement for induction of protection against leishmaniasis.

In this work, we have investigated the protective effect of vaccination with the recombinant A2 (rA2) protein against *L.* (*L.*) *chagasi* infection in dogs. Our findings demonstrated that immunization with rA2 induced type I immune responses, protected a significant number of dogs from developing symptoms and delayed symptoms on those that developed CVL. Finally, vaccination with rA2 did not convert serological tests using promatigote antigens, allowing for discrimination between vaccinated and infected animals, an important requirement for a public campaign control of CVL.

2. Materials and methods

2.1. Animals

The study included twenty-one 3–9-months-old beagle dogs, purchased from dog breeders located in non-endemic areas of Brazil. After a quarantine period, only healthy and well-fed animals under constant scrutiny for health problems by veterinarian, presenting normal hematological and biochemical parameters and that had received their routine vaccinations against leptospirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and parvovirosis were included in this study. Dogs were also treated with anti-helmintic drugs. The dogs were maintained according to the International Guiding Principles for Biomedical Research Involving Animals. The experimental design and all the conditions of animal maintenance and handling were approved by the Brazilian Public Animal Health and Agriculture authorities (MAPA 21028.007698/2003-15). Animals were maintained under conditions designed to exclude any possible contaminating *Leishmania* infections, after approval of experimental design and conditions by the local public health authorities. Kennel was sprayed with pyrethroid insecticide and all its extension was protected throughout appropriated and security stainless steel gauze as recommended by Brazilian Ministry of Health. During all the experiments, veterinarians followed animals and all the invasive procedures were performed following the rules of ethical procedures in animal experimentation and biosafety. All of them were negative for the presence of *Leishmania* antibodies as detected by indirect immunofluorescence (IFI titles lower than 1:40 dilutions) and ELISA. Assays were performed using negative and positive control sera. In ELISA, cut off values were determined from average optical densities plus two standard deviations, obtained from negative sera.

2.2. Immunization

Dogs ($n = 14$) were immunized subcutaneously on days 0, 21 and 42 with a vaccine, consisting of 100 μg of A2 recombinant protein (rA2), purified as previously described [14], and of 250 μg of Saponin (Riedel). Of those, 7 animals were challenged, constituting the group VI and 7 remained uninfected, composing the group VNI. Control animals received either PBS ($n = 4$) or Adjuvant ($n = 3$), composing the groups PBS and Infected and Adjuvant and Infected.

2.3. Parasites, total protein extract (LcPA) preparation and infection

Promastigotes of *L.* (*L.*) *chagasi* (MCAN/BR/2000/BH400) were grown at 23 °C in α-MEM medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 200 U of penicillin/ml (Sigma), 100 μg of streptomycin/ml (Sigma) at pH 7.4. Total protein extracts (LcPA) of *L.* (*L.*) *chagasi* promastigotes (LcPA) were prepared from stationary phase promastigotes, submitted to 5 cycles of freezing (liquid N_2) and thawing (42 °C), followed by ultrasonication (Ultrasonic processor, GEX600), with cycles of 10 s for 2 min at 35 MHz. The extracts were then submitted to centrifugation at 8000 × *g* for 20 min at 4 °C. The supernatant was collected and stored at −70 °C.

For challenge infection, 5×10^7 late-log-phase promastigotes of *L.* (*L.*) *chagasi* were injected intravenously into each dog, four weeks after the last vaccine dose.

2.4. ELISA for specific IgG, IgG1 and IgG2 isotypes

Levels of specific immunoglobulin IgG, IgG1 and IgG2 isotypes were measured by ELISA before vaccination, after each vaccine dose and at each three months over a period of 14 months after vaccination. Briefly, 96-well plates (Costar®) were sensitized with either rA2 (250 ng/well) or total protein extracts (LcPA) of *L.* (*L.*) *chagasi* promastigotes (1 μg/well), in coating buffer pH 9.6 (Na_2CO_3 0.015 M, $NaHCO_3$ 0.03 M) overnight at 4 °C. Plates were blocked with PBS/2% casein at 37 °C for 1 h and treated successively with 1:100 dilutions of canine serum samples for 2 h at 37 °C. Peroxidase-labeled antibodies specific to canine IgG, IgG1 or IgG2 isotypes (Sigma) were diluted at 1:5000 or 1:6000, respectively, and added for 2 h at 37 °C. The plates were washed six times with PBS/Tween 20 0.05% and incubated with H_2O_2 and *o*-phenylenediamine for reaction development. Reactions were stopped by the addition of 20 μl of H_2SO_4 2N (Merck). Optical densities were determinated at 492 nm in ELISA reader (BioRad, Model 2550, CA, USA).

For evaluation of specificity of anti-A2 total IgG and IgG2 antibodies elicited in vaccinated animals, 96-well plates (Costar®) were sensitized with 8 μg/well of an A2 derived peptide (VGPQSVG-PLSVGPQSVGPLS), at 37 °C for 1 h and then overnight at 4 °C. Sera of vaccinated animals were diluted 1:20 and peroxidase-labeled antibodies specific to canine total IgG or IgG2 isotypes were diluted 1:6000.

2.5. Peripheral blood mononuclear cell isolation and cytokine production assay

Levels of gamma interferon (IFN-γ) and IL-10 were assessed in vaccinated and control animals one month after the three vaccine doses, before infection and also seven months after challenge infection. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood, homogenized 1:1 with PBS at room temperature, layered over Ficoll (Histopaque®; Sigma) and submitted to centrifugation at 800 × *g*, for 20 min at 18 °C. Red blood cells were lysed by addition of cold sterile water for 45 s, followed by

the addition 3× PBS to stop cellular lyses. Cells were submitted to centrifugation at 600 × *g*, for 15 min at 4 °C. The white blood cells ring was collected and ressuspended in DMEM medium (Dulbecco's Modified Eagle's Medium; Sigma), supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamina, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) (Sigma), 50 μM 2-mercaptoethanol, 200 UI/ml penicillin and 100 μg/ml streptomycin (all from Sigma) at pH 7.2. 1 ml of cell suspension (2×10^6 ml^{-1}) was plated in duplicated 24-well culture plates (Nunc). Isolated PBMC were incubated for 48 h in the presence of 5 μg/ml of ConA (Sigma), 10 μg/ml of antigen A2, 50 μg/ml of LcPA or in the absence of exogenous stimuli at 37 °C and 5% CO_2. After incubation, the supernatant was collected and stored at −70 °C. Levels of IFN-γ and IL-10 in supernatants were assessed by sandwich ELISA (Duoset ELISA Canine IFN-γ and Duoset ELISA canine IL-10; R&D Systems). A titration curve was performed to determine the best supernatant dilutions for cytokine assays. For IFN-γ measurements, culture supernatants were diluted 1:5. Detection limits are 62.5–2000 pg/ml for the canine IFN-γ and 15.6–500 pg/ml for IL-10, according to the manufacturer.

2.6. Clinical and laboratorial evaluation of dogs

Biochemical and haematological analysis were preformed in animals monthly and considered by veterinarians for clinical evaluation of the animals. Whole blood cell counting were performed by a semi-automated blood cell counter (Abaccus Júnior, Diatron, Austria) and differential leukocyte counting were analyzed in blood smear stained with May Grünwald-Giemsa®, according to Jain [17]. Albumin, alpha-(α), beta-(β) and gamma-(γ) globulin fractions and the ratio albumin/globulin were evaluated in dog's sera and references values given by Amusategui et al. [18], were used to calculate protein concentrations. Serum levels of alanine-amino transferase (ALT) and aspartate aminotransferase (AST) and creatinine and urea were measured to evaluate liver and renal functions, respectively. References values to interpret the parameters of the liver and kidney functions were from Kaneko et al. [19]. All the biochemical analysis were performed by the Cobas® equipment using the diagnostic kits from LabTeste® (Brazil). Independent clinical veterinarians carried out blindly the examination of the dogs for endpoint determination of CVL. Animals were clinically classified, according to Mancianti et al. [20], and Lima et al. [21], as follows: (1) symptomatic dogs exhibited classical signs of the disease such as cutaneous alterations (alopecia, dry exfoliative dermatitis or ulcers), onychogryphosis, keratoconjunctivitis, cachexia and anemia; (2) oligosymptomatic dogs exhibited some clinical signs of the disease and/or lesions such as lymphadenopathy, moderate weight loss and/or dull brittle hair accompanied by cutaneous lesions; (3) asymptomatic dogs showed none of the above clinical symptoms.

2.7. DNA extraction

DNA was extracted either from blood samples collected in FTA® cards (Whatman International Ltd.) or from ear skin biopsy specimens collected from each dog, 6 and 9 months after infection. Skin biopsy specimens, measuring approximately 1.0 mm^3, were mounted in paraffin blocks. For DNA extraction from blood samples, punches obtained from FTA® cards were washed twice with 200 μl FTA Purification Reagent and twice with 200 μl TE buffer (Tris 10 mM, EDTA 1 mM; Sigma), as recommended by the manufacturer (Whatman International Ltd.). Skin biopsy slices from paraffin-mounted samples were obtained and washed in ethanol (Merck) and then xylol (Merck). Biopsy fragments were incubated with proteinase K (Life Technologies) at a final concentration of 100 μg/ml, for 3 h at 56 °C, followed by heat inactivation at 100 °C for 15 min. DNA was extracted using a Wizard TM Genomic Purification Kit (Promega), according to the manufacturer's instructions.

2.8. Polymerase chain reaction (PCR)

PCR reactions performed with primers derived from a repetitive sequence of the *L.* (*L.*) *infantum* genome and that are specific for *L.* (*L.*) *infantum*, *L.* (*L.*) *chagasi*, and *L.* (*L.*) *donovani* (5′-ACG AGG TCA GCT CCA CTCC-3′ and 5′-CTG CAA CGC CTG TGT CTA CG-3′), according to Piarroux et al. [22]. The reaction mixture consisted of 1× DNA polymerase buffer (Invitrogen) containing 1.5 mM of $MgCl_2$, 5 mM KCl, 75 mM Tris–HCl, pH 9.0, 2.0 mM $(NH_4)_2SO_4$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 μM of each primer (Promega) and Taq polymerase (Invitrogen) (1.5 units), in a final volume of 20 μl. The conditions for PCR amplification were as follows: 94 °C for 5 min, 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. In all assays, a negative control without DNA was included. PCR samples were submitted to electrophoresis on 6% polyacrylamide gel and silver-stained.

2.9. Culture of bone marrow aspirated fluid

Bone marrow samples were collected 6 and 9 months after challenge infection from each dog. Samples were mixed with 3 ml of culture medium (10% heat inactivated fetal bovine serum; Sigma®) in "Novy-MacNeal-Nicolle" NNN medium. The mixture was then incubated for 7 days at 23 °C and was examined by microscopy. When no *Leishmania* cells were found, 1 ml of the culture samples was sub-cultured onto the same medium for another 7 days at 23 °C. This procedure was repeated once more before confirming a negative result.

2.10. Statistical analysis

All data comparisons were tested for significance by using Kruskal–Wallis or accurate Fisher tests. Differences were considered statistically significant when p values < 0.05.

3. Results

3.1. Vaccine and Leishmania-specific serological responses

Levels of anti rA2 or anti-LcPA antibodies were examined in sera samples collected before vaccination, after each vaccine dose and over a period of one year after vaccination. Levels of anti-A2 specific total IgG (Fig. 1A) and IgG2 (Fig. 1B) antibodies increased after each vaccine dose and infection in the groups of animals vaccinated with rA2 and the higher levels were observed 1 month after infection and at the end of the study, but not in the control-infected animals that received PBS or only adjuvant. Interestingly, significantly increased levels of anti-A2 specific IgG2 antibodies ($p = 0.0001$) were detected in vaccinated groups shortly after vaccination (after the second dose). Moreover, as shown in Fig. 1C, anti-A2 IgG2 antibodies present in sera of vaccinated animals recognized an A2-derived peptide, when compared to sera of animals before vaccine doses ($p = 0.002$), attesting for the specificity of the antibody response elicited by vaccination. Similar results were obtained for anti-A2 total IgG antibodies (data not shown). In contrast, no significant alterations in anti-A2 IgG1 (Fig. 1D) antibodies were detected either in VNI (vaccinated and non-infected dogs) and VI (vaccinated and infected) groups or I (infected) and ADI (adjuvant and infected) control groups, suggesting the development of Th1 specific immune responses in vaccinated animals.

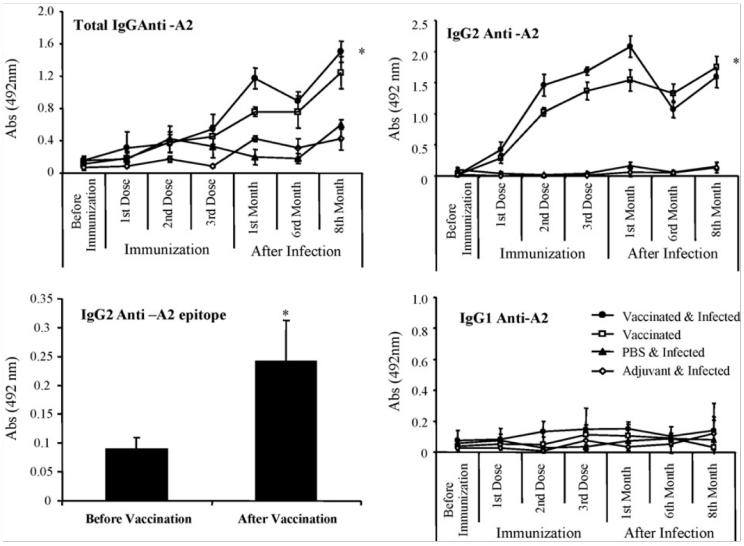

Fig. 1. Vaccine specific serological responses. Panels (A, B and D) show the evolution of levels of anti-A2 IgG, IgG2 and IgG1 antibodies, respectively, before and after vaccination and after challenge, as detected by ELISA. Plates were coated with 250 ng/well of rA2 and sera samples were diluted 1:100. Each point represents average ± standard error. Panel (C) shows levels of anti-A2 IgG2 antibodies, as detected by ELISA using an A2-derived peptide (8 μg/well) and sera samples diluted 1:20. Each bar represents average ± standard deviation of optical densities. The asterisks indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

During vaccination, no significant increases in anti-LcPA total IgG antibodies (Fig. 2A) were observed in any of the study groups. Increased levels of anti-LcPA total IgG antibodies ($p = 0.01$) were detected only after 3 months p.i. in animals of the infected groups (values above cut off appeared in the first month p.i. in animals of the I group and after 3 months p.i. in animals of the VI and ADI groups). Six months p.i., significant differences ($p = 0.01$) were observed for levels of anti-LcPA total IgG antibodies either between vaccinated and vaccinated-infected, vaccinated and PBS-infected or vaccinated and adjuvant-infected groups. A similar pattern ($p = 0.03$) was observed for levels of anti-LcPA IgG2 antibodies (Fig. 2B). In contrast, increased levels of anti-LcPA IgG1 (Fig. 2C)

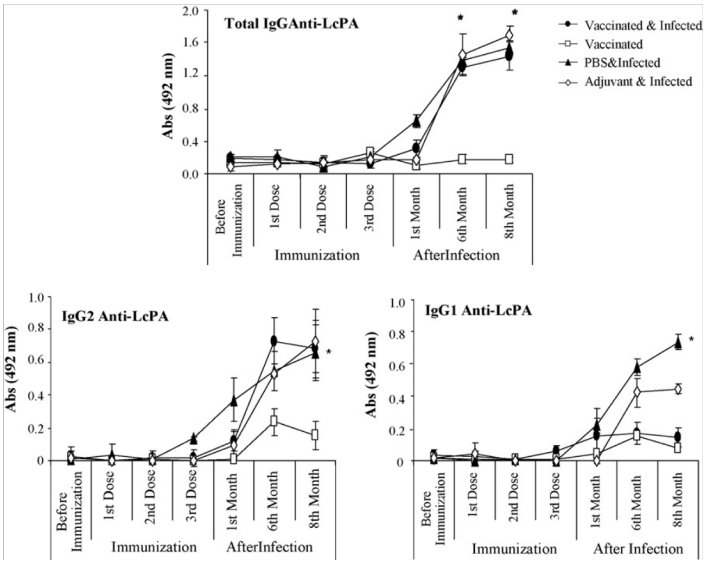

Fig. 2. Leishmania-specific serological responses. Panels (A–C) show the evolution of levels of anti-LcPA IgG, IgG2 and IgG1 antibodies, respectively, before and after vaccination and after challenge, as detected by ELISA. Plates were coated with 1 μg/well of LcPA and sera samples were diluted 1:100. Each point represents average ± standard error. The asterisks indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

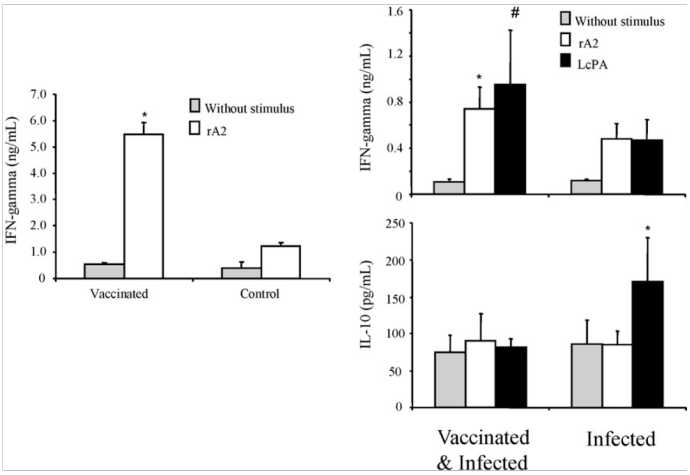

Fig. 3. Cytokine levels detected in culture supernatants of canine PBMC. Panel (A) shows IFN-γ levels detected after immunization and before infection in PBMC culture supernatants stimulated with rA2. Panel (B) shows levels of IFN-γ detected in PBMC culture supernatants produced in response to rA2 or LcPA, seven months after infection. Panel (C) shows levels of IL-10 in response to rA2 or LcPA seven months after infection. The asteriscs and # indicate that differences are statistically significant ($p < 0.05$ Kruskal–Wallis test).

antibodies were detected only in animals of the adjuvant-infected ($p = 0.025$) and PBS-infected groups ($p = 0.03$). Thus, animals that received rA2 and remained negative for anti-LcPA antibodies after vaccination, while those that were vaccinated but not infected remained negative during the follow-up period. These findings indicate that vaccination with rA2 allows the serological distinction between vaccinated and infected dogs.

3.2. IFN-γ and IL-10 productions by peripheral blood mononuclear cell (PBMC) of immunized dogs

We determined whether immunization with rA2 resulted in increased IFN-γ or IL-10 production in response to rA2 or LcPA stimulation. As shown in Fig. 3A, PBMC from dogs vaccinated with rA2, before infection but after three vaccine doses, secreted significantly higher levels of IFN-γ ($p = 0.003$) when stimulated with A2 than PBMC collected from control infected dogs (I and ADI groups). Before challenge, IL-10 was not detected in vaccinated or in control animals in response to rA2 or LcPA. Seven months after infection, significantly higher levels of IFN-γ, as compared to control animals, were also produced by PBMC from dogs vaccinated with rA2, when stimulated either with rA2 ($p = 0.025$) or LcPA ($p = 0.003$) (Fig. 3B), indicating that these animals displayed not only a sustained IFN-γ production after vaccination, but responded with an increased IFN-γ production to total parasite antigens after infection. Additionally, the production of IL-10 (Fig. 3C) was not significantly higher in the rA2-vaccinated dogs as compared to control animals, following stimulation with rA2. In contrast, seven months after infection (Fig. 3C), stimulation with LcPA resulted in a significantly increased IL-10 production ($p = 0.03$) by PBMC of control-infected animals, as compared to the vaccinated-infected group.

3.3. Biochemical, haematological, clinical and parasitological findings

The main clinical and parasitological features presented by all animals are summarized in Table 1. Clinical signs of VL appeared earlier (3–6 months p.i.) in control-infected animals (I) as compared to the Vaccinated and Infected (VI) group (1 year p.i.). In addition, 71.5% of the control-infected dogs were symptomatic, displaying more intense VL signs such as profuse blood diarrhea, intense loss of weight and cachexia. Some animals in this group presented a progressive form of the disease, showing multiple and severe symptoms that required, in some cases, that the animal be killed ($n = 3$) at nine months after infection. In contrast, in the VI group only 28.5% of the dogs showed signs of the active disease. In fact, they presented a moderate loss of weight, alopecia and mild lymphadenopathy, symptoms that appeared 12 months p.i. The remaining VI animals were considered asymptomatic. Asymptomatic animals in this group remained in this condition to the end of the follow-up period (14 months after challenge).

Experimental infection also induced alterations in biochemical and haematological parameters. As shown in Table 2, significant differences were observed only in the control infected animals (I) for average leukocyte counts ($p = 0.005$), albumin/globulin ratios ($p = 0.022$) and AST levels ($p = 0.018$) when pre-challenge average values were compared to the post-challenge determinations. No significant differences were detected for ALT, urea and creatinine levels and platelets or erythrocytes counting by comparing pre- and post-challenge values in both groups (not shown).

Parasitological evaluation consisted in culture of bone marrow aspirates and PCR of *Leishmania* DNA extracted from ear skin biopsy and blood samples, performed six and nine months after challenge. As shown in Table 1, nine months after challenge cultures resulted positive from bone marrows collected from all (100%) the dogs of the control infected groups, while 57.14% of the Vaccinated and Infected (VI) animals had positive results. *Leishmania* DNA was more frequently found in blood from the control infected dogs (71.5%) than in the rA2-vaccinated animals (28.5%). A positive correlation was thus observed between dogs developing clinical findings and presence of *Leishmania* DNA in peripheral blood. The ratio of PCR positive animals was significantly higher among animals that were Oligo/Symptomatic than in those that remained asymptomatic ($p = 0.048$; accurate Test of Fisher). Vaccinated asymptomatic dogs did not present *Leishmania* parasites in

Table 1
Clinical and parasitological evaluation of animals

Animal	Group	Bone marrow[a]	Peripheral blood[a]	Symptoms
1	VI	Positive	Positive	Asymptomatic
2	VI	Positive	Negative	[b]Symptomatic: loss of weight, alopecia of the left ear
3	VI	Positive	Positive	[b]Symptomatic: moderate loss of weight, alopecia, lymphadenopatia
4	VI	Negative	Negative	Asymptomatic
5	VI	Negative	Negative	Asymptomatic
6	VI	Positive	Negative	Asymptomatic
7	VI	Negative	Negative	Asymptomatic
Total		4/7	2/7	2/7
1	I	Positive	Negative	Asymptomatic
2	I	Positive	Positive	[c]Symptomatic: intense loss of weight, profuse bloody diarrhea, cachexia
3	I	Positive	Negative	Asymptomatic
4	I	Positive	Positive	[c]Symptomatic: alopecia, intense loss of weight, diarrhea
5	I	Positive	Positive	[c]Symptomatic: skin lesions, onychogryphosis, intense loss of weight, alopecia, cachexia
6	I	Positive	Positive	[c]Sympotmatic: intense loss of weight, profuse diarrhea, cachexia, purulent eye secretion
7	I	Positive	Positive	[c]Symptomatic: intense loss of weight, profuse diarrhoea, onychogryphosis, cachexia
Total		7/7	5/7	5/7

VI, Vaccinated and Infected dogs; I, dogs that received PBS or Adjuvant and were infected.
[a] Parasites were detected by culture of bone marrow aspirates or by of peripheral blood collected 9 months p.i.
[b] Symptoms appeared 1 year p.i.
[c] Symptoms appeared 3–6 months p.i.

skin, as detected either by PCR or by specific immunocytochemical analysis (data not shown).

4. Discussion

In the present investigation, we tested, in dogs, a vaccine formulation against CVL consisting of the recombinant protein A2 in combination with saponin. Saponin has been extensively used in other vaccine formulations and was shown to preferentially induce type 1 immune responses in animal models [23,24]. In high concentrations, however, saponin may induce adverse reactions such as local pain. In this study, animals tolerated quite well the vaccine doses, with no signs of local pain, abscess, ulceration or fever, may be due to the low saponin concentration used.

Clinical follow-up of the animals during the study allowed the classification of dogs as asymptomatic or symptomatic. In terms of clinical manifestations, the percentages of animals scored asymptomatic by the end of the study were higher in the rA2 immunized group than in control groups immunized with adjuvant only or PBS, resulting in 42.86% of efficacy. These results indicate that rA2 was able to induce partial protection against *Leishmania* infection, although due to the low number of animals per group, it was difficult to establish whether such differences were statistically significant.

The outcome of CVL following natural and experimental infection is highly variable [25]. A proportion of infected dogs develop symptomatic, ultimately fatal infection, while other dogs remain asymptomatic. Progression of clinical CVL is associated with establishment of large numbers of parasites in different tissues, although *Leishmania* DNA found in bone marrow might be significantly higher than that measured in blood samples, in both experimental and natural infections [26]. A sequential parasite dissemination pattern, in which lymph reticular organs harbored higher numbers of parasites at earlier stages of infection than the skin, has been also reported [27,28]. Although, in the present study not all animals in the control groups developed clinical signs of CVL, bone marrow cultures were positive for all of them. A high proportion of these animals were also positive for the presence of *Leishmania* DNA in peripheral blood. Although the parasitological methods may differ in sensitivity, both of them indicated a higher proportion of parasitized animals in control groups (PBS/Adjuvant) than in rA2-vaccinated dogs. It is worth noting that three serial passages were performed before bone marrow cultures were considered negative. Rodríguez-Cortés et al. [28] reported that *Leishmania* DNA was more frequently found in blood from experimentally infected dogs developing clinic-pathological findings than in blood from asymptomatic dogs and that the intensity of *Leishmania* infection in target organs and clinical status might be positively correlated, as in naturally infected dogs [29,30]. In agreement, a significant positive correlation ($p = 0.008$) was observed in the present study between dogs developing clinical findings and presence of *Leishmania* DNA in peripheral blood.

The evaluation of immune responses suggested that rA2-immunized animals mounted a type 1 immune response. In the humoral response, the first observed Ig was A2-specific IgG2 antibodies and no anti-A2 IgG1 antibodies were detected. This humoral response correlated with increased levels of IFN-γ in response to rA2 before and in response to both rA2 and total parasite antigens, after infection. In contrast, detectable levels of IgG1 antibodies and IL-10 in response to total parasite antigens were observed only

Table 2
Leukocyte counts and albumin/globulin ratios in experimental groups

Group	Leukocyte counts (average ± standard deviations)		Albumin/globulin ratio (average ± standard deviations)		AST levels (average ± standard deviations)	
	Pre-challenge	Post-challenge[a]	Pre-challenge	Post-challenge[a]	Pre-challenge	Post-challenge[a]
VI	10.994 ± 1.750	8.489 ± 2.625	1.70 ± 0.33	1.25 ± 0.40	42.42 ± 6.29	49.85 ± 14.80
I	12.968 ± 3.108	6.945 ± 2.588*	1.86 ± 0.59	1.12 ± 0.44*	31.28 ± 0.59	52.82 ± 16.50*

VI, Vaccinated and Infected dogs; I, dogs that received PBS or Adjuvant and were infected.
[a] Nine months post-challenge.
* Indicates significant differences ($p < 0.05$) when compared to pre-challenge determinations.

in control-infected animals. Although immunological and parasitological parameters associated with asymptomatic infection or active disease in CVL are only partially defined, resistance in CVL has been associated with the development of a *Leishmania*-specific, cell-mediated immune response (CMI) and increased levels of IFN-γ, whereas active disease has been associated with high antibody levels and suppressed CMI [28,31–33]. Recently, increased IL-10 expression has been associated with increase in parasitic loads and progression of the disease [34,35].

In this study, detection of anti-LcPA IgG1 antibodies correlated with appearance of symptoms in control-infected groups, suggesting an association between this Ig isotype and active disease. Several studies have attempted to link *Leishmania*-specific IgG1 and IgG2 levels with clinical status and disease progression [36–38], leading contradictory results. This is likely due to genetic heterogeneity of dog populations and in timing course of infection in naturally infected animals. On the other hand, in the present study, under experimental conditions, only the control-infected animals, many of them presenting severe symptoms, produced IgG1, suggesting an association between this isotype and the development of the disease.

It has been proposed that adoption of multiple and simultaneous actions to control vector populations and reservoir infection are required to significantly decrease VL incidence [5]. Culling of sero-positive dogs, although a matter of intense debate is a major VL control measure adopted in endemic areas in Americas. In this complex scenario, a CVL vaccine is certainly an important tool, but must fit some requirements, such as the discrimination between vaccinated and infected dogs by means of inexpensive serological tests. One of the major findings of the present study indicated that vaccinated dogs remained negative for anti-LcPA antibodies during the vaccination period, thus allowing the distinction between vaccinated and infected dogs. This is a relevant issue regarding the interface among VL control measures adopted in Brazil, although additional data, obtained after vaccination of a larger number of dogs in natural populations, are required to confirm this finding. It would be also of interest if vaccination could lead to reduction of zoonotic transmission. Considering that symptomatic dogs are more infectious to sand flies than the oligosymptomatic and asymptomatic animals [27,39,40] and the reduction of symptomatic animals as demonstrated for the rA2-vaccinated animals, it is plausible that vaccination with rA2 would impact favorably, reducing the parasite burden in the reservoir and transmission in endemic areas. It is worth noting that parasites were not detected neither by PCR nor immunocytochemical analysis in the skin of rA2-vaccinated animals. However, additional studies are required to clarify this issue.

A major difficulty associated with phase II vaccine trials against CVL is the lack of an adequate infection model. Important variations in the course of infection following either infection with different parasite doses, different infective stages of the parasites or different routes of infections have been evidenced [25]. Increased numbers of symptomatic dogs are generally observed after infection with high parasite doses (>10^7 promastigotes via endovenous injection), resulting in a very rigorous infection model as compared to natural infection conditions [25]. In the present study, a high dose was used of a highly infective parasite strain, which was isolated from a symptomatic dog. This challenge infection conditions proved to be a good method to induce severe symptoms of CVL in 5 out of 7 control animals (PBS and adjuvant inoculated animals). On the other hand, considering previous attempts of vaccination against CVL, the prevention of severe disease in the majority of animals is clearly difficult to achieve. However, the highly infective challenge conditions may have overpowered a protective response present in the vaccinated dogs. Thus, the partial protection obtained in the present study confirms the capacity of recombinant protein A2 to limit parasite replication and prevent severe disease even under high dose experimental challenge infection. These findings strongly support further evaluation of efficiency of vaccination with rA2 against CVL in natural infection conditions.

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3.3. Artigo 3. Improved Canine and Human Visceral Leishmaniasis Immunodiagnosis Using Combinations of Synthetic Peptides in Enzyme-Linked Immunosorbent Assay.

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PLoS NEGLECTED TROPICAL DISEASES

Improved Canine and Human Visceral Leishmaniasis Immunodiagnosis Using Combinations of Synthetic Peptides in Enzyme-Linked Immunosorbent Assay

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Abstract

Background: Zoonotic visceral leishmaniasis (VL) is a severe infectious disease caused by protozoan parasites of the genus *Leishmania* and the domestic dogs are the main urban parasite reservoir hosts. In Brazil, indirect fluorescence antibody tests (IFAT) and indirect enzyme linked immunosorbent assay (ELISA) using promastigote extracts are widely used in epidemiological surveys. However, their sensitivity and specificity have often been compromised by the use of complex mixtures of antigens, which reduces their accuracy allowing the maintenance of infected animals that favors transmission to humans. In this context, the use of combinations of defined peptides appears favorable. Therefore, they were tested by combinations of five peptides derived from the previously described *Leishmania* diagnostic antigens A2, NH, LACK and K39.

Methodology/Principal Findings: Combinations of peptides derived A2, NH, LACK and K39 antigens were used in ELISA with sera from 44 human patients and 106 dogs. Improved sensitivities and specificities, close to 100%, were obtained for both sera of patients and dogs. Moreover, high sensitivity and specificity were observed even for canine sera presenting low IFAT anti-*Leishmania* antibody titers or from asymptomatic animals.

Conclusions/Significance: The use of combinations of B cell predicted synthetic peptides derived from antigens A2, NH, LACK and K39 may provide an alternative for improved sensitivities and specificities for immunodiagnostic assays of VL.

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Introduction

Zoonotic visceral leishmaniasis (VL) caused by *Leishmania infantum* is an important emerging parasitic disease found in countries around the Mediterranean basin, in the Middle East, and in Latin America [1,2]. In these areas, wild canids constitute major sylvatic reservoirs, and domestic dogs are the principal urban reservoir hosts [3,4]. Hence, euthanasia of seropositive dogs has been adopted as a mainstay control measure in some countries [5]. However, domestic reservoir control programs may fail because of the high incidence of canine infection, the insensitivity of the diagnostic tests to detect infectious dogs and time delays between diagnosis and **euthanasia** by public health services [4]. Although adopted in European countries, treatment of infected dogs is not allowed in Brazil, based on the assumption that treated dogs may also remain as a source of parasites for sand fly infection. In this context, sensitive diagnostic tests, applicable to field conditions, are becoming increasingly necessary to facilitate and improve the control of disease [6].

Enzyme-linked immunosorbent assays (ELISAs) [7] and indirect fluorescence antibody tests (IFAT) [8] are widely used for serological diagnosis of VL. However, these tests present relative low sensitivity and specificity, which underestimates the actual rate of infection and allows the maintenance of infected animals and transmission. Several defined *Leishmania* antigens have been tested to overcome these difficulties and to improve both sensitivity and specificity [9]. Immunochromatographic tests for the diagnosis of leishmaniasis using the rK39 antigen has been evaluated in several countries, with variable results [6,10,11]. Development of effective diagnosis is also critical for control and possible eradication of visceral leishmaniasis and sensitive and specific rapid tests may be especially helpful to achieve this goal [12]. Therefore, there are

Author Summary

Visceral leishmaniasis is endemic in many areas of tropical and subtropical America where it constitutes a significant public health problem. It is usually diagnosed by enzyme-linked immunosorbent assays (ELISA) using crude *Leishmania* antigens, but a variety of other immunological methods may also be applied. Although these approaches are useful, historically their sensitivity and specificity have often been compromised by the use of complex mixtures of antigens. In this context, the use of combinations of purified, well-characterized antigens appears preferable and may yield better results. In the present study, combinations of peptides derived from the previously described *Leishmania* diagnostic antigens A2, NH, LACK and K39 were used in ELISA against sera from 106 dogs and 44 human patients. Improved sensitivities and specificities, close to 100%, for both sera of patients and dogs was observed for ELISA using some combinations of the peptides, including the detection of VL in dogs with low anti-*Leishmania* antibody titers and asymptomatic infection. So, the use of combinations of B cell predicted synthetic peptides derived from antigens A2, NH, LACK and K39 may provide an alternative for improved sensitivities and specificities for immunodiagnostic assays of VL.

still much room for improvement of serological diagnosis of VL, including identification and combination antigens and test formats.

B cell epitopes prediction by bioinformatics analysis of protein sequences has been proposed as a good alternative to select peptides for diagnostic tests [13,14]. In the present study, we tested, in ELISA against sera from 44 patients and 106 dogs, combinations of predicted B cell peptides derived from A2, NH, LACK and K39, which have been previously evaluated as antigens for serodiagnosis of visceral leishmaniasis [15–21]. Improved sensitivity for detection of asymptomatic and symptomatic canine visceral leishmaniasis (CVL), including canine sera with low anti-*Leishmania* antibody titers as detected by IFAT, and active disease in human patients was demonstrated for the majority of the peptide combinations.

Methods

Ethics Statement

Sera of dogs were obtained from already-existing collections (Sera collection of the Laboratory of Molecular Biology of the Faculty of Pharmacy, Federal University of Minas Gerais). Approval to use the samples was obtained from institutional review board (IRB) - Comitê de Ética em Experimentação Animal (CETEA) from Universidade Federal de Minas Gerais (UFMG), under the protocol 20/2010.

Sera of human patients were also obtained from an already-existing collection (Sera collection of the Laboratory of Immunoparasitology of the Research Center René Rachou, Fundação Oswaldo Cruz). IRB approval to use the samples was obtained from the Institutional Committee on Ethics of Human Research of Fundação Oswaldo Cruz, under the protocol 12/2006. All samples were analyzed anonymously.

Mapping B-cell Epitopes

The aminoacid sequence of A2 (amastigote stage-specific S antigen homolog of *L. donovani*), k39 (kinesin related protein of *L. chagasi*), LACK (*Leishmania* analogue of the receptor kinase C) and NH (nucleoside hydrolase) proteins were subjected to analysis with software available online at http://www.expasy.org/tools/protscale.html. The analyses generate numerical and graphical scores to predict the position of linear B-cell epitopes. Peptides that fulfilled, at least in part, the criteria of high hydrophilicity (Hoop &Woods), high alpha-helix structures (Chou & Fasman), low coil (Deleage & Roux), low beta-sheet structures (Chou & Fasman), high percentage of accessible residues and low beta-turn structures (Chou & Fasman) were selected for synthesis and screening. The selected peptides were also submitted to BepiPred software (http://www.cbs.dtu.dk/services/BepiPred/) to predict the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method [13]. BepiPred software scores peptides according to hydrophilicity values, secondary structures and the probability of a aminoacid is located in certain positions as compared to other mapped B cell epitopes. Peptides displaying scores higher than 0.35 may be, therefore, considered putative B cell epitopes.

Synthetic peptides

Peptides were synthesized according to a standard N-9-ethyloxycarbonyl (Fmoc) strategy on a PSSM8 multispecific peptide synthesizer (Shimadzu, Kyoto, Japan) by solid-phase synthesis and were purified by high performance liquid chromatography and confirmed with a Micromass Q-Tof Micro (Micromass MS Technologies, Division of Waters, Milford, MA) and the peptides obtained by this method were all C-terminal amides.

Dog sera and infection status

A panel of 106 canine sera was used in the study. Serum samples were divided into three groups based on history of exposure and infection status. Group 1 contained negative control sera from 14 healthy blood donor pets of various ages and breeds (previously classified as seronegative dogs after ELISA-based assays for the detection of antibodies against parasite-specific recombinant antigens rK39, rK26, and rA2) that attended a veterinary clinic in Minas Gerais, Brazil. Group 2 contained 30 serum samples from clinically symptomatic ($n = 17$) and asymptomatic ($n = 13$) dogs in which *L. infantum* visceral infection was proven by the demonstration of the presence of the parasites in bone marrow specimens and/or necropsy tissue samples as previously reported [21]. All infected dogs enrolled in this group were selected during a longitudinal epidemiological survey of CVL carried out in a rural area of endemicity (Pancas, ES; 2003–2004) in southeast Brazil [22]. Group 3 contained sera from 62 dogs with *L. infantum* infection from CVL endemic areas in Brazil. They had been previously tested in IFAT and ELISA. Sera presenting IFAT titers >1:40 dilutions and ELISA optical densities>cut off values (cut off values were determined by the mean of OD of 14 negative canine control sera plus two standard deviations) were considered positive (IgG) for CVL. All 62 samples had their status confirmed by parasitological analyses which included the search for parasites in bone marrow aspirates by PCR, microscopic examination of Giemsa stained smears and culturing in NNN/LIT medium at 23°C, as previously described [23]. Since a significant correlation was observed between IFAT and ELISA tests for all sera samples (data not shown), positive sera in group 3 were further grouped, according to their previous reactivity in IFAT, regardless its clinical status, as low ($n = 20$) (<1:320 dilutions), intermediate ($n = 20$) (>1:320 <1:640) and high ($n = 22$) (>1:640) IFAT titers.

Human sera

Human VL sera were obtained from patients with active visceral leishmaniasis ($n = 28$). Diagnosis of VL was defined when, besides clinical and epidemiologic features, amastigotes were seen at Giemsa stained smears of bone marrow aspirates or promastigote forms were identified on culture of peripheral blood or bone marrow aspirates. In the presence of suggestive clinical and epidemiologic characteristics, negative parasitological findings, but positive anti-*Leishmania* antibodies by IFAT or ELISA, definitive diagnostic was firmed after successful specific treatment. Control sera ($n = 16$) were obtained from individuals living in Vale do Jequitinhonha (in cities: São Pedro do Jequitinhonha, Caju, Virgem das Graças e Melquíades), a rural region of Minas Gerais State in southeast Brazil. None of the individuals presented signs of visceral leishmaniasis at clinical examination. All of them had negative results for specific *Leishmania* PCR in sera samples. Sera samples were also submitted to ELISA with the crude extract of the parasite to confirm that they were negative.

Enzyme-linked immunosorbent assays ELISA for canine sera

Levels of total IgG immunoglobulin were measured by ELISA. Briefly, 96-well flexible PVC plates (BD Biosciences, San Jose, CA) were sensitized with 5 µg/mL of each synthetic peptide diluted in water (100 µL per well). The sensitized plate was left in the oven until dry and then was left overnight at 4°C. Plates were blocked with PBS-2% casein at 37°C for 1 h and treated successively with 1:200 dilutions of canine serum samples for 1 h at 37°C. Peroxidase labeled antibodies specific to canine IgG (Sigma, St. Louis, MO) were diluted at 1:5000 and added for 1 h at 37°C. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) in citrate buffer containing hydrogen peroxide. Reactions were stopped by the addition of H_2SO_4 2N. Optical densities were determined at 450 nm in ELISA reader (BioRad, Hercules, CA). Each sera sample was assayed in triplicate. The lower limit of positivity (cut off) was determined by the mean of OD of 14 negative canine control sera plus two standard deviations.

Enzyme-linked immunosorbent assays ELISA for human sera

Sensitization of the plates followed the same as described above. However, plates were sensitized with 40 µg/mL of each synthetic peptide diluted in water (100 µL per well, resulting in 4 µg/well). When 2 peptides were tested simultaneously (peptides 13 and 47, 13 and 19, 18 and 19, 17 and 47 and 19 and 47), plates were sensitized with 20 µg/mL of each synthetic peptide. After antigen sensitization, the plates were blocked with 2% BSA at 37°C for 2 h and treated successively with 1:100 dilutions of patients serum samples for 1 h at 37°C. After washing step, biotinilated labeled antibodies human-IgG (Sigma, St. Louis, MO) were diluted at 1:5000 and added to the plate for 1 h at 37°C. Then, add the streptavidin-peroxidase conjugate diluted 1:1000 for 30 minutes at 37°C. After three washes, the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) in citrate buffer containing hydrogen peroxide were added to the plate. Reactions were stopped by the addition of H_2SO_4 2N. Optical densities were read at 450 nm in ELISA reader (BioRad, Hercules, CA). Each sera sample was assayed in triplicate. The lower limit of positivity (cut off) was determined by the mean of OD of 16 negative human control sera plus two standard deviations.

Statistical analysis

One-Way ANOVA test was used to compare the performances of the assays. A p value of less than 0.05 was considered significant. Sensitivity and specificity were calculated by binary classification test. The sensitivity and specificity for each test were calculated by using the formulas: Sensitivity = True positive/(True positive+False negative)×100% and Specificity = True negative/(True negative+False positive)×100%.

Results

Bioinformatics analysis

The study of the structure of proteins (ProtScale) allowed the selection of five peptides, as shown in Table 1: TPAVQKRVKEVGTKP and TTVVGNQTLEKVT, corresponding to numbers 17 and 18 peptides, respectively, derived from Nucleoside Hydrolase antigen, VVSTSRDGTAISWK, corresponding to peptide 19, derived from LACK protein and ESTTAAKMSAEQDRESTRATLE, corresponding to peptide number 13, derived from K39 protein. Additionally, in Table 1 is represented the peptide derived from the A2 protein, corresponding to peptide 47 (VGPQSVGPLSVGPQSVGPLS). However, the inclusion of this peptide was based on previous analysis of epitope prediction and reactivity with sera of BALB/c mice vaccinated with the A2 antigen [24]. Among the peptides select, 4 (peptides 13, 17, 19 and 47) also showed linear B-cell epitopes with significant values in the analysis by BepiPred, with emphasis on peptides 13 and 47, derived from K39 and A2 antigens, which presented higher scores. By contrast, the peptide 18 showed a low score for the presence of linear B-cell epitopes.

Reactivity of dogs and human visceral leishmaniasis sera with the selected peptides

Initially, to test the reactivity of the selected peptides with antibodies present in dogs' sera with VL, the spot synthesis technique, followed by immunoassay was applied (data not shown). The membranes were incubated with a pool of sera from infected and control animals. Four of the five peptides showed high intensity reactions with sera of dogs with confirmed VL as compared with the control group. Peptide 19 presented reactivity with both positive and negative sera. However, considering the use of pooled sera on the spot synthesis experiment, peptide 19 was also included in a more discriminatory analysis through ELISA with individual sera samples.

All peptides were then tested against Group 2 sera, which included samples collected from symptomatic and asymptomatic animals (Figure 1). As shown in figure 1, all peptides were able to detect as positives all the sera samples from both asymptomatic ($n = 13$) and symptomatic animals ($n = 17$) Figure 1. No significant differences were observed in sensitivity between the two groups.

The reactivity of the 5 peptides was further evaluated with a larger panel of sera from parasitological positive (Group 3) or control dogs (Group 1), which included sera samples classified according to IFAT reactivity as low (<1:320 dilutions), intermediate (>1:320 <1:640) and high (>1:640) titers (Figure 2). High sensitivity (100%) was observed for all peptides to detect infection in dogs with high IFAT antibody titers (22 animals), when the peptides were tested individually (Figure 2/Table 2). However, decreased sensitivities (varying between 55% and 90%) were observed for all peptides when tested against sera of dogs with IFAT intermediate (>1:320 <1:640) (20 animals) or low antibody titers (1:80 >1:320) (20 animals) (Table 2). Concerning specificity, a value of 100% was observed for peptide 17 and 92% for the other peptides, as shown in Table 2.

Table 1. Bioinformatic analysis of A2, K39, LACK and NH proteins to predict linear B-cell epitopes.

ExPASy	Parameters	Peptide 13	Ppetide 17	Peptide 18	Peptide 19	Peptide 47
Av (Max;Min)/Prot	Alpha-helix	1.1 (0.6; 1.3)	1 (0.7; 1.2)	0.96 (0.7; 1.2)	0.88 (0.7; 1.2)	0.77 (0.7; 1.2)
	Beta-turn	0.9 (0.6; 1.3)	0.83 (0.6; 1.3)	0.9 (0.6; 1.3)	0.91 (0.7; 1.3)	1.13 (0.6; 1.2)
	Beta-sheet	0.85 (0.6; 1.2)	1.1 (0.8; 1.3)	1.1 (0.8; 1.3)	1 (0.7; −1.3)	1 (0.7; 1.4)
	Coil	0.95 (0.8; 1.1)	1 (0.8; 1.2)	0.93 (0.8; 1.2)	0.92 (0.8; −1.1)	1.13 (0.8; 1.15)
	% Ac. residues	6.6 (4.2; 8)	6.4 (4; 7)	6.2 (4; 7)	5.8 (3.6; 7.4)	6.08 (4.2; 7)
	Hydrophilicity	0.73 (−1.2; 2)	0.6 (−1.2; 1.3)	−0.1 (−1.2; 1.3)	0.12 (−1; 1.2)	−0.4 (−1.4; 0.6)
BepiPred	**Parameters**	**Peptide 13**	**Ppetide 17**	**Peptide 18**	**Peptide 19**	**Peptide 47**
Max; Min/aa	Score>0.35	0.12; 1.53	0.33; 1.33	0.03; 0.51	0.01; 1.08	0.69; 1.64

Av: Average, Max: Maximum, Min: Minimum, Prot: Protein, aa: Aminoacid, Ac: Acid.
doi:10.1371/journal.pntd.0001622.t001

Since decreased sensitivity was detected for each peptide individually with sera of dogs with intermediate and low IFAT titers, we tested the hypothesis that the sensitivity to detect VL would increase by combining peptides in the same reaction. Assuming this would increase sensitivity by broaden instead of simply increasing the number of available epitopes for reaction, half of the concentration was used for each peptide instead of double the total peptide concentration. Combinations of two peptides were then tested against dog sera with low and intermediate antibody titers (Figure 3/Table 2). For sera with low antibody titers, the best results, i.e, improved sensitivities as compared to peptides tested individually, ranged from 90% to 95%, were obtained with combinations between the peptides 13 and 47, 13 and 19, 18 and 19, 47 and 17 and 47 and 18. Specificity was also improved, reaching 100% for all of these combinations. The sensitivity of peptide 47 was not altered when combined with peptide 17, which in contrast, had its sensitivity improved from 85% to 90% (Figure 3/Table 2). For sera with intermediate antibody titers, improved sensitivities for both peptides were observed for combinations between peptides 13

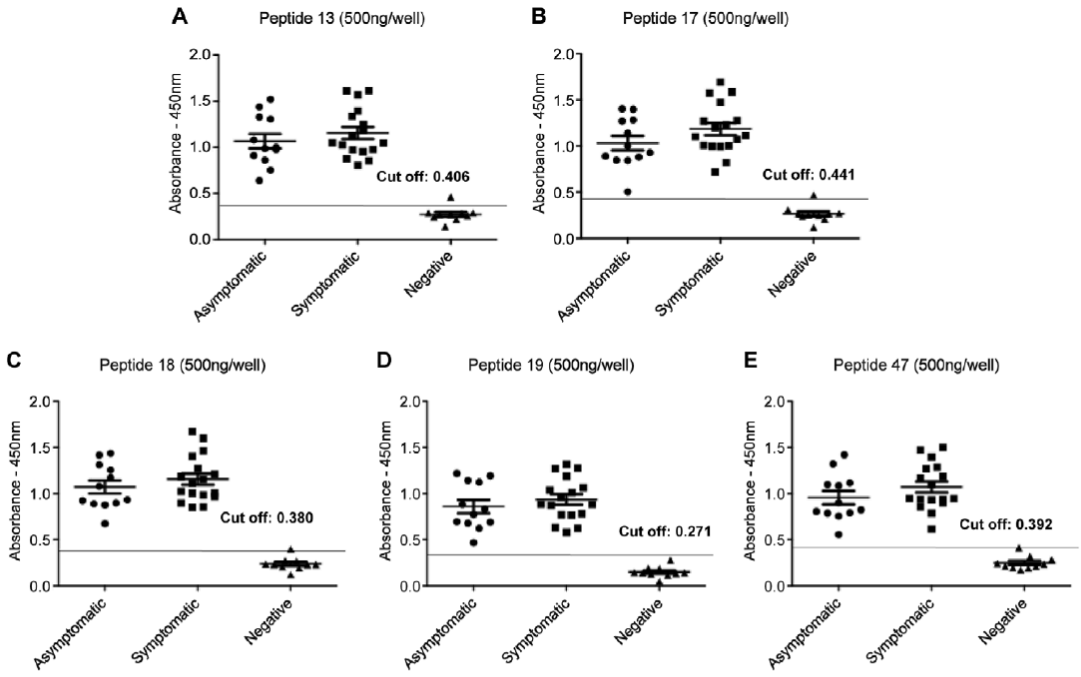

Figure 1. ELISA with individual synthetic peptides for detection of IgG-antibodies in dogs with *L. infantum* infection. Detection of anti-leishmanial total IgG antibodies with synthetic peptides by ELISA assay using sera from asymptomatic ($n = 13$) and symptomatic ($n = 17$) dogs and control group ($n = 14$). It was used 500 nanograms/well of peptides 13 (panel **A**), 17 (panel **B**), 18 (panel **C**), 19 (panel **D**) and 47 (panel **E**). The sensitivity and specificity of asymptomatic and symptomatic dogs ranged between 100 and 90%, respectively, for both.
doi:10.1371/journal.pntd.0001622.g001

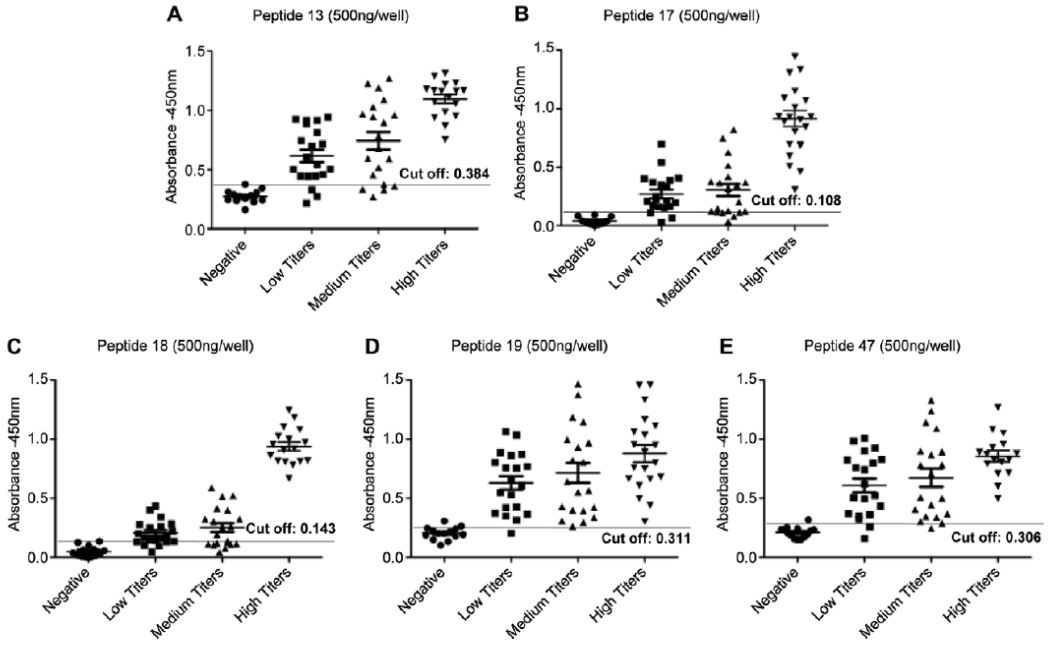

Figure 2. Reactivity of sera from infected dogs displaying different IFAT antibody titers with peptides. Detection of anti-leishmanial total IgG antibodies with synthetic peptides by ELISA assay using canine samples classified according to IFAT reactivity as low (<1:320 dilutions), intermediate (>1:320 <1:640) and high (>1:640) titers and 500 nanograms/per well of peptides 13 (panel **A**), 17 (panel **B**), 18 (panel **C**), 19 (panel **D**) and 47 (panel **E**).
doi:10.1371/journal.pntd.0001622.g002

and 19, 18 and 19, 47 and 18, varying from 80% to 95%. The sensitivity of peptide 47 (85%) was not affected when combined with peptide 13 and decreased when associated to peptide 17 (Figure 3/Table 2).

Peptides were also tested (individually and combined) against human sera ($n=44$) including patients with active visceral leishmaniasis ($n=28$) and healthy individuals with previous negative results in ELISA to *Leishmania* ($n=16$). The results obtained are shown in Figure 4 and 5/Table 3. Sensitivity values of 82%, 93% and 96% were observed for peptides 18, 47 and 13, respectively. And ELISA with peptides 17 and 19 gave the best results, displaying sensitivities of 100%. The specificity for the peptides tested individually ranged from 81% to 94%. The combination of peptides brought an improvement in sensitivity

Table 2. Performance of ELISA employing synthetic peptides and canine sera classified according to IFAT reactivity.

Peptides	Low Titer* (n = 20)	Intermediate Titer (n = 20)	High Titer (n = 22)	Uninfected Dogs (n = 14)
	Se (%)	Se (%)	Se (%)	Sp (%)
13	85	75	100	92
17	85	75	100	100
18	70	55	100	92
19	90	85	100	92
47	90	85	100	92
13 and 47	95	85	ND	100
13 and 19	95	95	ND	100
18 and 19	95	90	ND	100
47 and 17	90	80	ND	100
47 and 18	95	95	ND	100

Se: sensitivity, Sp: specificity.
*canine sera samples were classified according to IFAT reactivity as low (<1:320 dilutions) and intermediate (>1:320 <1:640) titers and high (>1:640) antibody titers.
doi:10.1371/journal.pntd.0001622.t002

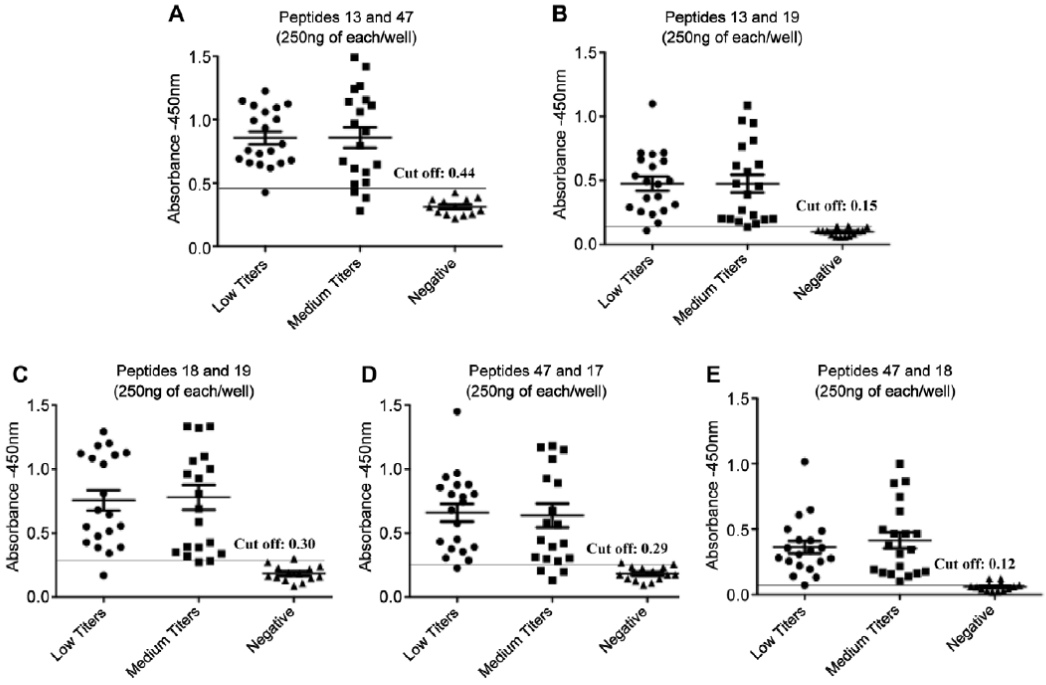

Figure 3. Reactivity of sera from infected dogs displaying different antibody titers with combined peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using canine samples classified according to IFAT reactivity as low (<1:320 dilutions) and intermediate (>1:320 <1:640) titers and 250 nanograms/per well from each peptide. Peptides were combined in pairs in the same reaction as following: peptides 13 and 47 (panel **A**), 13 and 19 (panel **B**), 18 and 19 (panel **C**), 47 and 17 (panel **D**) and 47 and 18 (panel **E**).
doi:10.1371/journal.pntd.0001622.g003

and specificity between peptides 13 and 19, 18 and 19, 47 and 13, and 19 and 47 where we observe a sensitivity of 100% for the first three combinations and 95.65% for the last one, respectively. Only for the combination between peptides 17 and 47 a reduction in sensitivity to 70.83% was observed. Specificity values for all combination ranged from 93.10% to 100% (Figure 4 and 5/ Table 3).

Discussion

In the present work, the *Leishmania* proteins A2, K39, LACK and NH were submitted to B cell epitope prediction and the derived synthetic peptides were evaluated through ELISA against sera of dogs and patients for the serodiagnosis of VL. Using the Protscale software, six different parameters were evaluated for each protein to select peptides. Considering the scores for these parameters, an adequate profile was observed for the majority of peptides, as compared to the minimal and maximum scores for the corresponding proteins, except for peptide 47. Peptide 47 displayed the lower values for hydrophilicity and presence of alpha helix, which are expected to be high for B cell epitopes, and the highest values for coil and beta turn structures, which in contrast are expected to be low. On the other hand, prediction using BepiPred resulted in scores higher than 0.35 for all peptides, except for peptide 18. Altogether, our results indicate that the two analyses may be complementary to each other and that this strategy is useful for selecting diagnostic antigens.

Accurate diagnosis of canine leishmaniasis is essential towards a more efficient control of this zoonosis, but it remains problematic due to the high incidence of asymptomatic infections [25]. Initially, we tested the five peptides with sera from dogs clinically classified as asymptomatic and symptomatic (Group 2). It is noteworthy that the sera samples of Group 2 have been previously tested in ELISA using SLA or the recombinant proteins rA2, rK39 and rK26 [21]. The retrospective analysis of the data obtained by Porrozzi et al. (2007) revealed that 4, 9, 5 and 4 out of 13 sera from asymptomatic animals included in the present study, were not reactive with SLA, rA2, rK39 and rK26, respectively, whereas all the symptomatic samples were positive when either rK26, rK39 or SLA were used as antigens. Using rA2, 6 symptomatic sera samples were identified as negative. Moreover, the majority of samples that were not reactive with these antigens were obtained from asymptomatic animals presenting low antibody titers in IFAT (≤1:80). In the present analysis, for both asymptomatic and symptomatic VL canine sera, sensitivities and specificities of 90% and 100%, respectively, were observed. Therefore, improved sensitivity was observed for assays using the synthetic peptides as compared to SLA and the recombinant proteins, especially for sera of asymptomatic animals. In this sense, our results largely confirm and improve the potential of these antigens for serodiagnosis of leishmaniasis.

Detection of infection in animals with low or intermediated anti-*Leishmania* antibody titers, regardless their clinical status, is critical for diagnosis and control of VL. The failure to detect infection in these animals may contribute to the maintenance of parasite's

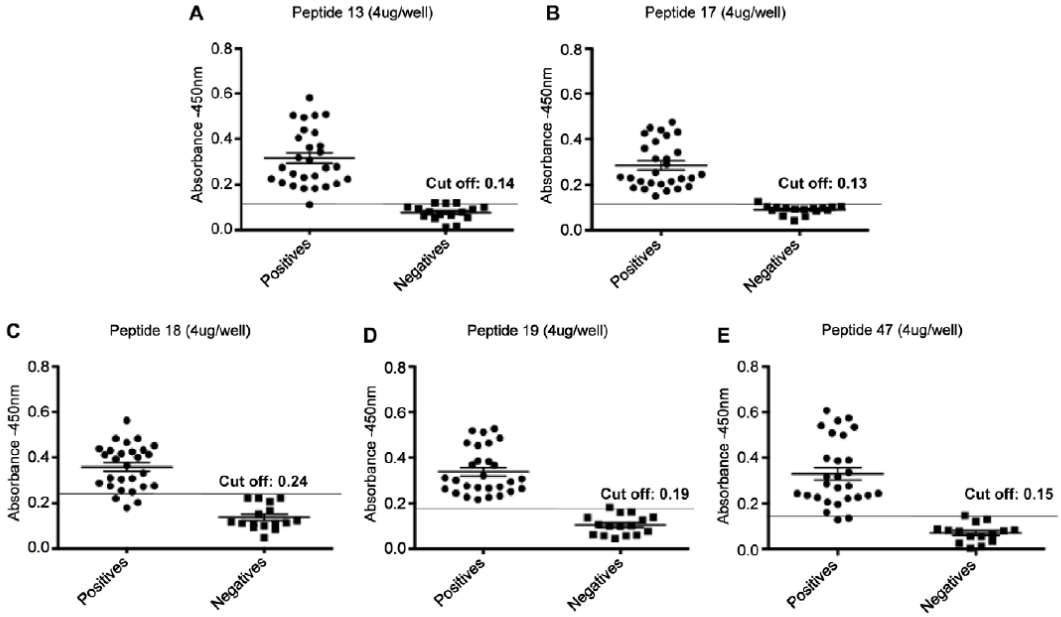

Figure 4. Detection of IgG-antibodies in sera of human patients with visceral leishmaniasis with individual peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using sera from patients with active visceral leishmaniasis ($n = 28$) and healthy individuals with previous negative results in ELISA to *Leishmania* ($n = 16$) and 4 μg/per well peptides 13 (panel **A**), 17 (panel **B**), 18 (panel **C**), 19 (panel **D**) and 47 (panel **E**).
doi:10.1371/journal.pntd.0001622.g004

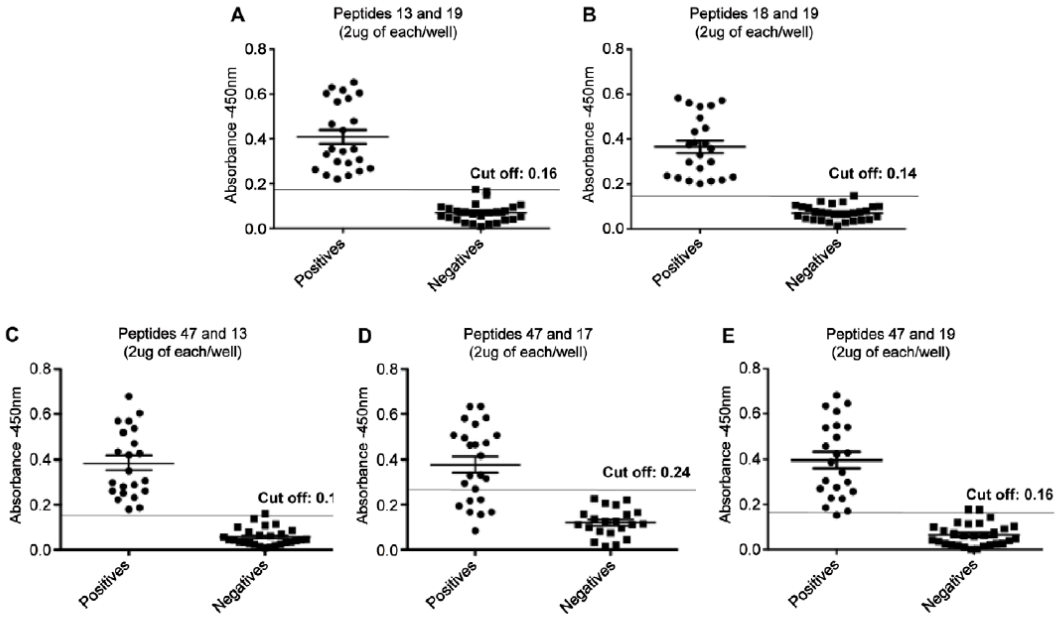

Figure 5. Detection of IgG-antibodies in sera of human patients with visceral leishmaniasis with combined peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using sera from patients with active visceral leishmaniasis ($n = 28$) and healthy individuals with previous negative results in ELISA to *Leishmania* ($n = 16$) and 2 μg/per well from each peptide. Peptides were combined in pairs in the same reaction as following: peptides 13 and 19 (panel **A**), 18 and 19 (panel **B**), 47 and 13 (panel **C**), 47 and 17 (panel **D**) and 47 and 19 (panel **E**).
doi:10.1371/journal.pntd.0001622.g005

Table 3. Performance of ELISA employing synthetic peptides and sera of patients with visceral leishmaniasis.

Total IgG	Acute phase of infection (n = 28)	Healthy Control Individuals (n = 16)
	Se (%)	Sp (%)
Peptide 13	96	81
Peptide 17	100	94
Peptide 18	82	81
Peptide 19	100	94
Peptide 47	93	94
Peptides 13 and 19	100	93
Peptides 18 and 19	100	100
Peptides 13 and 47	100	96
Peptides 17 and 47	71	100
Peptides 19 and 47	97	93

Se: sensitivity, Sp: specificity.
doi:10.1371/journal.pntd.0001622.t003

transmission for both canine and human populations, one of the major factors that hinder control strategies. On the other hand, highly sensitive diagnosis may require combined antigens. As indicated by immunoproteomic approaches, *Leishmania* parasites display extensive variability in antigenic composition, and apparently absence of immunodominant antigens when individual sera samples are analyzed [26], suggesting that single antigen diagnostic tests may display decreased sensitivities. Indeed, rK39 based tests may lack sensitivity for canine sera with low antibody concentration [19].

By combining two peptides, increased sensitivities (90–95%) and specificity (100%) were observed for dog sera with low IFAT antibodies titers. Similar findings were also observed for sera with intermediated antibodies titers (80–95% of sensitivity and 100% specificity). Improved sensitivities may have resulted from increased number of reactive epitopes, leading to increased OD readings and numbers of positive sera as compared to the reactivity with a single peptide. These findings will be particularly useful for diagnosis of dogs with low and intermediate titers of antibodies, since most current tests fail in this task.

Since early and sensitive diagnosis is seen as a critical aspect for management and, possibly, eradication of human visceral leishmaniasis [12,27–29], we have also investigated the reactivity of the peptides with sera of patients with active VL. Similarly, improved results have been observed when the combinations of peptides were tested against sera of human patients with active disease, suggesting that the epitopes selected were also recognized individually by human sera and that their serological reactivity may be independent and complementary, leading to an additive effect. Therefore, the association of peptides is an alternative to broaden the epitopes to be detected by antibodies, improving sensitivity. On the other hand, the absence of improved sensitivity for association between the peptides 17 and 47 may be explained by the presence of low levels of *Leishmania* specific antibodies in the control negative sera, since healthy controls were selected from endemic area and previous exposure of these individuals to parasite antigens may not be completely ruled out.

In many endemic areas, VL frequently overlaps with the occurrence of other forms of leishmaniasis or even with other infectious diseases, such as tuberculosis and leprosy. Cross-reactivity with antibodies raised against other infectious diseases consists in an additional shortcoming for development of specific visceral leishmaniasis diagnosis. Cross-reactivity of synthetic peptides with sera of patients presenting other infections was not assessed in the present work. Therefore, additional investigations are further warranted to better determine peptides specificity.

In conclusion, the combination of synthetic peptides, identified through B cell epitope predicition, may be useful for the development of highly sensitive and specific serodiagnosis for VL. The peptides identified may be especially interesting for the development sensitive immunochromatographic tests. Since these test format do not require sophisticated laboratory facilities or trained personnel staff to be routinely performed, and antibody quantification is not required for diagnosis of VL, they are more practical and easily applied, allowing rapid diagnosis in field conditions in endemic areas of difficult access to laboratory facilities [11,12,30–32]. Therefore, these peptides coupled to immunochromatographic tests may allow sensitive and early detection of infected dogs and their fast withdraw from transmission areas, regardless their antibody levels and clinical status, improving the control of VL in endemic areas.

Author Contributions

Conceived and designed the experiments: RTG APF. Performed the experiments: MMC MP MSS DD EF. Analyzed the data: MMC DD MSMM GG RTG APF. Contributed reagents/materials/analysis tools: MSMM GG APF. Wrote the paper: MMC GG RTG APF.

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3.4. Artigo 4. Analysis of *Leishmania chagasi* by 2-D gel eletrophoresis (2-D DIGE) and immuno-proteomic: Identification of novel candidate antigens for diagnostic tests and vaccine.

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Analysis of *Leishmania chagasi* by 2-D Difference Gel Eletrophoresis (2-D DIGE) and Immunoproteomic: Identification of Novel Candidate Antigens for Diagnostic Tests and Vaccine

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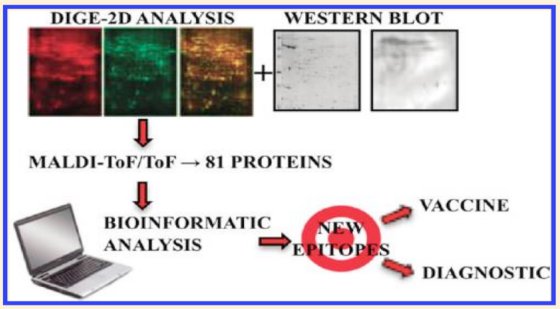
Supporting Information

ABSTRACT: Identification of novel antigens is essential for developing new diagnostic tests and vaccines. We used DIGE to compare protein expression in amastigote and promastigote forms of *Leishmania chagasi*. Nine hundred amastigote and promastigote spots were visualized. Five amastigote-specific, 25 promastigote-specific, and 10 proteins shared by the two parasite stages were identified. Furthermore, 41 proteins were identified in the Western blot employing 2-DE and sera from infected dogs. From these proteins, 3 and 38 were reactive with IgM and total IgG, respectively. The proteins recognized by total IgG presented different patterns in terms of their recognition by IgG1 and/or IgG2 isotypes. All the proteins selected by Western blot were mapped for B-cell epitopes. One hundred and eighty peptides were submitted to SPOT synthesis and immunoassay. A total of 25 peptides were shown of interest for serodiagnosis to visceral leishmaniasis. In addition, all proteins identified in this study were mapped for T cell epitopes by using the NetCTL software, and candidates for vaccine development were selected. Therefore, a large-scale screening of *L. chagasi* proteome was performed to identify new B and T cell epitopes with potential use for developing diagnostic tests and vaccines.



KEYWORDS: leishmaniasis, proteome, antigens, diagnosis, vaccine

INTRODUCTION

Leishmaniasis occurs in 88 countries with approximately 12 million infected individuals and 350 million people at risk of contracting the infection (http://www.who.int/en/). There are several clinical manifestations of the disease, which differ according to the *Leishmania* species and the host immune response. These manifestations are the cutaneous, mucocutaneous, diffuse and visceral diseases. Visceral leishmaniasis (VL), the more severe form of disease, is an anthroponosis in India and Central Africa, and a zoonosis in the Mediterranean and Latin America. Infection with *Leishmania infantum*, also named *Leishmania chagasi* in Latin America, represents 20% of the global human cases (100 000 cases per year) of zoonotic VL, and its incidence is increasing in urban and peri-urban areas in the tropics.[1] Although available, the drugs used to treat VL lead to severe side effects, and clinical presentation of the disease is not sufficiently specific to guide treatment.[2] Moreover, the existing diagnostic tests and vaccines for VL need to be improved.[3,4] Since dogs are the main reservoirs for *Leishmania* parasites, they are important targets for control of parasite transmission in countries, where VL is a zoonosis. Hence, development of accurate serodiagnostic tests, as well as more effective vaccines for canine VL, are highly desirable to control transmission and disease dissemination in Mediterranean and Latin American countries.

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The development of new serodiagnostic tests and vaccines for VL is largely hampered by insufficient knowledge about the complexity of the immune responses as well as the identification of proteins that are immunogenic for B and T lymphocytes. Indeed, only a limited number of *Leishmania* proteins have been tested in diagnostic tests and vaccine formulations for VL.[2,5−8] In this context, the identification of new immunogenic proteins derived from *Leishmania* species that cause VL, is highly desirable for the development of more sensitive/specific serodiagnostic tests, as well as effective vaccines. Thus, proteomics is a useful approach to obtain a more complete list of immunogenic proteins from *Leishmania* parasites. Currently, there are several studies in *Leishmania* proteomics, including *L. infantum*.[9−14] Nevertheless, this is the first proteomic study designed to identify antigens of potential use in development of diagnostic tests and vaccine for canine VL. For this purpose, we used a highly virulent strain of *L. chagasi* isolated from a VL dog in Brazil.[15] While *L. chagasi* and *L. infantum* are considered the same species,[16,17] their insect vector and wild reservoirs are distinct in Europe and Latin America.[18,19] These differences in parasite life cycle may result in variation in protein expression, leading to distinct biological behavior, as is the case of parasite virulence and higher number of severe cases in Latin America.[20,21]

Precisely, we performed a wide screen to search for immunogenic proteins from both promastigotes and amastigotes forms of *L. chagasi*. As criteria of antigen selection for serodiagnostic tests, we identified proteins that were recognized by antibodies present in sera from dogs with VL. We then mapped both B-cell and $CD8^+$ T cell epitopes from the immunogenic proteins. B cell epitopes were experimentally selected by a second screen employing peptide arrays and sera from infected dogs. $CD8^+$ T cell epitopes were further selected, *in silico*, based on their high affinity and ability to bind to various HLA haplotypes. Importantly, we identified 19 hypothetical as well as 7 putative proteins, which were among the antigens with highest immunogenic scores. Thus, our study allowed the identification of previously undefined *L. chagasi* proteins, which are strong candidates for developing novel immunological based diagnostic tests and vaccines for VL.

MATERIALS AND METHODS

Ethics Statement

Experiments with dogs were performed in accordance to guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation (Comitê de Ética em Experimentação Animal, CETEA) from the Universidade Federal de Minas Gerais, protocol 211/07 approved in 03/12/2008.

L. chagasi

The *L. chagasi* amastigotes (MCAN/BR/2000/BH400) were purified from spleens of hamsters 90 days postinfection. For purification of amastigotes from hamster spleens, we used a modified version of the method described by Chang (1980).[22] Immediately after the spleen removal, imprint smears were prepared and stained with GIEMSA for visualization of amastigotes by optic microscopy. The organ was then macerated in Schneider medium (Gibco BRL, Paisley, U.K.) employing a daucer, and centrifuged at 100*g* for 10 min. The residual red cells in the supernatant were disrupted with the addition of 0.05% saponine (w/v) for 5 min, followed by centrifugation at 2000*g* for 10 min. The pellet was then resuspended in 5 mL of Schneider medium. The suspension was passed through a 26-G needle, gently added to 5 mL of Percoll solution (Sigma, St. Louis, MO), and centrifuged at 2000*g* for 40 min to separate the amastigote from the cellular ring. The purity of our preparation was verified by microscopic inspection, and no intact host cells were found in the amastigote suspension. The amastigote suspensions were pelleted and frozen at −70 °C until use.

L. chagasi promastigotes (MCAN/BR/2000/BH400) were grown at 23 °C in Schneider's medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 200 U/mL of penicillin (Sigma), and 100 μg/mL of streptomycin (Sigma) at pH 7.4. Promastigotes from the logarithm phase were submitted to centrifugation at 8000*g* for 20 min at 4 °C and the pellet was collected and stored at −70 °C.

Canine Sera

For the initial screening of *Leishmania* antigens, we used a pool of sera from 20 animals per experimental group, that is, acutely infected, chronically infected, and uninfected control dogs. The acutely infected dogs were challenged intravenously with 10^7 amastigotes of *L. chagasi* and blood was collected 30 days postinfection. The chronically infected dogs were naturally infected with *Leishmania* in the metropolitan region from Belo Horizonte, rescued and maintained in our facility for laboratorial and clinical evaluation. VL in chronically infected dogs was certified by the presence of clinical symptoms, and parasitological tests in bone marrow cells examined by optical microscopy. The uninfected dogs were negative in parasitological as well as serological tests for VL and used as negative controls in our study. Blood was withdrawn and maintained at room temperature for 3 h to obtain serum. Individual sera were tested for serology employing immunofluorescence and ELISA tests for anti-*Leishmania* antibodies. Animals were included in our study as acutely infected, chronically infected, and uninfected when the diagnostic tests were IgM (+), IgG (+)/IgM (−), and IgG (−)/IgM (−), respectively. One hundred microliters from individual serum of 20 dogs selected per group were deposited in a single tube, in order to obtain a pool of sera that was representative of acutely infected, chronically infected, and uninfected dogs.

Protein Extract

The parasite were suspended in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 40 mM Tris base, and a protease inhibitor mix; GE Healhtcare, San Francisco, CA) in a proportion of 500 μL of lysis buffer for 10^9 parasite. Samples were incubated for 1 h at room temperature, with occasional vortexing, and then centrifuged for 15 min at 20 000*g* and room temperature. The supernatant (protein extract) was kept at −70 °C until analysis. The protein content was measured using the 2D-Quant kit (GE Healthcare) according to the manufacturer's instructions.

Two-Dimensional Gel Electrophoresis (2-DE)

To identify differentially expressed proteins between amastigote and promastigote forms, we used Differential Gel Electrophoresis (DIGE). Briefly, 150 μg of sample was labeled with 400 pmol of *N*-hydroxysuccinimidyl-ester-derivates of the cyanine dyes (Cy2, Cy3 and Cy5; GE Healthcare) following the manufacturer's protocol. The reaction was quenched with 1 mL of 10 mM lysine for 10 min on ice and in the dark. A mixture of protein extracts from amastigote and promastigote forms was labeled with Cy2 as an internal standard. Protein extracts from promastigote and amastigote forms were labeled with Cy3, and

Cy5, respectively. Experiments were performed with three biological replicates and a dye-swap for both parasite forms. Differently labeled extracts were pooled, reduced with 2% DTT, complemented with 2% ampholytes (pH 4−7), adjusted to a final volume of 350 μL with sample buffer (7 M urea, 2 M thiourea and 4% CHAPS), and incubated for 10 min on ice and in the dark. The isoelectric focusing voltage was increased gradually to 8000 V and run for 60 000 Vh at 20 °C and a maximum current of 50 μA/strip in Immobilized pH Gradient (IPG) 18 cm, pH 4−7 (GE Healthcare). Focused IPG strips were incubated for 15 min in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM DTT) and then alkylated for further 15 min in an equilibration solution containing 13.5 mM iodoacetamide instead of DTT. Strips were transferred onto a 12% SDS-PAGE gel and second-dimensional focusing was performed at 15 °C using 20 mA/gel for 1 h, followed by 50 mA/gel, with an Ettan DALT 6 unit (GE Healthcare). Gels were scanned on a Typhoon Trio laser imager (GE Healthcare) with excitation/emission wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm).

Images were analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare). Normalized spot volume data were log10-transformed before analysis, in order to eliminate distributional skew and improve the normal approximation for validity of *p*-values. Analysis of variance (ANOVA) was performed on log10-normalized spot volumes. Estimated differences between amastigotes and promastigotes were obtained from the model as differences in least-squares and exponential means (linear contrasts). Significance testing was performed at the 5% level. All statistical analyses were accomplished using SASs V9.1 software. To manually remove the selected spots after scanning in Typhoon, the DIGE gels were also stained with colloidal Coomassie Brilliant Blue (CBB) G-250 following procedures described elsewhere.[23]

Western Blot−2-DE

For Western blot analysis, the 500 μg protein extract from promastigote forms were used and fractionated in 2-DE gel. First-dimension IEF and second-dimension SDS-PAGE were performed as described above, including the isoelectric focusing voltage and the IPG of 18 cm, pH 4−7 (GE Healthcare). The samples were determined in individual gels and no fluorescent dye was employed. The proteins from unstained 2D were transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) in a trans-blot semidry transfer Unit (GE Healthcare) by applying a current of 1.6 mA/cm^2 for 2 h. The membranes were rinsed with TBS−Tween buffer (20 mM Tris, 500 mM NaCl, pH 7.4) and incubated with blocking buffer (5% low fat milk powder in Tris-buffered saline) at 4 °C overnight. The blotted membranes were incubated with pools of canine sera (i.e., acutely and chronically infected or uninfected control dogs) diluted 1:500 in blocking buffer, for 2 h at room temperature. After washing three times in 0.05% TBS−Tween for 15 min, the membranes were incubated with either anti-total dog IgG, anti-IgG1, anti-IgG2 or anti-IgM-peroxidase conjugates (Sigma) diluted 1:10 000 in blocking buffer, for 2 h at room temperature. Membranes were washed three times with TBS−Tween buffer for 5 min and three times with TBS for 5 min. Finally, nitrocellulose sheets were washed with a mixture of 6 mg of 3,3′-diaminobenzidine DAB (Sigma) in 12 mL of TBS buffer and 12 mL of a solution containing 10 mL of the phosphate-buffered saline, 10 μL of hydrogen peroxide and 2 mL of methanol (Sigma). Blots were incubated with this solution for 1−3 min. The reaction was interrupted with water, and the blots were dried with paper towels and stored at room temperature.[24] Spots that were recognized only by sera (total IgG, IgG1, IgG2 and/or IgM) from infected animals, but not from uninfected dogs were cut in gels. To select the spots, the images from membranes and gels with protein extracts were analyzed using the ImageMaster 2D Platinum 6.0 (GE Healthcare). The selected spots were removed for identification by mass spectrometry.

Identification of Proteins−MALDI-TOF/TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry)

Protein spots were manually excised from stained 2-D gels. The gel pieces were washed three times with 100 μL of 25 mM ammonium bicarbonate containing 50% (v/v) acetonitrile. After drying, gel pieces were rehydrated for 30 min at 4 °C with 10 μL of trypsin solution (Promega, Madison, WI) containing 20 ng/μL in 25 mM ammonium bicarbonate. Excess protease solution was then removed and replaced by 20 μL of 25 mM ammonium bicarbonate. Digestion was performed for 16 h at 37 °C. Peptide extraction was performed twice for 15 min with 30 μL of 50% acetonitrile/5% formic acid solution. Trypsin digests were then concentrated in a SpeedVac (Savant Instruments, Inc., Farmingdale, NY) concentrator to about 10 μL and desalted using ZipTip (C18 resin; P10, Millipore Corporation, Bedford, MA). Peptides were eluted from the column with 50% acetonitrile/0.1% trifluoroacetic acid.

Roughly 0.3 μL of the sample solution was mixed with an equal volume of a saturated matrix solution [10 mg/mL *R*-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in 50% acetonitrile/0.1% trifluoroacetic acid] on the target plate and allowed to dry at room temperature. Raw data for the identification of proteins were obtained on the 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Both MS and MS/MS data were acquired with a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1600 shots were accumulated for spectra in the S mode, while 2400 shots were accumulated for spectra in the MS/MS mode. Six of the most intense ion signals with a signal-to-noise ratio above 30 were selected as precursors for MS/MS acquisition, with the exclusion of common trypsin autolysis peaks and matrix ion signals. External calibration in MS mode was performed using a mixture of four peptides: des-Arg1-Bradykinin (m/z 904.468); angiotensin I (m/z 1,296.685); Glu1-fibrinopeptide B (m/z 1,570.677); and ACTH (18−39) (m/z 2,465.199). MS/MS spectra were externally calibrated using known fragment ion masses observed in the MS/MS spectrum of angiotensin I. Following data acquisition, a peak list was obtained from the raw MS/MS data using the "Peaks to Mascot" function in the 4000 Series Explorer software (Applied Biosystems).

Database Search. Uninterpreted tandem mass spectra were searched against the nonredundant protein sequence database from the National Center for Biotechnology Information (NCBI) using the Mascot (version 2.1) MS/MS ion search tool (http://www.matrixscience.com). The search parameters were as follows: no restriction of protein molecular weight, one missed trypsin cleavage allowed, nonfixed modifications of methionine (oxidation) and cysteine (carbamidomethylation); pyroglutamate formation at the N-terminal glutamine of peptides with no other post-translational modifications being taken into account. Mass tolerance for the peptides in the searches was 0.8 Da for MS spectra and 0.6 Da for MS/MS spectra. Peptides were considered

to be identified when the scoring value exceeded the identity or extensive homology threshold value calculated by Mascot. In cases of protein identification based on a single peptide, the minimum threshold for the probability-based Mascot score was 40. Otherwise, mass spectra with lower scores, but presenting a reasonable tandem mass spectrum, were manually verified.[25]

Mapping T-cell and B-cell Epitopes, Peptide Synthesis, and Immunoassay

All *Leishmania* proteins identified during the course of the study were screened for potential T cell epitopes using the NetCTL algorithm (Web service for prediction of cytolitic T cell epitopes in protein sequences).[26] NetCTL was the method of choice for cytotoxic T-lymphocyte epitope prediction because it integrates predictions for different steps involved in MHC class I presentation: proteosomal cleavage, TAP transport efficiency and MHC class I affinity. More importantly, NetCTL method was shown to have a higher predictive performance than the SYFPEITHI, the BIMAS HLA Peptide Binding Prediction, EpiJen, MAPPP, MHC-pathway, and WAPP methods using a data set containing approximately 300 experimentally validated CTL epitopes.[27] Various studies have also used this method for CTL epitope predictions that were experimentally validated.[28−33] In the present analysis, a score cutoff of 0.75, which corresponds to a good compromise between sensitivity (0.8) and specificity (0.97) was used. A total of 10 HLA supertypes were tested.

The immunogenic proteins selected by Western blot were mapped by BEPIPRED (B cell epitope prediction) software to predict the presence and location of linear B-cell epitopes. We used the BEPIPRED method because it uses the propensity scale methods (as other linear B-cell epitope predictors) and also incorporates hidden Markov model (HMM). The combination of the two best propensity scale methods (Parker and Levitt) with HMM resulted in a performance significantly better than a number of individual tested propensity scales (Parker, Chou and Fasman, Levitt, Emini).[34] Our group has successfully used this method in several other studies for prediction of B-cell epitopes followed by experimental validation. Moreover, the presence of the peptide signal (http://www.cbs.dtu.dk/services/SignalP/) and the N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/) was evaluated. Peptides formed by 12 consecutive amino acids with score higher than 2.0 were selected and tested in cellulose membranes by the SPOT synthesis membrane (peptide arrays on cellulose support generated using SPOT synthesis technology).

The SPOT synthesis was employed using a method for preparation of immobilized peptides with 12 amino acids.[35] The assembly of the peptides was performed utilizing the previously described Fmoc-chemistry.[36] The reactivity of the SPOT membrane was evaluated according to the protocol described by Soutullo et al.[37] Sera from either chronically or acutely infected dogs diluted at 1:500 (v/v) were used as primary antibodies. The anti-total dog IgG or anti-IgM-alkaline phosphatase (AP) conjugates (Bethyl Laboratories, Montgomery, TX) were used as secondary antibodies, at a dilution of 1:5000 (v/v). All experiments using different combinations of primary and secondary antibodies were performed in triplicate.

RESULTS

Differential Expression of Proteins between Amastigote and Promastigote Forms of *L. chagasi*

Differential expression of proteins between amastigotes and promastigotes forms of *L. chagasi* were analyzed by DIGE, and the representative images are presented in Figure 1. Green spots (Figure 1A) indicate promastigote proteins, and red spots (Figure 1B) reveal proteins from amastigote form. An overlay of panels A and B is shown in Figure 1D, and yellow spots (Figure 1C) are mix of proteins from both, amastigotes and promastigotes. Approximately 900 spots were detected in extracts from each parasite stage. All differentially expressed proteins (spots), in addition to those that were abundant in both samples, were excised from gel. A total of 113 spots were excised from the gels: (i) 56 spots only from promastigote forms; (ii) 43 spots only from amastigotes; and (iii) 14 spots present in extracts from both stages were selected and identified by MS.

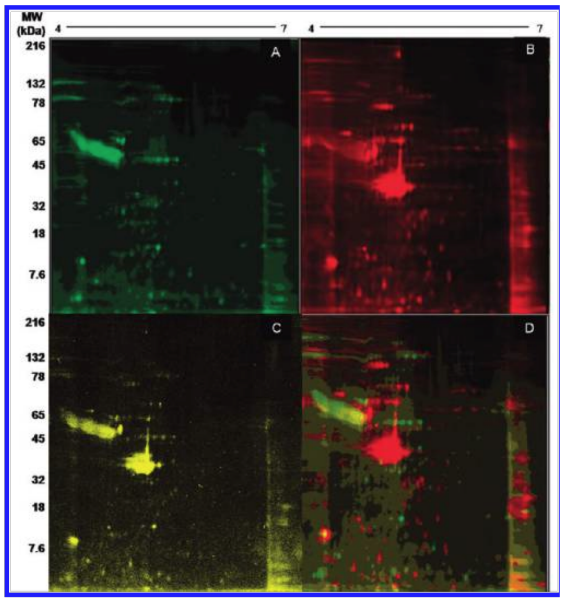

Figure 1. 2D-DIGE analysis of promastigote and amastigotes extracts from *L. chagasi*. Proteins of promastigotes and amastigotes forms, and a pooled internal standard were labeled with CyDyes Cy3, Cy5 and Cy2, respectively, mixed and separated on a 2D gel using 18 cm pH 4−7 (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second dimension. The standards for Cy2 as well as molecular weight and p*I* were defined in the same gel stained with Coomassie Blue and software analysis, respectively. Gels were scanned to obtain single images of (A) promastigotes (Cy3, green), (B) amastigotes (Cy5, red), or (C) the internal standard (Cy2, yellow). (D) An overlay of the two dyes (Cy3, Cy5) is shown in yellow.

The 56 spots selected from promastigote forms corresponded to 25 different proteins from *Leishmania*. In addition, we identified 10 proteins that had similar expression in both promastigote and amastigote stages (two Hypothetical proteins, Eukaryotic translation initiation factor 3 subunit, Translation elongation factor 1-beta, ATP synthase, epsilon chain, Eukaryotic initiation factor 5a, Adenosine kinase, Ribonucleoprotein p18, mitochondrial precursor, Adenosylhomocysteinase and Trypanothione reductase). From 43 spots from amastigote extract, 18 proteins were identified, and only 5 were derived from *Leishmania* (Alpha tubulin, Hypothetical protein, Phosphomannomutase, Prostaglandin f2-alpha synthase and Translation elongation factor 1-beta). The other proteins were proteins from the hamster, from which actin was by far the dominant contaminant.

Thus, from the 28 proteins identified in amastigote extracts, 15 (~54%) were from *Leishmania* and the remaining 13 from hamster. The criteria used to determine the origin of different proteins were the levels of homology to protein sequences deduced from genes found in the *L. chagasi* versus hamster genome. All *Leishmania* proteins identified are presented in Supplementary Table 1. Proteins that are differentially expressed in each sample are highlighted in Figure 2A,B, and those with similar expression in Figure 2C,D. Most of the 900 spots found on DIGE were common to both promastigote and amastigote stages and we assume that they are derived from *Leishmania* parasites. The Venn diagram with the most abundant proteins of amastigote an promastigote stages of *L. chagasi* identified by 2D-DIGE and mass spectrometry is presented in Figure 3.

Immunogenic Proteins Identified by Western Blot−2D Gel

To identify additional immunogenic proteins from *L. chagasi*, we used pool of sera from animals at different stage of infection, that is, acutely infected, chronically infected, or uninfected controls. Details of selected animals and pool of sera are described in Materials and Methods. We have chosen not to use individual sera, because the repertoire of immunoglobulin varies from animal to animal. Thus, using a pool of sera we should have a better representation of antibody specificity to *Leishmania* antigens, than in individual sera. This approach was shown effective, since we found a series of novel antigens and epitopes that were recognized by some, but not other sera from infected dogs. Furthermore, particular peptides derived from the newly identified antigens were recognized by individual sera from the vast majority of dogs with VL, but not from uninfected dogs (data not shown).

The gel from promastigote forms of *L. chagasi* were stained with Coomassie Blue (Figure 4A) or transferred to nitrocellulose membrane and incubated with sera from dogs undergoing acute leishmaniasis (30 days after infection) (Figure 4B) or from uninfected control dogs (Figure 4C) to identify proteins recognized by serum IgM. Three proteins (Mannose-1-phosphate guanyltransferase, heat shock protein 83-1 and α-tubulin) were identified (Supplementary Table 2).

Immunoblots were also performed to identify antigens recognized by total IgG, as well as IgG1 and IgG2 isotypes present in sera from dogs chronically infected with *L. chagasi*. The gel from promastigote forms were stained with Coomassie Blue (Figure 5A), transferred to nitrocellulose membrane, incubated with sera from chronically infected (Figure 5B−D) or uninfected control dogs (Figure 5E), and probed with anti-total IgG (Figure 5B), anti-IgG1 (Figure 5C) or anti-IgG2 (Figure 5D)

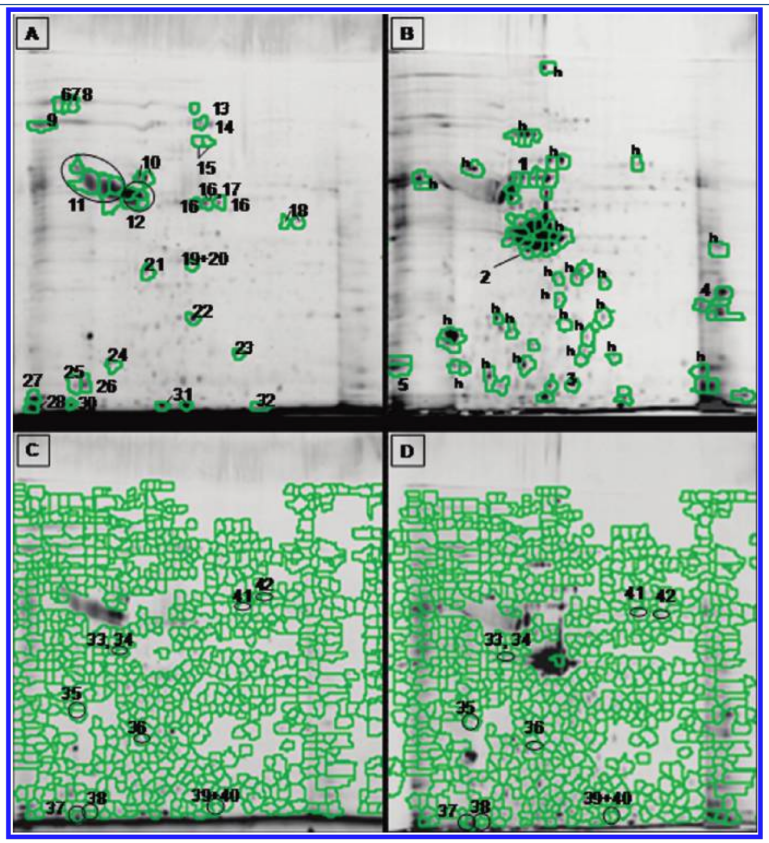

Figure 2. Proteins highly expressed by promastigotes (A) and amastigotes (B) forms derived from *L. chagasi*. The level of protein expression was determined using ImageMaster 2D Platinum 6.0 software, please see details in the Materials and Methods section. The "h" letter (in panel B) represents hamster-derived proteins present in amastigotes sample. Panels C and D show spots with similar expression between promastigote and amastigote, respectively. The numbers refer to the spot identification used in the Supplementary Table 1.

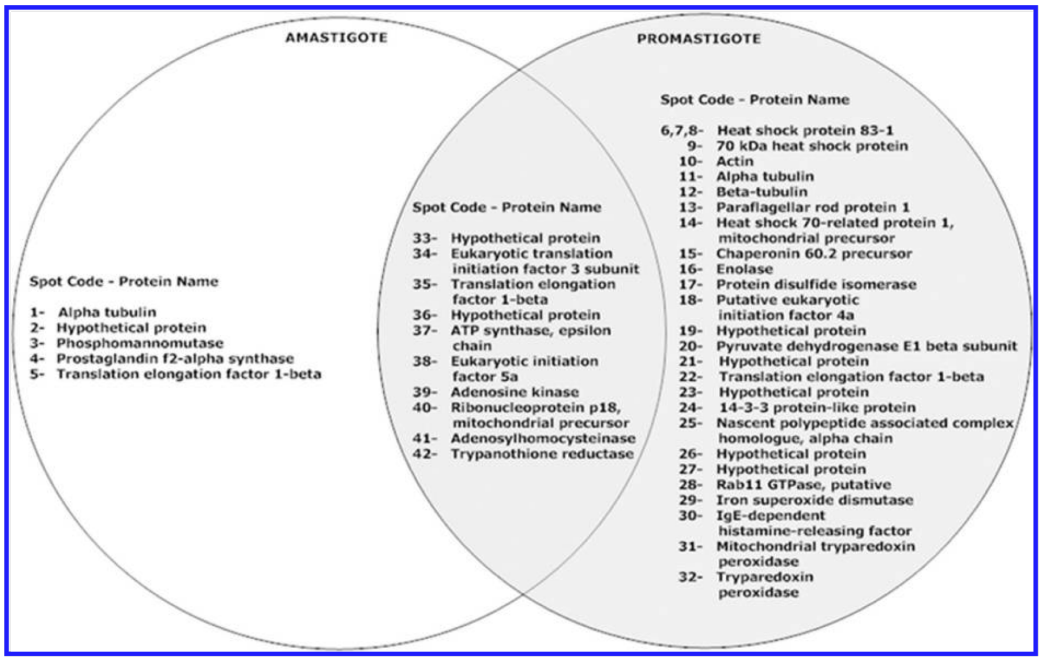

Figure 3. Venn diagram showing proteins expressed in promastigote and amastigote forms from *L. chagasi*.

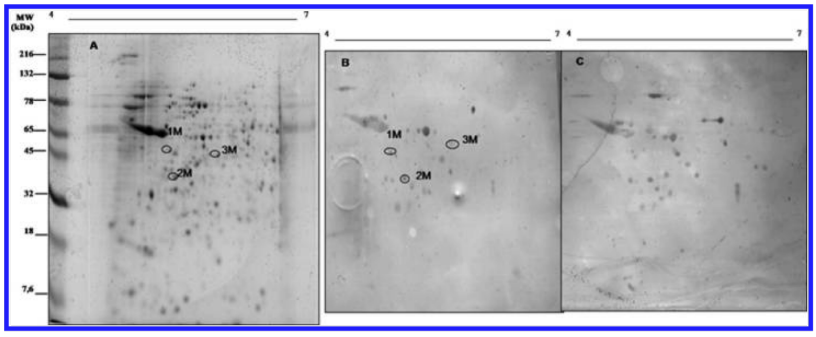

Figure 4. Proteins from promastigotes forms of *L. chagasi* recognized by IgM from sera of acutely infected dogs. Promastigote extract were fractionated using 18 cm pH 4−7 (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second dimension. (A) Gel stained with Coomassie Blue; (B) gel transferred to nitrocellulose membrane and incubated with sera of dogs in the acute phase of infection; or (C) uninfected dogs; and developed with anti-IgM conjugated with peroxidase. The spots recognized only by infected animals and identified by MS are highlighted. The numbers refer to the spot identification used in the Supplementary Table 2.

antibodies conjugated with peroxidase. A large number of antigenic spots were detected in the acidic pH range (4−7). The antigens that were specifically recognized by immune sera from infected dogs, and not by sera from control animals, were selected and further analyzed by MS-MS. Forty-four spots were recognized by total IgG. From those, 12 spots were recognized by both IgG1 and IgG2, 9 recognized by IgG2, and 6 recognized by IgG1 subclasses of immunoglobulin (Supplementary Table 2). Seventeen spots from *Leishmania* extract were recognized by total IgG, but not by IgG1 and IgG2 antibodies. We believe that this is a question of protein concentration and higher sensitivity of the immunoblot, when we employed a secondary antibody for total IgG, versus secondary antibodies specific for IgG1 or IgG2 isotypes. The spots identified by each subclass and the number of spots found between the subclasses of immunoglobulin are shown in Figure 6.

Peptides and Immunoassay in SPOT Synthesis Technique as Candidates for Serodiagnosis

A total of 180 peptides (12 amino acids length) with a high score were selected after BEPIPRED analysis and submitted to SPOT synthesis. The cellulose membranes containing the peptide array were incubated with pools of sera from chronically infected (positive) and uninfected (negative) dogs and developed with anti-total IgG secondary antibody. The intensity of the spots was determined by overlapping membranes incubated with

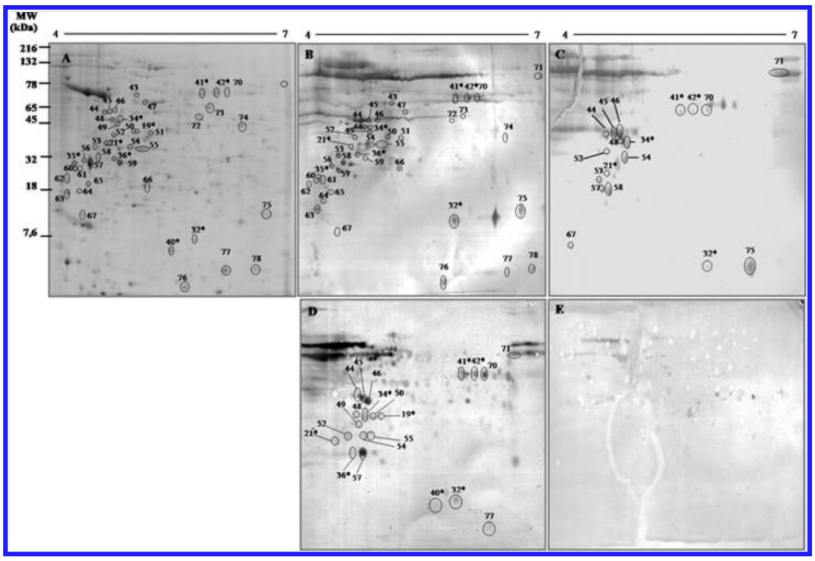

Figure 5. Proteins from promastigotes forms of *L. chagasi* recognized by IgG, IgG1 and IgG2 from sera of chronically infected dogs. Extracts from *L. chagasi* promastigotes were fractionated using 18 cm pH 4−7 (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second dimension. (A) Gel stained with Coomasssie Blue; (B−D) transferred to nitrocellulose membrane and incubated with sera of chronically infected, (E) or uninfected dogs. Immunoblot was developed with either anti-total IgG (B and E), anti-IgG1 (C), or anti-IgG2 (D) conjugated with peroxidase. The spots recognized only by sera of infected animals and identified by MS are highlighted. The numbers refer to the spot identification used in the Supplementary Table 2.

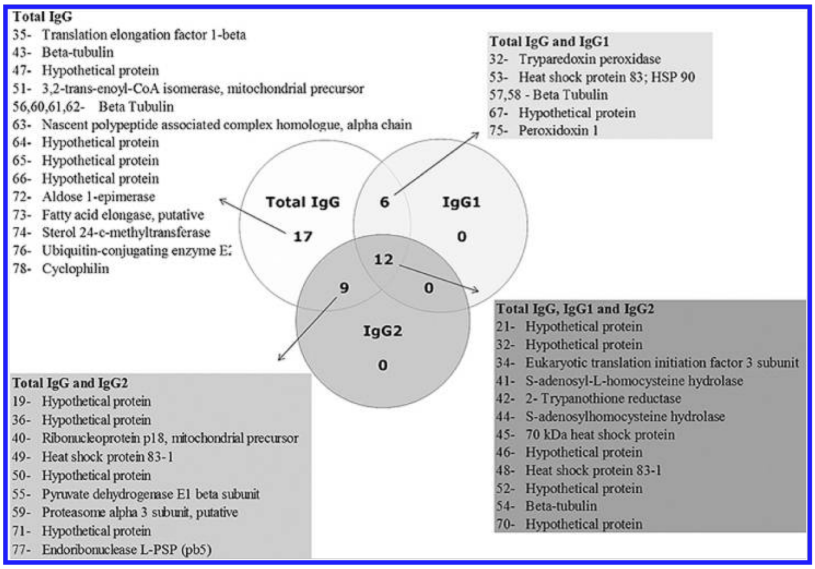

Figure 6. Venn diagram showing intersections of subclasses of immunoglobulins that recognize spots identified by MS after Western blot analysis with sera from infected dogs.

positive and negative sera, as indicated by analysis employing Image-Master software. We selected all spots (peptides) with Relative Intensity (RI) greater than 2.0 (Table 1 and Figure 7). On the basis of immunoassay, we selected 25 peptides with no false positive according to the RI determined in the SPOT image. The 25 peptides were derived from 8 different proteins: Heat Shock Protein-83 (1 peptide), 3,2-*trans*-enoyl-CoA isomerase, mitochondrial precursor (2 peptides), Ribonucleoprotein p18, mitochondrial precursor (1 peptide), Aldose 1-epimerase (2 peptides) and other four hypothetical proteins presenting 2, 3, 7, and 7 peptides each (Table 1 and Figure 7).

Table 1. List of Proteins and Their Respective Immunogenic Peptides (B Cell Epitopes) Selected by BEPIPRED Algorithm and Reactivity with IgG Antibodies Present in Sera from Dogs Infected with *L. chagasi*[a]

B Cell Epitopes			
spot*code	peptide code	protein name	peptide sequence
40	40A	Ribonucleoprotein p18, mitochondrial precursor	EAPSKQDKPVEN
46	46A	Hypothetical protein	TERPEGANFATP
	46B		VEGERVETT
	46C		LTMNTNQPRMPQ
47	47A	Hypothetical protein	TRGVKSSSKLPA
	47B		RDDPHKVTPSDM
49	49A	Heat shock protein 83-1	VTKEYEVQNK
51	51A	3,2-*trans*-enoyl-CoA isomerase,mitochondrial precursor	FQSQPPPGVPQG
	51B		RHQDTNAAPAGS
64	64A	Hypothetical protein	ANIKGVPTRAET
	64B		DSDDTEEGEDEG
	64C		EGTAGEPKPPAM
	64D		MRTSTDMPSQHI
	64E		TRQTSQEPTPVS
	64F		PLATQSYGFGSD
	64G		GVAPPGWYDPPVQ
66	66A	Hypothetical protein	PHRAGETSAAGL
	66B		SQQAPAVPPLPQ
	66C		QGMMSPGRSEEK
	66D		VPKGDKAVSSPP
	66E		GERRRGDAEDGR
	66F		SSRPSPPSKVSS
	66G		AAAAASSPSIAP
72	72A	Aldose 1-epimerase	GYPKNPEEAYAD
	72B		LPASGGPGQRYA

[a] From 41 proteins selected by 2D gel and Western blot analysis, 180 peptides were identified with the BEPIPRED software and synthesized. Twenty-five immunogenic peptides (B cell epitopes) were selected from eight proteins derived from *L. chagasi* based on their ability to discriminate sera of infected dogs from sera from uninfected dogs. These peptides present relative intensity corresponding to 2 or greater, when comparing the reactivity of sera from infected dogs to sera from uninfected dogs (see Figure 7) in a SPOT synthesis membrane.

Prediction of MHC Class I Binding Peptides Using the NetCTL Software

All *Leishmania* proteins selected in the DIGE gel as well as in the immunoblot (Supplementary Tables 1 and 2) were screened for potential T cell epitopes using the NetCTL software. In our analysis, we used a score cutoff of 0.75, which corresponds to a good compromise between sensitivity (0.8) and specificity (0.97).[26] After analysis by the NetCTL software, the following criteria were used to select T cell antigens/epitopes: (i) the ability of a given peptide to bind from three to five HLA haplotypes; (ii) the high score in the prediction for HLA binding; and (iii) the number of T cell epitopes present in a single protein.

We tested the ability of each peptide/protein to bind to 10 different HLA supertypes (A1, A2, A3, A24, B7, B8, B27, B44, B58 and B62) by virtual analysis. The *in silico* analysis indicate that the largest proportion of nanomers were found to bind A24 (17.5%) allele, followed by A3 (14.9%), and then B62 (11.9%), and then by the others. A majority of the nanomers were predicted to bind to only one HLA, but some seemed to be promiscuous and bind to multiple supertypes. The largest number of supertypes to which a given peptide could bind is five. On the average, around 78% of the predicted peptides were found to bind to only one supertype, 16.6% of the peptides reacted to two HLAs, 4.3% peptides to three HLAs, 1.1% peptides to four HLAs, and 0.06% peptides to five supertypes. The sequence of peptides that bind HLA predicted to bind from three to five HLA supertypes and the name of proteins they belong to are presented in Supplementary Table 3.

Finally, we determined the number of immunogenic peptides present in *Leishmania* proteins (Supplementary Table 4). A hypothetical protein (Spot Code 64) contains the highest number of predicted peptides with score >1.5 (166 in total). In part, this is due to the fact that this is the largest protein in the data set (4873 amino acids). Nevertheless, this protein is found among the one-third top proteins with the highest percentage of predicted peptides (25%), which takes into account the total number of predicted peptides and the total number of amino acids in the protein. More importantly, we found that the putative protein Fatty acid elongase (Spot Code 73), which has only 299 amino acids, presented the highest percentage of predicted peptides (33%) (Supplementary Table 4). Proteins and peptides with greater potential to be recognized by CD8+ T cell epitopes are shown in Table 2 and deserve further investigation as a vaccine candidate.

DISCUSSION

In this study, we performed proteomic analysis of the promastigote and amastigote stages of *L. chagasi*, aiming to identify

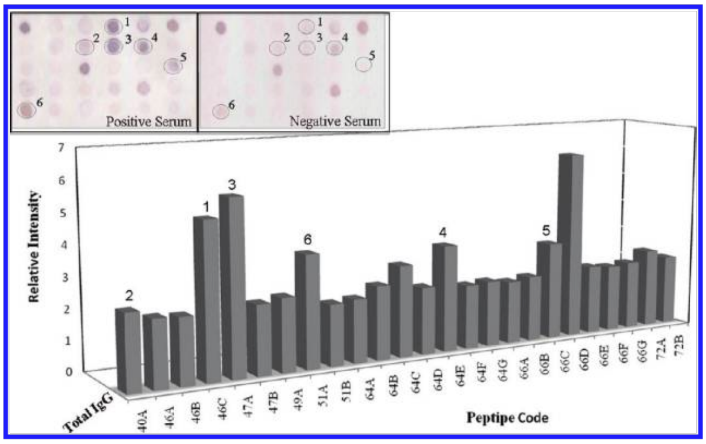

Figure 7. Peptides recognized by sera from chronically infected dogs. To evaluate the antibody reactivity in SPOT synthesis membranes, the relative intensity of the signal was estimated based on comparison of reactivity in immunoblots with sera from chronically infected dogs to the background levels, determined by reactivity with sera from uninfected dogs. A signal was scored as reactive when Relative Intensity (RI) ≥ 2. Only peptides with RI ≥ 2 are shown. Inset shows representative results of immunoblot employing a SPOT synthesis membrane and pools of sera from chronically infected dogs (positive serum) as well as from control uninfected dogs (negative serum). The reaction was revealed with secondary anti-total IgG antibody. Spots 1−6 correspond, respectively, to peptides 46C, 40A, 47A, 64E, 66C, and 51A. Protein (GI) and the sequences of peptides are shown in Table 1.

immunogenic proteins with potential application in the development of immunodiagnostic tests and vaccine for canine VL.[7,8,38,39] To identify immunogenic B cell epitopes, we employed sera from dogs infected with *L. chagasi*. A total of 3 and 38 antigens were specifically recognized by IgM and IgG, respectively. The identification of antigens was followed by *in silico* analysis to predict B cell epitopes. One hundred and eighty putative immunogenic peptides were screened on a peptide array, leading to the selection of 25 peptides that are able to discriminate sera of infected from control uninfected dogs. We also performed *in silico* analysis of proteins identified by DIGE or Western blot techniques to predict epitopes recognized by $CD8^+$ T lymphocytes (CTL), which are thought to be important elements in host resistance to VL. Importantly, the approach described above led to the discovery of various hypothetical and putative proteins that are strong candidates for developing new immunological based diagnostic tests and vaccine for VL.

This study represents several novel aspects in terms of proteomic analysis and antigen discovery for *Leishmania* species. While *L. chagasi* and *L. infantum* are considered the same species,[16,17] differences in their life cycle, such as the insect vector and wild reservoirs, may result in variations on protein expression, leading to different behavior.[18,19] For instance, the number of cases and death due VL is much higher in Brazil than in Europe.[20,21] Here, we perform proteomic analysis of a highly virulent strain of *L. chagasi*, originally isolated from a dog with VL in Belo Horizonte, Brazil.[15] Importantly, this is the first proteomic study designed to identify antigens that are immunogenic for dogs and can be directly applied to develop new immunodiagnostic test as well as vaccine for canine VL. Furthermore, this study represents a thorough proteomic analysis, in which selection of 81 proteins by DIGE and 2D immunoblots was followed by identification of B and potential T cell epitopes, strengthening our search of antigens. Finally, we identified 19 hypothetical as well as 7 putative proteins, which were not previously described.

The lack of access to good-quality diagnostic tests for *Leishmania* infection contributes to the enormous burden of ill health in the world. Since the clinical manifestations of VL lack specificity, confirmatory tests are required to identify dogs infected with *L. chagasi*. This is critical for control of disease, since dogs are the main reservoir of *L. chagasi*, and therefore, critical for transmission and spread of VL to humans. Several antibody-detection tests employing parasite preparations or recombinant proteins have been developed for laboratory and field diagnosis of VL. However, a diagnostic method with high specificity and sensitivity, to guide the management and control of VL in dogs, remains to be developed. We believe that a new generation of diagnostic tests with the expected high sensitivity and specificity should be composed of various linear B-cell epitopes, which have been mapped from *Leishmania* antigens.[2,34,38]

Proteomic maps have been generated for different species that cause cutaneous leishmaniasis, that is, *Leishmania major*,[40] *Leishmania braziliensis*[41] and *Leishmania mexicana*,[10,42,43] as well as for *Leishmania donovani*[14,44,45] and *L. infantum*[7,9,11] that are the etiological agents of VL. Precisely, it was evaluated the differential expression of proteins in axenic amastigotes and promastigotes forms of *L. donovani*.[13,14] In addition, studies mapping *L. donovani* antigens were performed using 2-D Western blot with human sera and parasites isolated from VL patients in India.[44,45] Another study performed a high-resolution proteome analysis of *L. infantum* promastigotes and allowed the identification of immunogenic proteins recognized by a hyperimmune serum from rabbits.[7]

The early diagnosis and treatment has an important role in preventing the development of long-term complications or interrupting transmission of the infectious agent. Three proteins, that is, Mannose-1-phosphate guanyltransferase, alpha tubulin and heat shock protein (HSP) 83-1, were recognized by IgM present in sera from acutely infected dogs. From these proteins, only one peptide from Mannose-1-phosphate guanyltransferase was recognized by IgM antibodies. The HSP 83-1 has a predicted site for glycosylation and carbohydrates may be the main B cell epitope recognized by IgM present in sera from acutely infected dogs.

Sera from chronically infected animals were also used to identify immunogenic proteins recognized by anti-*Leishmania* specific IgG. All immunogenic proteins were analyzed to predict

Table 2. List of *L. chagasi* Derived Proteins and Peptides with Greater Potential for Binding with High Affinity to Multiple HLA Supertypes[a]

T Cells Epitopes								
selected proteins				selected peptides				
spot code	protein name	% predicted peptides	no. of predicted peptides with score >1.5	spot code	protein name of selected peptides	peptide sequence aa position	no. of supertypes	supertypes
73	Fatty acid elongase, putative	33.0	16	18	Putative eukaryotic initiation factor 4a	223-FMRDPVRIL-231	5	A2, A24, B7, B8, B62
53	Heat shock protein 83; HSP 90	29.5	16	47	Hypothetical protein	486-WSSQSPKSF-494	5	A1, A24, B8, B58, B62
74	Sterol 24-c-methyltransferase	29.3	22	64	Hypothetical protein	1326-RMMGVLFDY-1334	5	A2, A3, A24, B27, B62
32	Tryparedoxin peroxidase	29.3	11	72	Aldose 1-epimerase	84-FTLDGVKYY-92	5	A1, A3, A24, B58, B62
75	Peroxidoxin 1	29.1	10	3M	Mannose-1-phosphate guanyltransferase	75-WSRKLGVSF-83	5	A24, B7, B8, B58, B62
65	Hypothetical protein	28.6	9	1	Alpha tubulin	164-KSKLGYTVY-172	4	A1, A3, B58, B62
2	Hypothetical protein	27.6	17	2	Hypothetical protein	93-FVQKVMMPL-101	4	A2, B7, B8, B24
24	14−3−3 protein-like protein	27.6	12	3	Phosphomannomutase	539-GTEPKIKWY-547	4	A1, A3, B58, B62
30	IgE-dependent histamine-releasing factor	27.2	9	9	70 kDa heat shock protein	83-ITNPQSTFY-91	4	A1, A3, B58, B62
49	Heat shock protein 83-1	26.3	23	10	Actin	162-HTVPIYEGY-170	4	A1, A3, B58, B62

[a] NetCTL algorithm was used to identify T cell epitopes. The proteins were chosen based on the number of peptides predicted to bind with high affinity to multiple HLAs. Peptides were selected based on their ability to bind with high affinity to more than three type of HLA.

B cell epitopes, and a peptide array used in an immunoblot for seeking immunogenic peptides.[46−48] Twenty-five peptides from 8 proteins were recognized specifically by sera from infected dogs: HSP-83 (1 peptide), 3,2-*trans*-enoyl CoA isomerase (2 peptides), Ribonucleoprotein p18, mitochondrial precursor (1 peptide), Aldose 1-epimerase (2 peptides) and another four hypothetical proteins presenting 2, 3, 7, and 7 peptides, respectively (Table 1).

A number of studies have demonstrated that different vaccine formulations can induce significant protection against infection with *Leishmania* spp. in a variety of animal models.[5,49,50] Currently, two vaccines for canine VL are commercially available in Brazil, one vaccine composed of parasite extracts,[51] and one composed of a amastigote specific recombinant protein, named A2.[15] However, the efficacy of these vaccines remains partial, and it is necessary to develop a new vaccine formulation with greater efficacy. We also sought to apply bioinformatic methods to identify proteins from *L. chagasi* that contain immunogenic T cell epitopes[52,53] and are candidates for a vaccine against VL. Considering the polymorphic nature of the HLA, promiscuous T cell epitopes are of interest for vaccine design, and the first step of selection of $CD8^+$ T cell epitopes was the ability to bind at least to 3 HLAs. We also considered the affinity of peptide binding to various HLAs, and the number of T cell epitopes in a single protein. On the basis of these parameters, we generated a list of native proteins as well as peptides (Table 1) that could be used to generate chimera proteins for the development of a VL vaccine.

While both $CD4^+$ and $CD8^+$ T lymphocytes are important components for host resistance to VL,[54] algorithms to identify $CD4^+$ T cell epitopes are more error-prone, and we planned to identify these epitopes experimentally. To predict $CD8^+$ T cell epitopes, we used the NetCTL program[26] that predicts peptide binding to 10 HLA class I supertypes (A1, A2, A3, A24, B7, B8, B27, B44, B58 and B62). The NetCTL program also integrates other routines to predict proteasomal C-terminal cleavage and transport efficiency by the transporter associated with antigen processing (TAP).[55] Because dog MHC I polymorphism is not well-known and therefore not incorporated into the predictors, we used human HLA to predict potential dog MHC I peptide binders. Nevertheless, we recognize that this study can be directly applied to elaborate a vaccine for dogs, as a major overlap of dog and humans HLA has been described.[56]

The amastigote is the parasite stage found in the mammalian hosts. Thus, proteins expressed in this developmental form represent potential candidates for vaccine development. Indeed, experiments indicate that immunization with proteins expressed by amastigote stage can provide effective protection against infection.[15,57] In our DIGE, we found only five proteins which were amastigote-specific, that is, phosphomannomutase, prostaglandin f2-alpha synthase, elongation factor-1a, alpha tubulin, which have been previously reported to be abundant in *Leishmania* amastigotes,[9−11,13] and a newly described hypothetical protein. Importantly, from five proteins identified as amastigote-specific, four of them presented high content of T cell epitopes. Three of those proteins contained promiscuous epitopes, which

potentially bind to four different supertypes and were selected as antigens with greater potential to be immunogenic for T cells and vaccine development (Table 2). Furthemore, a vast majority of antigens found in the DIGE were common to amastigotes and promastigotes, and thus, potential vaccine candidates.

Different studies have shown complex antigenic patterns in VL when accessed by Western blot technique.[58−61] Some of these antigens, such as HSP70, gp63, HSP83, several ribosomal proteins, histones, KMP11 or LACK, are well characterized and have been used in either diagnostic tests and vaccination protocols.[62] Other antigens are still waiting to be identified and characterized. For example, 3,2-*trans*-enoyl CoA isomerase and Aldose-1-epimerase are enzymes that catalyze the geometrical or structural changes within one molecule, and the existence of proteins in *Leishmania* protozoa has so far been inferred from homology. Importantly, among the B cell as well as T cell antigens analysis, we identified various antigens previously defined as hypothetical proteins. These hypothetical proteins have conserved homology in other species of *Leishmania* (*L. infantum*, *L. major*, and *L. braziliensis*). Some of these proteins were among the best candidates as B cell antigens for diagnostic tests (Table 1, spot codes 46, 47, 64 and 66), and T cell antigen for vaccine formulations (Table 2, spot codes 2, 47, 64, and 65).

In conclusion, using proteomic and *in silico* analysis, we were able to identify novel proteins that are important targets for humoral and T cell responses against *Leishmania* parasites that cause VL. Further studies employing some of these native as well as chimera proteins shall be employed to develop an accurate diagnostic test and an effective vaccine, to identify infected hosts as well as to prevent transmission and development of canine VL.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Table 1, detailed list of proteins expressed in either or both promastigote or amastigote stages of *L. chagasi*, as revealed by DIGE analysis; Supplementary Table 2, detailed list of proteins revealed by immunoblot analysis; Supplementary Table 3, sequence of peptides, derived from *L. chagasi* proteins, selected by virtual analysis employing the NetCTL and shown to bind to at least three HLA supertypes; Table 4, list of proteins which contain high number of peptides able to bind to different HLAs with a high score, as determined by virtual analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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3.5. Artigo 5. High-throughput analysis of synthetic peptides for the immunodiagnosis of canine visceral leishmaniasis.

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PLoS NEGLECTED TROPICAL DISEASES

High-Throughput Analysis of Synthetic Peptides for the Immunodiagnosis of Canine Visceral Leishmaniasis

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Abstract

Background: Visceral leishmaniasis is the most severe form of leishmaniasis. Approximately 20% of zoonotic human visceral leishmaniasis worldwide is caused by *Leishmania infantum*, which is also known as *Leishmania chagasi* in Latin America, and disease incidence is increasing in urban and peri-urban areas of the tropics. In this form of disease, dogs are the main reservoirs. Diagnostic methods used to identify *Leishmania* infected animals are not able to detect all of the infected ones, which can compromise the effectiveness of disease control. Therefore, to contribute to the improvement of diagnostic methods for canine visceral leishmaniasis (CVL), we aimed to identify and test novel antigens using high-throughput analysis.

Methodology/Principal Findings: Immunodominant proteins from *L. infantum* were mapped in silico to predict B cell epitopes, and the 360 predicted peptides were synthesized on cellulose membranes. Immunoassays were used to select the most reactive peptides, which were then investigated with canine sera. Next, the 10 most reactive peptides were synthesized using solid phase peptide synthesis protocol and tested using ELISA. The sensitivity and specificity of these peptides were also compared to the EIE-LVC Bio-Manguinhos kit, which is recommended by the Brazilian Ministry of Health for use in leishmaniasis control programs. The sensitivity and specificity of the selected synthesized peptides was as high as 88.70% and 95.00%, respectively, whereas the EIE-LVC kit had a sensitivity of 13.08% and 100.00% of specificity. Although the tests based on synthetic peptides were able to diagnose up to 94.80% of asymptomatic dogs with leishmaniasis, the EIE-LVC kit failed to detect the disease in any of the infected asymptomatic dogs.

Conclusions/Significance: Our study shows that ELISA using synthetic peptides is a technique with great potential for diagnosing CVL; furthermore, the use of these peptides in other diagnostic methodologies, such as immunochromatographic tests, could be beneficial to CVL control programs.

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Introduction

Leishmaniasis, which is one of the major parasitic diseases recognized by the World Health Organization, affects approximately 1–2 million individuals annually. Dogs are considered the main domestic reservoir of *Leishmania infantum* (also known as *L. chagasi*) [1], which is the causative agent of zoonotic visceral leishmaniasis (VL) in both the Old and New Worlds [2]. In endemic areas, up to 85% of infected dogs may be asymptomatic [3], and they serve as reservoir for vector transmission to susceptible animals and humans [4].

The epidemiological control of VL in Brazil involves the elimination of infected dogs, widespread insecticide use and the systematic treatment of human cases [5]. Reliable diagnostic tests for *L. infantum* detection are essential to prevent disease transmission and the unnecessary culling of dogs. Given the frequency of asymptomatic infections in dogs and the difficulty of direct parasite detection, the development of rapid and accurate indirect diagnostic methods for canine infection is essential for VL surveillance programs. The principal serodiagnostic tests include the immunofluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA). These conventional tests employ crude antigen preparations of either whole promastigotes or their soluble extracts, which limits assay standardization and result reproducibility [6].

An alternative method for the production of antigens for immunoassays is the synthesis of peptides. These peptides are relatively simple to synthesize and are cheaper to produce compared to the production of whole proteins [7]. In general, the use of synthetic peptides increases the specificity of immunoassays compared to crude antigens [8].

Author Summary

Globally, the number of new human cases of visceral leishmaniasis (VL) is estimated to be approximately 500,000 per year. This is the most severe of all forms of leishmaniasis, and the zoonotic form of VL, caused by *Leishmania infantum* (also known as *Leishmania chagasi*), represents 20% of human visceral leishmaniasis worldwide; additionally, its prevalence is increasing in urban and peri-urban areas of the tropics. In Brazil, the identification and elimination of infected dogs, which act as a reservoir for *Leishmania* parasites, is a control measure employed in addition to the use of insecticides against the vectors and the identification and treatment of infected humans. Currently, the diagnostic methods employed to identify infected animals are not able to detect all of these dogs, which compromises the effectiveness of control measures. Moreover, one of the most important issues in controlling VL is the difficulty of diagnosing asymptomatic dogs, which act as parasite reservoirs. Therefore, to contribute to the improvement of the diagnostic methods for CVL, we aimed to identify and characterize new antigens that were more sensitive and specific and could be applied in epidemiologic surveys.

In previous studies, we identified almost 50 immunodominant proteins of *L. infantum*, mapped their B cell epitopes and submitted 180 peptides to Spot synthesis and immunoassay. A total of 25 peptides showed promising characteristics for serodiagnosis of visceral leishmaniasis [9]. Here, we increased the B cell epitopes mapping, performed a high-throughput analysis of 360 peptides and selected the top 10 peptides for ELISA testing. When assessed, the specificity and sensitivity of the selected peptides was as high as 88.7% and 95.0%, respectively. These new antigens represent solid candidate peptides for the diagnosis of VL with great accuracy, especially in asymptomatic animals.

Methods

Ethics Statement

Experiments with dogs were performed in compliance with the guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation (Comitê de Ética em Experimentação Animal – CETEA, national guidelines Lei 11.794, de 8 de outubro de 2008) from Universidade Federal de Minas Gerais (UFMG); protocol 211/07 was approved on 03/12/2008.

Canine Sera

For the initial screening of *Leishmania* antigens on cellulose membranes, we used a pool of sera from ten animals per experimental group, *i.e.*, chronically infected dogs and uninfected control dogs. The chronically infected dogs were naturally infected with *Leishmania*, and they were found in the metropolitan region of Belo Horizonte, Minas Gerais state, Brazil, rescued and maintained in our facility for laboratory and clinical evaluations. VL in chronically infected dogs was certified by the presence of clinical symptoms and parasitological tests that were conducted on bone marrow cells examined by optical microscopy. The uninfected dogs were negative based on parasitological as well as serological tests for VL; these animals served as negative controls in our study. Blood from all the dogs was withdrawn and maintained at room temperature for 3 h to obtain serum. For each animal in a group, 100 µL of serum was deposited in a single tube to obtain a pool of sera that was representative of chronically infected or uninfected dogs; each group was comprised of 10 dogs.

For the ELISA tests, we used the serum described above and 62 serum samples from 23 symptomatic and 39 asymptomatic dogs from Pancas, Espírito Santo state, Brazil. Dogs were scored for 6 typical signs of canine visceral leishmaniasis: alopecia, dermatitis, chancres, conjunctivitis, onychogryphosis and lymphadenopathy. Each sign was scored on a semi-quantitative scale from 0 (absent) to 3 (severe), and these scores were added together to give an overall clinical score. Dogs with a total score of 0-2 were arbitrarily classed as asymptomatic, 3-6 as oligosymptomatic and 7-18 as symptomatic [10]. Additionally, several serum samples from dogs that were experimentally infected with other pathologies were also tested; samples from dogs that were seropositive for *Trypanosoma cruzi* by RIFI (n = 15) and positive for *Leishmania braziliensis* based on parasitological examination and molecular identification (n = 20) were kindly provided by Prof Dr. Ricardo Toshio Fujiwara of UFMG and Prof Dr. Alexandre Barbosa Reis of UFOP, respectively.

Epitope Identification and Spot Synthesis

Previously, we identified almost 50 immunodominant proteins from *L. infantum*, performed the mapping of their B cell epitopes using BepiPred program that is based on propensity scale methods (http://www.cbs.dtu.dk/services/BepiPred/[11]), and a total of 180 peptides were submitted for Spot synthesis and immunoassays, and 25 peptides were shown to be of interest for use in VL serodiagnosis [9]. Here, we completed the mapping of the same proteins [9] using two different programs: ABCPred, based on machine learning methods that apply a recurrent neural network (http://www.imtech.res.in/raghava/abcpred [12]) and BCPreds, which is also based on machine learning methods but involve those that apply a support vector machine (http://ailab.cs.iastate.edu/bcpreds/[13]). Epitopes that were predicted by the two programs simultaneously (excluding those previously identified [9]) were synthesized using the Spot synthesis method [14] on derivatized cellulose membranes with an Ala-Ala linker; peptide size ranged from 9 to 14 amino acids [15].

Immunoassays with cellulose-bound peptides

Initially, the selection of the most immunoreactive peptides was performed using immunoassays of pooled canine serum (previously described in [9]) and alkaline phosphatase-conjugated goat anti-dog immunoglobulin G. We tested all of the peptides mapped in both studies. The relative intensity of the spots representing each peptide was determined by overlapping positive and negative membranes with ImageMaster™ Platinum program. Peptides with relative intensity values of 2 or greater (RI $\geq$ 2) were considered potential candidate antigens [16]. Next, new membranes were synthesized with only the selected peptides, and they were tested with individual canine sera. To evaluate these assays, cut off values were calculated for each peptide using the mean color intensity + 2 standard deviations (SD) from 5 known negative individual canine sera. All assays were performed in duplicate.

Synthesis of soluble peptides

Based on the results of immunoassays conducted using cellulose-bound peptides that were probed with canine sera, 10 peptides that exhibited reactivity with the largest number of serum samples from infected animals were chemically synthesized using 9-florenyl-methoxy-carbonyl (Fmoc) chemistry [17] in an automated synthesizer, model PSSM8 (Shimadzu, Kyoto, Japan). Peptide purity was assessed with reverse-phase high performance liquid chromatography (HPLC) and mass spectrometry (MALDI-TOF-TOFAutoFlexIII™, BrukerDaltonics, Billerica,

Massachussets, USA). The synthetic peptides were diluted in PBS and used as antigens in ELISA assays.

ELISA

All ELISA procedures were optimized in terms of antigen concentrations and the dilutions of serum and conjugated immunoglobulins to develop a reproducible and robust assay. The optimal antigen concentration was 20µg/mL. A clear separation between sera from *L. infantum*-infected and uninfected animals was possible using 1:100 dilutions for sera and 1:5,000 dilutions for the conjugated immunoglobulins.

Falcon flexible microtitration plates purchased from Becton Dickinson Labware Europe (Becton Dickinson, France S.A.) were coated for 16 h approximately with 100 µL/well of synthetic peptides (20 µg/mL) in PBS. Wells were then blocked with 5% powdered skim milk in PBS/T (PBS containing 0.05% Tween20) at 37°C for 1 hour. Serum samples, diluted 1:100 in PBS/T containing 0.5% powdered skim milk, were added and incubated at 37°C for 1 hour. Plates were washed three times with PBS/T and then incubated with peroxidase-conjugated anti-dog immunoglobulin G (Sigma-Aldrich, St. Louis, MO) diluted 1:5,000 in PBS/T containing 0.5% powdered skim milk at 37°C for 1 h. After washing three times with PBS/T, reactions were developed with Fast-OPD™ (Sigma-Aldrich, St. Louis, MO). Plates were incubated for 30 min in the dark. The reactions were stopped with 2 M H_2SO_4, and the plates were read at 492 nm in a Multiskan Plate Reader (MCC/340).

The results of the ELISA using synthetic peptides as antigens (EP) were compared with those obtained with the EIE-LVC Bio-Manguinhos kit, which is based on immunoenzymatic detection of canine visceral leishmaniasis. The EIE-LVC Bio-Manguinhos kit uses crude antigens and is currently recommended by the Brazilian Ministry of Health for the screening of seroreactive animals [18]. To do this comparison, the same serum samples were tested using both assays; the EIE-LVC kits were used according to the manufacturer's instructions and also calculating a different cut off, employing ROC curve and the same control serum samples already described.

Statistical analysis

A cut off point for optimal sensitivity and specificity was determined using ROC analysis [19], and the area under the curve (AUC) was calculated to assess the performance of the tests. All of the statistical analyses were performed using GraphPad Prism™ (version 5.0) and MedCalc™ (version 7.3).

Agreement beyond chance between the tests was assessed using the Cohen Kappa (κ) coefficient [20] and interpreted according to the following scale: 0.00–0.20, negligible; 0.21–0.40, weak; 0.41–0.60, moderate; 0.61–0.80, good and 0.81–1.00, excellent [21]. The accuracy of each test was evaluated according to the AUC referent to the ROC curve and the Youden index J [22].

Results

Epitope identification, Spot synthesis and immunoassays with cellulose-bound peptides

ABCPred and BCPreds programs simultaneously predicted 191 distinct peptides. However, 11 peptides predicted by both programs were previously predicted by BepiPred [9]. All of these peptides (n = 360) were synthesized in cellulose membranes and submitted to immunoassays. Among these 360 peptides, there were 48 with RI ≥ 2, which are presented in Table S1.

All of the 48 selected peptides were synthesized onto new cellulose membranes and subjected to immunoassays with individual canine serum samples obtained from 5 uninfected dogs and 20 *L. infantum*-infected dogs. The pattern of recognition of the various serum samples was similar against the same peptide; furthermore, uninfected serum samples always showed lower reactivity compared to serum samples from infected animals. In this step, peptides that were reactive with pooled sera from *T. cruzi*-infected animals were excluded (data not shown).

Ten peptides were reactive against multiple individual canine serum samples (from 35% to 75% of samples) and also did not cross-react with pooled sera from *T. cruzi*-infected animals (presented in Table S2). Coincidently, these 10 peptides resulted from the simultaneous prediction by two programs (ABCPred and BCPreds). These peptides were then synthesized in a soluble form using solid phase technique to be used in ELISA tests.

ELISA

The antigens (peptides) selected for ELISA testing were named as follows: PSLc1, PSLc2, PSLc3, PSLc4, PSLc5, PSLc6, PSLc7, PSLc8, PSLc9 and PSLc10. All of the peptides were mixed into a single solution (Mix10), which was used as another antigen in ELISA testing.

Peptide sensitivity and specificity were calculated using parasitological results as a gold standard. Most of the peptides were able to detect a large percentage of symptomatic and asymptomatic infected dogs, which was not observed with EIE-LVC kit. Diagnostic performances of the EP and EIE-LVC kit for canine serum samples are shown in Table 1. Based on the accuracy of the EP, those peptides with higher AUC and Youden index J values were selected. Thus, PSLc6, PSLc8 and PSLc10, as well as the Mix10, were tested again with a higher number of serum samples.

The next stage of testing was performed with 107 serum samples from *L. infantum*-infected dogs and the same 20 serum samples that were used as the negative controls in the previous assays. Additionally, 15 serum samples from *T. cruzi*-infected dogs and 20 from *L. braziliensis*-infected dogs were also tested. Figure 1 shows the results obtained with the three selected peptides and the Mix10 tested separately. Some cross-reactivity with *T. cruzi* and *L. braziliensis* occurred for all of the antigens, being cross-reactivity with *L. braziliensis* more frequent. For PSLc6, 40% of *T. cruzi* and 70% of *L. braziliensis*-infected serum samples were considered positive (Figure 1A). PSLc8 exhibited cross-reactivity with 26.6% of *T. cruzi* and 85% of *L. braziliensis*-infected serum samples (Figure 1B), whereas 93.3% of *T. cruzi* and 95.0% of *L. braziliensis*-infected samples reacted with PSLc10 (Figure 1C). Mix10 showed a similar pattern, with 80% of *T. cruzi*-infected serum samples and 70% of *L. braziliensis*-infected serum samples testing positive (Figure 1D).

The same serum samples were tested using the kit recommended by the Brazilian Ministry of Health (EIE-LVC kit). The cut off value obtained as recommended by manufacturer (negative control absorbance multiplied by two) was very high, and was therefore outside the detection range for many of the infected dogs (Figure 2A). Furthermore, when ROC curve was applied using the previous described control serum samples, the cut off obtained was lower, which increased sensitivity, but cross-reactivity occurred with 53.3% of *T. cruzi* infected sera and with 40.0% of *L. braziliensis* infected sera (Figure 2B).

The sensitivity, specificity, AUC and Youden index J values were calculated for the investigated serological tests (Table 2). The sensitivity of the widely used EIE-LVC kit performed according to the manufacturer was 13.08%, but when ROC curve was applied to calculate cut off value, the sensitivity was 87.85%. EP showed sensitivities that ranged between 71.03% and 84.10%, depending

Table 1. Diagnostic performance of EP and EIE-LVC kit in serum samples from symptomatic and asymptomatic dogs.

Antigen/test	Se %	Sp %	AUC	Youden index J	Sym + (%)	Asym + (%)	Total+ (%)
PSLc1	72.50	75.00	0.754	0.475	17 (73.9%)	28 (71.7%)	45 (72.5%)
PSLc2	75.80	70.00	0.769	0.458	16 (69.5%)	31 (79.4%)	47 (75.8%)
PSLc3	74.10	70.00	0.778	0.441	22 (95.6%)	24 (61.5%)	46 (74.1%)
PSLc4	75.80	70.00	0.797	0.458	11 (47.8%)	36 (92.3%)	47 (75.8%)
PSLc5	70.96	55.00	0.642	0.259	16 (69.5%)	28 (71.7%)	44 (70.9%)
PSLc6	79.00	85.00	0.904	0.64	16 (69.5%)	33 (84.6%)	49 (79.0%)
PSLc7	85.40	80.00	0.863	0.654	19 (82.6%)	34 (87.1%)	53 (85.4%)
PSLc8	82.26	95.00	0.944	0.772	17 (73.9%)	34 (87.1%)	51 (82.2%)
PSLc9	79.00	75.00	0.847	0.540	12 (52.1%)	37 (94.8%)	49 (79.0%)
PSLc10	88.70	85.00	0.947	0.737	20 (86.9%)	35 (89.7%)	55 (88.7%)
Mix10	75.81	95.00	0.916	0.708	13 (56.5%)	34 (87.1%)	47 (75.8%)
EIE-LVC kit *	8.00	100.00	NA	0.08	5 (21.7%)	0 (0%)	5 (8.0%)

Samples from symptomatic (n = 23), asymptomatic (n = 39) *L. infantum*-infected dogs and healthy dogs (n = 20) were tested. Se: sensitivity; Sp: specificity; Sym: number of symptomatic dogs diagnose as positive; Asym: number of asymptomatic dogs diagnose as positive;
*cut off obtained according to the manufacturer.
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upon the peptide utilized. The EP Mix10 presented the highest AUC value (0.902), showing a high accuracy [23], followed by the EP PSLc10 with an AUC value of 0.888. The Youden index J values for these two tests were also the highest, with values of 0.585 and 0.641, for the EP Mix10 and EP PSLc10, respectively. Specificity rates were determined for all uninfected dogs. The specificity of the kit was 100%, as a result of the high cut off value. In contrast, the specificity of EP ranged from 55% to 80%, depending on the peptide used.

When the ROC curves obtained from all of the investigated tests with synthetic peptides were combined, it was possible to observe that EP PSLc6 had the lowest AUC value, showing the

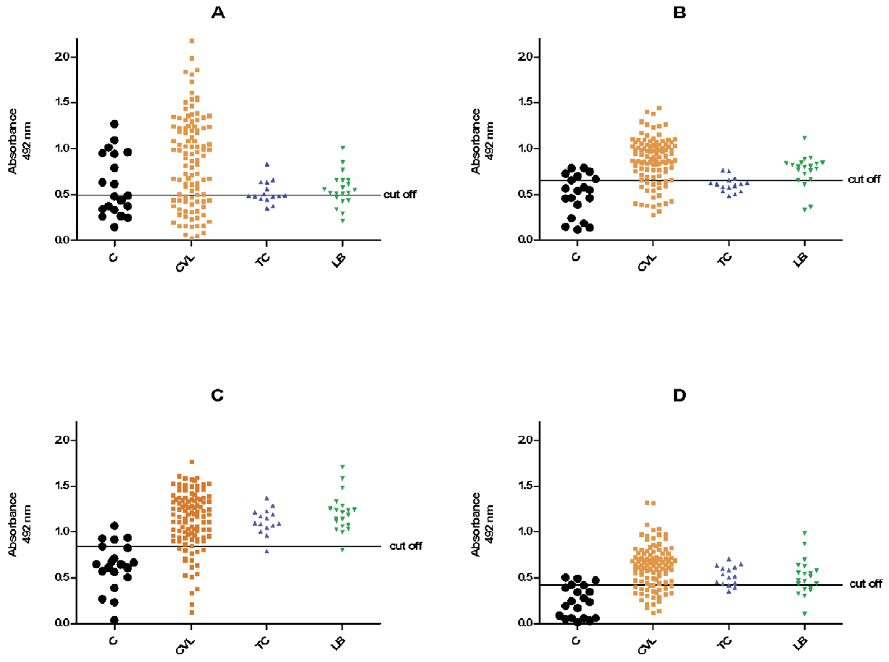

Figure 1. Comparison of ELISA reactivity of canine sera against PSLc6, PSLc8, PSLc10 and Mix10. ELISA was performed in different groups of dogs (C, control group, n = 20; CVL, *L. infantum* group, n = 107; TC, *T. cruzi* group, n = 15; LB, *L. braziliensis* group, n = 20) against PSLc6 (A), PSLc8 (B), PSLc10 (C) and Mix10 (D).
doi:10.1371/journal.pntd.0001310.g001

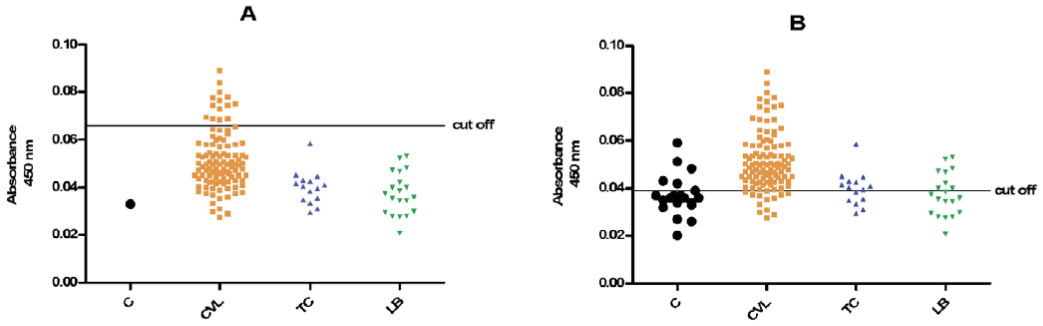

Figure 2. Comparison of ELISA reactivity of canine sera assessed by EIE-LVC kit. ELISA was performed in different groups of dogs (C, control group, n = 1, given by the kit; CVL, *L. infantum* group, n = 107; TC, *T. cruzi* group, n = 15; LB, *L. braziliensis* group, n = 20). In A, cut off was performed according to the manufacturer; in B, it was performed using ROC curve with serum samples from control group.
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most ineffective performance of all of the tests. In contrast, the other tests presented similar performances, showing overlapping ROC curves (Figure 3).

A good agreement beyond chance (κ index) ranging from 0.402 to 0.751 was obtained when the results from each peptide (PSLc6, PSLc8, PSLc10) and Mix10 were cross-tabulated (Table 3). Among these results, the κ index value for PSLc6 and Mix10 was the lowest (0.402), showing weak agreement. The correlation between PSLc8 and Mix10 had the highest κ index value (0.751), showing a good agreement. When the EIE-LVC kit performed according to the manufacturer was cross-tabulated with the synthetic peptides, the agreement was very poor. The index ranged between 0.031 (compared to Mix10) to 0.064 (compared to PSLc8). All of these correlations indicate a negligible agreement between these tests. When EIE-LVC kit performed using ROC curve and 20 control serum samples was cross-tabulated with the synthetic peptides, the agreement was weak. It ranged from 0.213 (compared to PSLc6) to 0.406 (compared to PSLc10).

Discussion

Despite the efforts to search for new diagnostic methods, a method with satisfactory CVL diagnosis efficiency is not yet available. The use of accurate methods that are easy use in the field and are cheap is crucial for diagnosis and consequently, for disease control. Therefore, the identification of new antigens is an important research area for VL disease control. In this work, new antigens for CVL serological diagnosis were investigated using bioinformatic tools and successive screenings with immunoassays.

Using bioinformatics, 360 epitopes were predicted from 47 immunogenic proteins that were identified by bidimensional electrophoresis and Western blot [9]. B cell prediction showed great efficiency because, in the immunoassays, the majority of the cellulose-bound peptides were immunogenic. Of the 48 peptides that could be used to differentiate infected and uninfected dogs, we selected the ten most specific ones.

The ten best peptides resulted from the prediction of two programs simultaneously (BCPreds and ABCPred), and none resulted from using BepiPred considering 2 as a minimum score. However, we observed that 60% of the ten best peptides were also predicted as epitopes based on BepiPred's default score (0.35). These results suggest that using the default score of prediction programs associated with the overlapping predictions by more than one program can be better than using a single type of prediction. Several authors have already shown that combined T cell epitopes generated using consensus predictions are believed to be more accurate [24,25]. It is important to mention that ABCPred and BCPreds approaches are similar (machine learning methods), whereas the BepiPred approach is based on propensity scale methods. This difference could potentially explain the differences between the epitope predictions made by BepiPred alone compared to those made by the ABCPreds and BCPreds programs together.

After the immunoassays in cellulose membranes, the ten peptides that reacted with multiple tested serum samples were

Table 2. Diagnostic performance of EP and EIE-LVC kit in a larger panel of canine serum samples.

Serological test	Sensitivity in CVL group % (95% CI)	Specificity in control group % (95% CI)	AUC	Youden index J
EP PSLc6	71.03 (61.4–79.3)	55.0 (31.5–76.9)	0.661	0.260
EP PSLc8	81.3 (72.6–88.1)	70.0 (45.7–88.1)	0.876	0.513
EP PSLc10	84.1 (75.7–90.4)	80.0 (56.3–94.2)	0.888	0.641
EP Mix10	78.5 (69.5–85.8)	80.0 (56.3–94.2)	0.902	0.585
EIE-LVC kit *	13.08 (7.3–20.9)	100.0 (2.5–100.0)	NA	0.130
EIE-LVC kit #	87.85 (80.12– 93.3)	75.0 (50.9–91.3)	0.848	0.620

Samples from *L. infantum*-infected dogs (n = 107) and healthy dogs (n = 20) were tested. CI: confidence interval; AUC: area under the curve; NA: not applicable;
*cut off obtained according to the manufacturer;
#cut off obtained by ROC curve using 20 control serum samples.
doi:10.1371/journal.pntd.0001310.t002

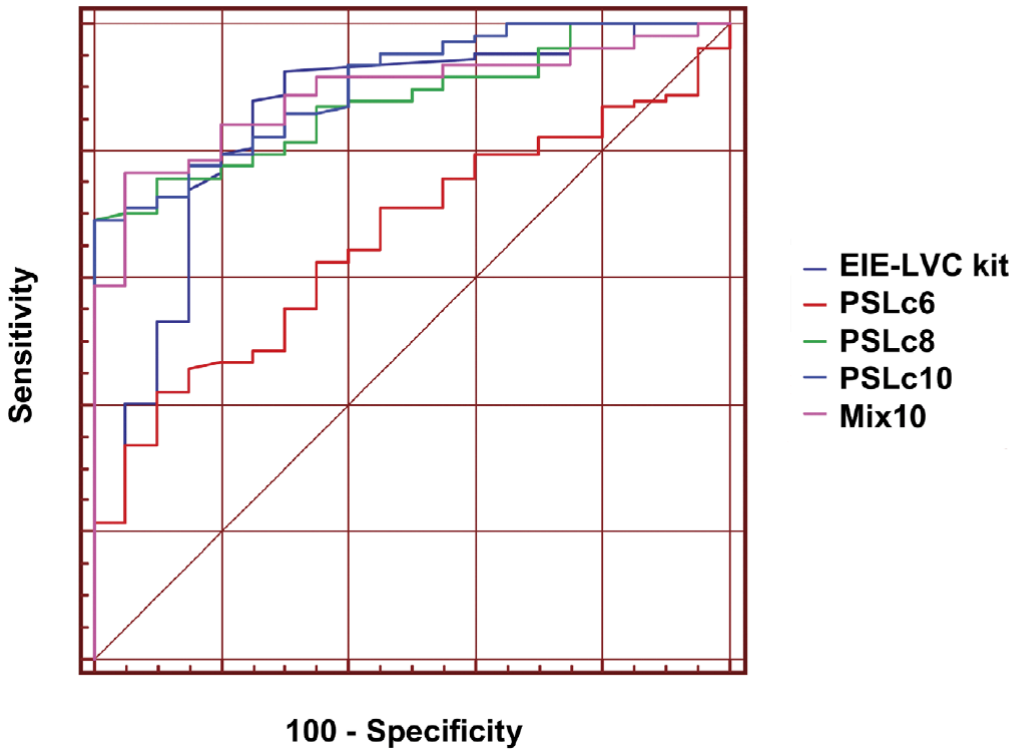

Figure 3. Comparison of ROC curves obtained from all the tests. The curves were used to determine ELISA cut off, sensitivity, specificity and AUC.
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synthesized using Fmoc technique. These peptides showed good reactivity as antigens and were able to recognize specific antibodies in ELISA. The performance observed for the new antigens presented in this work (maximum of sensitivity and specificity of approximately 84.1% and 80.0% respectively) is compatible with the performance of many antigens, mainly recombinant proteins, that have been developed in recent years for CVL diagnosis [26-28] with the advantage of being cheaper and faster to produce.

However, there are other ELISA methods described for CVL that have greater sensitivity or specificity compared to EP. A study conducted in Brazil investigated sera from negative controls (n = 30) and from *L. infantum*-infected dogs (n = 60) in a fucose-mannose ligand based ELISA. Using ROC curve, the sensitivity was 90% and specificity was 93.3% in oligosymptomatic dogs [29]. Another Brazilian study analyzed 209 sera samples from *L. infantum*-infected dogs in a recombinant cysteine proteinase based ELISA. The cut off value was obtained by adding two standard deviation values to the mean absorbance of 22 sera samples from healthy dogs. It provided values of 98% and 96% from sensitivity and specificity respectively [30]. In Europe, some researchers investigated soluble antigens derived from promastigote or amastigote-like stages of *L. infantum* in ELISA. The cut off value was the arithmetic mean plus 3 standard deviations of 48 negative controls. When a group of 47 *L. infantum*-infected dogs was tested, sensitivity varied from 94.1 to 100%, and specificity varied from 96 to 100% [31]. All of these findings show that much has been done to improve CVL diagnosis, but the results are similar. Higher values of sensitivity and specificity are motivations for our antigen improvement.

We compared the results obtained in EP with those obtained with the widely used kit recommended by the Brazilian Ministry of Health, the EIE-LVC kit. Evaluation of EIE-LVC kit, used as recommended by manufacturer, revealed a sensitivity of 13.08% and a specificity of 100%. This low sensitivity could be explained by the high cut off value, which missed many infected dogs. However, following this manufacturer's instruction, test accuracy is related to the composition of only one negative control provided by the kit. Then, we used also 20 serum samples from known uninfected dogs, and the cut off values were obtained using ROC curves. This way, it was possible to obtain sensitivity of 87.8% and specificity of 75.0%. The performance of this kit, when evaluated by other authors is variable. Values of sensitivity of approximately 87.5% and specificity of approximately 100% were obtained elsewhere; 15 false negative results were reported, being 11 in asymptomatic dogs, in a group of 120 samples. Therefore, the prevalence of CVL was underestimated [32]. However, using the same kit, another study showed a sensitivity of 72% and a specificity of 87.5%. These authors indicated the use of EIE-LVC kit in parallel with another kit produced by Bio-Manguinhos, which employed indirect immunofluorescence, to minimize the number of false negatives [33].

Previous data have shown that serologic test performance in CVL depends on infection status [34,35] and an important limitation in CVL control programs is the inability to identify

Table 3. Kappa index (κ) between paired results of diagnostic tests.

Serological test		Serological test														
		EIE-LVC kit *			EP PSLc6			EP PSLc8			EP PSLc 10			EP Mix10		
		P (+)	N (−)	T	P (+)	N (−)	T	P (+)	N (−)	T	P(+)	N(−)	T	P(+)	N(−)	T
EIE-LVC kit #	P(+)	14	87	101	73	28	101	81	20	101	84	17	101	76	25	101
	N(-)	0	26	26	12	14	26	12	14	26	10	16	26	12	14	26
	T	14	113	127	85	42	127	93	34	127	94	33	127	88	39	127
κ index (95% CI)		0.062 (-0.049–0.172)			0.213 (0.011–0.415)			0.306 (0.097–0.514)			0.406 (0.208–0.605)			0.245 (0.041–0.450)		
EP Mix10	P(+)	11	77	88	70	18	88	84	4	88	81	7	88	-		
	N(-)	3	36	39	15	24	39	9	30	39	13	26	39			
	T	14	113	127	85	42	127	93	34	127	94	33	127			
κ index (95% CI)		0.031 (-0.098–0.161)			0.402 (0.227–0.578)			0.751 (0.622–0.878)			0.613 (0.458–0.769)					
EP PSLc 10	P(+)	13	81	94	75	19	94	85	9	94	-			-		
	N(-)	1	32	33	10	23	33	8	25	33						
	T	14	113	127	85	42	127	93	34	127						
κ index (95% CI)		0.060 (-0.061–0.182)			0.455 (0.280–0.629)			0.655 (0.503–0.808)								
EP PSLc8	P(+)	13	80	93	75	18	93	-			-			-		
	N(-)	1	33	34	10	24	34									
	T	14	113	127	85	42	127									
κ index (95% CI)		0.064 (-0.059–0.186)			0.477 (0.306–0.648)											
EP PSLc6	P(+)	11	74	85	-			-			-			-		
	N(-)	3	39	42												
	T	14	113	127												
κ index (95% CI)		0.041 (-0.094–0.175)														

Samples from *L. infantum*-infected dogs (n = 107) and healthy dogs (n = 20) were tested. P: positive; N: negative; T: total; CI: confidence interval;
*cut off obtained according to the manufacturer;
#cut off obtained by ROC curve using 20 control serum samples.
doi:10.1371/journal.pntd.0001310.t003

asymptomatic dogs because classic diagnostic tests are insufficiently sensitive [36]. Thus, a sensitive and specific antigen for the detection of asymptomatic dogs would be highly desirable because it would allow for effective control intervention in areas where CVL occurs. In our tests, the EIE-LVC kit was not able to detect any of the 39 serum samples from asymptomatic dogs, while the new antigens exhibited strong reactivity to the tested sera. For example, in symptomatic dogs, positive reactions reached up to 95.6% and in asymptomatic dogs, reached up to 94.8%, depending upon the used peptide.

Among the tested peptides, PSLc6, PSLc8, PSLc10 and Mix10 showed the highest accuracies when tested with serum samples from animals with defined clinical statuses. Thus, they were selected to be tested with more serum samples. Then, we observed that EP Mix10 showed the best performance, with an AUC of 0.902, characterizing the test as highly accurate. EP PSLc8 and EP PSLc10 had AUC values that characterized these tests as moderately accurate; only PSLc6 generated a test with low accuracy. The highest value of sensitivity (84.1%) also was obtained when PSLc10 was used as antigen. In a canine epidemiological screening, a test with high sensitivity is desirable.

In addition, all of the EP assays showed a good agreement according to the κ index, when they were cross-tabulated. However, when the EP tests were cross-tabulated with the EIE-LVC kit, the agreement was poor in both situations: as performed according to the manufacturer, it was negligible and as performed with our control serum samples, it was weak. It could be explained mainly by the different antigens employed in the tests. The EP appeared to be more sensitive, once it uses synthetic peptide while the EIE-LVC kit employs crude antigen. Thus, even using the ROC curve, the agreement still remained weak between EIE-LVC kit and EP tests.

The occurrence of cross-reactivity with *T. cruzi* and *L. braziliensis* in EP were observed with all synthetics antigens. These results corroborate other researchers' findings concerning to new antigens in CVL diagnosis [26,27,37,38]. In all of them, some cross-reactivity with these parasites occurred.

Although there has been speculation on the role of dogs in the zoonotic cycle of tegumentary leishmaniasis (TL) caused by *L. braziliensis*, only circumstantial evidence supports this hypothesis [39]. Indeed, the primary reservoirs of *L. braziliensis* are small mammals, particularly wild rodents [40], making the importance of this kind of cross-reactivity in the diagnosis of CVL controversial. Therefore, it could be considered that TL infected dogs have low antibodies levels similar to human TL infections. Some authors described the immune response in human TL infection as predominantly cellular, with low levels of circulating antibodies [41].

Importantly, canine infection with *L. braziliensis* is associated with rural areas. Our test has been developed with the intention of being used in urban surveys, as a diagnostic tool in the control of urban LV. Besides, the prevalence rates of *L braziliensis* in dogs are low (3.1%), when parasitological examination was performed in Brazil [42]. About the prevalence of *T. cruzi* in dogs, few data have been published. In a study conducted with 244 dogs in nine municipalities in Paraná state, Brazil, no dogs were found to be

infected with *T. cruzi* [43]. For this reason, it is difficult to establish the real importance of cross-reactivity with these trypanosomatids in EP tests.

The existence of false positives related to these tripanossomatids (*L. braziliensis* and *T. cruzi*) raises the suspicion of cross reaction with another parasites with higher prevalence in urban dogs, such as *Ehrlichia canis* and *Babesia canis*. Further investigation will be necessary in order to characterize the occurrence of potential cross reactions in EP.

Recent studies have evaluated multiple-epitope chimeric antigens as diagnostic markers for the serodiagnosis of CVL [26,44], which represents an interesting approach to our peptides. The development of an immunochromatographic strip would also be desirable, as it is a common approach in the diagnosis of CVL (rK39 strips) and other pathologies [45–47]. It would be interesting to combine multiple peptides to improve the accuracy, as CHEMBIO has done with the DPP™ immunochromatographic test that employs recombinant antigens K39 and K26 to diagnose CVL. It is feasible to use in the same strip test an antigen with good sensitivity and another with good specificity. Taken together, to improve the diagnostic specificity preventing the unnecessary culling of dogs, some alternatives employing the synthetic peptides should be further investigated. For example, structural changes in the antigens, such as the production of conjugated peptides would be an interesting approach in order to increase both sensitivity and specificity.

In conclusion, we have designed new synthetic peptides for the improved serodiagnosis of CVL. The synthetic peptides named PSLc6, PSLc8, PSLc10 and Mix10 afforded high accuracy in detecting CVL cases and are faster and cheaper to produce. Our findings indicate that synthetic peptides will be useful for serodiagnosis and allow for the detection of asymptomatic dogs. The development of an immunochromatographic test using these peptides would be a valuable tool for the rapid diagnosis of CVL, an important issue for the control of this neglected disease in endemic areas.

Supporting Information

Table S1 Peptides with relative intensity (RI) equal or greater than 2.
(XLS)

Table S2 Reactivity of serum samples to 48 different peptides.
(XLS)

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Author Contributions

Conceived and designed the experiments: ARF MMC RTG HMA. Performed the experiments: ARF MMC MSG MLOP. Analyzed the data: ARF GG RTG HMA. Contributed reagents/materials/analysis tools: GG MSG MLOP RTG HMA. Wrote the paper: ARF RTG HMA.

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3.6. Patente 1. Composição Imunogênica para vacina e kit para teste imunodiagnóstico de leishmaniose visceral.

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Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

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: Inst. de Ensino e Pesquisa

1.3 CNPJ/CPF: 17217985000104

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

☐ continua em folha anexa

2. Natureza: ● Invenção ○ Modelo de Utilidade ○ Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: PATENTE DE INVENÇÃO

3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):
"COMPOSIÇÃO IMUNOGÊNICA PARA VACINA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL"

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ○ interna ○ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Asinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: RICARDO TOSTES GAZZINELLI

6.2 Qualificação: PROFESSOR 6.3 CPF: 355.766.506-20

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6.5 CEP: 30160-030 6.6 Telefone: 31 3409-4774 6.7 Fax: 31 3409-6430

6.8 E-Mail: patentes@ctit.ufmg.br

☒ continua em folha anexa

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7. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

☐ 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

☐ em anexo

8. Declaração de divulgação anterior não prejudicial: (Período de Graça):
(art. 12 da LPI e item 2 do AN nº 127/97) ☐ em anexo

9. Procurador (74)

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço completo:

9.5 CEP: 9.6 Telefone: 9.7 Fax:

9.8 E-Mail:

10. Listagem de sequências Biológicas (documentos anexados) (se houver):

☒ Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).

☒ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☒ Declaração de acordo com o artigo 7 e 10 da Resolução INPI nº 228/09: 1 fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório descritivo	22 fls.
☐	11.2 Procuração	fls.	☒	11.6 Reivindicações	2 fls.
☐	11.3 Documentos de Prioridade	fls.	☒	11.7 Desenhos	2 fls.
☐	11.4 Doc. de contrato de trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros que não aqueles definidos no campo 11 (especificar) Anexo 1, Declaração, CNPJ, Portaria ; código de controle				8 fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): 36 fls.

13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

Belo Horizonte, 13 de agosto de 2010
Local e Data

Assinatura e Carimbo

Prof. Rubén Dario Sinisterra
Diretor da Coordenadoria de Transferência
e Inovação Tecnológica - Reitoria/CTIT/UFMG

Anexo 1

6.9 Nome: Hélida Monteiro de Andrade

6.10 Qualificação: Professora

6.11 CPF: 463.292.773-20

6.12 Endereço completo: R. Santa Maria do Itabira – Sion Belo Horizonte - MG,
6.13 CEP: 30310-600

6.14 Telefone: (31) 34094033

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

6.16 Nome: Míriam Maria Silva Costa Franco

6.17 Qualificação: Professora

6.18 CPF: 058.753.016-27

6.19 Endereço completo: Rua Pará, Bairro dos Professores Coronel Fabriciano-MG

6.20 CEP: 35.170-013

6.21 Telefone: (31) 34094033

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

6.23 Nome: Angélica Rosa Faria

6.24 Qualificação: Farmacêutica

6.25 CPF: 067.445.716-13

6.26 Endereço completo: Rua Macedo, n° 89, Bairro Floresta Belo Horizonte- MG

6.27 CEP: 31015-370

6.28 Telefone: (31) 34094033

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

Este anexo apresenta o código de controle da listagem de sequências biológicas de que trata a Resolução INPI 228 de 11/11/2009:

Código de Controle

Campo 1

973060FEBB19CB0A

Campo 2

B7BD9AF6F39BCBCA

Outras Informações:

- Nome do Arquivo: COMPOSIÇÃO IMUNOGÊNICA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL.txt
- Data de Geração do Código: 19-07-2010
- Hora de Geração do Código: 16:45:43
- Código de Controle:
    - Campo 1: 973060FEBB19CB0A
    - Campo 2: B7BD9AF6F39BCBCA

Gerado pelo Sistema de Listagem de Sequências Biológicas (SisBioList)

3.7. Patente 2. Peptídeos recombinantes, método e kit para teste imunodiagnóstico de leishmaniose visceral.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
PROTOCOLO GERAL
07/10/2010 014100003510
14:16 DFMG
0000221 5543997

Espaço reservado ao protocolo | Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

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: Instituição de Ensino e Pesquisa
1.3 CNPJ/CPF: 172179850001-04
1.4 Endereço Completo: Av. Antônio Carlos, 6627, Pampulha - Belo Horizonte - MG
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

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: PATENTE DE INVENÇÃO

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

"Peptídeos recombinantes, método e kit para teste imunodiagnóstico de Leishmaniose Visceral"

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: Ana Paula Salles Moura Fernandes
6.2 Qualificação: Bióloga 6.3 CPF: 623387576-53
6.4 Endereço completo: Rua Cônego Rocha Franco,120,Apto 1000,Bairro Gutierrer-BH-MG
6.5 CEP: 30441-045 6.6 Telefone: 31 3409-4774 6.7 Fax: 31 3409-6430
6.8 E-Mail: patentes@ctit.ufmg.br

☒ continua em folha anexa

INPI Formulário 1.01 – Depósito de Pedido de Patente ou de Certificado de Adição (folha 1/2)

7. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

☐ 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

☐ em anexo

---

8. Declaração de divulgação anterior não prejudicial: (Período de Graça):
(art. 12 da LPI e item 2 do AN nº 127/97)

☐ em anexo

---

9. Procurador (74)

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço completo:

9.5 CEP: 9.6 Telefone: 9.7 Fax:

9.8 E-Mail:

---

10. Listagem de sequências Biológicas (documentos anexados) (se houver):

☐ Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).

☐ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☐ Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

---

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório descritivo	19 fls.
☐	11.2 Procuração	fls.	☒	11.6 Reivindicações	3 fls.
☐	11.3 Documentos de Prioridade	fls.	☒	11.7 Desenhos	12 fls.
☐	11.4 Doc. de contrato de trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros que não aqueles definidos no campo 11 (especificar) Anexo 1, DOU), CNPJ, Portaria				4 fls.

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12. Total de folhas anexadas (referentes aos campos 10 e 11): 40 fls.

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13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

BH, 23/11/10
Local e Data

Assinatura e Carimbo

Prof. Renato de Lima Santos
Pro-Reitor de Pesquisa UFMG
Delegação de Competência
Portaria nº 042 / 2010

Anexo 1

6.9 Nome: Ricardo Tostes Gazzinelli
6.10 Qualificação: Professor
6.11 CPF: 355766506-20
6.12 Endereço: Rua Espírito Santo, 2444, Apto 701, Bairro Lourdes – Belo Horizonte - MG
6.13 CEP: 30160-032
6.14 Telefone: (31) 3409-4033
6.15 E-mail: patentes@ctit.ufmg.br

6.16 Nome: Miriam Maria Silva Costa Franco
6.17 Qualificação: Professora
6.18 CPF: 058753016-27
6.19 Endereço: Rua Prof. Domício Murta, 420, Apto 302, Bairro Ouro Preto – Belo Horizonte - MG
6.20 CEP: 31330-670
6.21 Telefone: (31) 3409-4033
6.22 E-mail: patentes@ctit.ufmg.br

3.8. Patente 3. Peptídeos recombinantes, método e kit para teste imunodiagnóstico de leishmaniose.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
PROTOCOLO GERAL
29/11/2010 014100004197 11:29 DEMG
00002210071383 44

Espaço reservado ao protocolo | Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

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: Instituição de Ensino e Pesquisa
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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

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: PATENTE DE INVENÇÃO

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

"Peptídeos recombinantes, método e kit para teste imunodiagnóstico de Leishmaniose"

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: Ana Paula Salles Moura Fernandes
6.2 Qualificação: Bióloga 6.3 CPF: 623387576-53
6.4 Endereço completo: Rua Cônego Rocha Franco,120,Apto 1000,Bairro Gutierrer-BH-MG
6.5 CEP: 30441-045 6.6 Telefone: 31 3409-4774 6.7 Fax: 31 3409-6430
6.8 E-Mail: patentes@ctit.ufmg.br

☒ continua em folha anexa

7. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

☐ 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

☐ em anexo

8. Declaração de divulgação anterior não prejudicial: (Período de Graça):
(art. 12 da LPI e item 2 do AN nº 127/97) ☐ em anexo

9. Procurador (74)

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço completo:

9.5 CEP: 9.6 Telefone: 9.7 Fax:

9.8 E-Mail:

10. Listagem de sequências Biológicas (documentos anexados) (se houver):

☐ Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).

☐ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☐ Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório descritivo	16 fls.
☐	11.2 Procuração	fls.	☒	11.6 Reivindicações	5 fls.
☐	11.3 Documentos de Prioridade	fls.	☒	11.7 Desenhos	3 fls.
☐	11.4 Doc. de contrato de trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros que não aqueles definidos no campo 11 (especificar) Anexo 1, DOU), CNPJ, Portaria				4 fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): 30 fls.

13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

BH, 23 / 11 / 10
Local e Data

Assinatura e Carimbo

Prof. Renato de Lima Santos
Pro-Reitor de Pesquisa UFMG
Delegação de Competência
Portaria nº 042 / 2010

6.9 Nome: Ricardo Tostes Gazzinelli
6.10 Qualificação: Professor
6.11 CPF: 355766506-20
6.12 Endereço: Rua Espírito Santo, 2444, Apto 701, Bairro Lourdes – Belo Horizonte - MG
6.13 CEP: 30160-032
6.14 Telefone: (31) 3409-4033
6.15 E-mail: patentes@ctit.ufmg.br

6.16 Nome: Miriam Maria Silva Costa Franco
6.17 Qualificação: Professora
6.18 CPF: 058753016-27
6.19 Endereço: Rua Prof. Domício Murta, 420, Apto 302, Bairro Ouro Preto – Belo Horizonte - MG
6.20 CEP: 31330-670
6.21 Telefone: (31) 3409-4033
6.22 E-mail: patentes@ctit.ufmg.br

4. DISCUSSÃO

As leishmanioses representam um grave problema de saúde pública, sendo consideradas pela Organização Mundial de Saúde, em conjunto, como uma das seis mais importantes doenças tropicais de países em desenvolvimento (WHO, 2002; Desjeux, 2004). O controle da LV no Brasil se faz principalmente por meio do controle das populações do inseto vetor e da eliminação dos reservatórios da doença, representado pelos cães infectados. Entretanto, há uma grande falha no controle da doença em função da ausência de uma vacina eficaz para os cães e de testes diagnósticos acurados para leishmaniose (Ashford, 2000; Desjeux, 2004; Chappuis et al., 2007; Boelaert et al., 2007). Estudos baseados em modelos matemáticos indicam, no entanto, que medidas de controle baseadas na detecção sorológica dos cães falham devido à alta incidência da infecção em cães, à alta infectividade dos parasitas, à falta de sensibilidade dos testes diagnósticos sorológico e à demora entre diagnóstico e eliminação dos cães infectados, entre outros aspectos (Ashford 2000; Barbiéri et al., 2006; Chappuis et al., 2007; Porrozzi et al., 2007; Boelaert et al., 2007).

A aplicação das medidas de controle da LV no Brasil é bastante complexa devido ao intricado ciclo de transmissão da doença, que envolve reservatórios domésticos, silvestres e insetos vetores, também devido à precária condição sócio-econômica da população em muitas regiões do país, à urbanização e conseqüente expansão da doença para áreas onde, até recentemente, ela não era registrada. Outros aspectos referem-se às diferenças epidemiológicas da doença em diferentes regiões do país, à baixa sensibilidade dos testes diagnósticos especialmente para a detecção de cães que se encontram nos estágios iniciais da doença. Nesse contexto, a identificação de novos antígenos vacinais e métodos diagnósticos que apresentem maior sensibilidade e especificidade é de grande importância (Ashford, 2000; Desjeux, 2004; Chappuis et al., 2007; Boelaert et al., 2007).

Vários estudos demonstraram o potencial do antígeno A2, específico do estágio amastigota de *Leishmania*, como antígeno vacinal e diagnóstico (Ghedin et al., 1997, Carvalho et al., 2002 Ghosh et al., 2002, Coelho et al, 2003 e Porrozzi et al, 2007). Estudos prévios, conduzidos em camundongos, mostraram que a imunização com o antígeno A2 induz proteção contra *L. (L.) amazonensis* (Coelho et al., 2003) e *L. (L.)*

donovani (Ghosh et al, 2001; Gosh et al, 2002) e permite a diferenciação sorológica entre animais vacinados e infectados, portanto, este antígeno apresenta grande potencial para o desenvolvimento de vacinas.

No presente estudo, utilizando este antígeno, administrado na forma de vacina de DNA, observamos proteção em camundongos contra a infecção por *L. (L.) chagasi* e *L. (L.) amazonensis* (Zanin et al, 2007). Em seguida, realizamos também um estudo de fase II, no qual o antígeno A2, igualmente, induziu proteção em cães beagles vacinados com a proteína A2 associada ao adjuvante saponina (constituindo a vacina Leish-tec®) contra a infecção por *L. (L.) chagasi* (Fernandes et al., 2008). Na avaliação da resposta humoral foi observado que a imunização com a vacina Leish-tec® não induziu a produção de anticorpos específicos ao parasita *Leishmania*, permitindo a distinção sorológica entre cães vacinados e infectados (Fernandes et al., 2008). Assim esta vacina preenche o critério proposto pelo Ministério da Saúde, ou seja, cães vacinados não apresentarão resultados positivos nos testes sorológicos, que é o principal método diagnóstico utilizado atualmente para controle da leishmaniose visceral no Brasil (WHO, 2010). Já a resposta imune celular foi avaliada através da dosagem de IFN-γ e IL-10, sob estímulo com a proteína A2-HIS e com extrato solúvel do parasita (LcPA), antes e após a infecção desafio. Foi observado um aumento significativo da produção de IFN-γ após as imunizações, que se manteve elevado sete meses após o desafio (Fernandes et al., 2008). Estudos sobre a resposta imune celular canina indicam que a produção de citocinas como IFN-γ, IL-12, IL-2 e IL-18 que caracterizam o perfil da resposta T_H1, foram detectadas em cães assintomáticos ou resistentes a infecção (Pinelli et al., 1994; Santos-Gomes et al., 2002 e Chamizo et al., 2005). Com relação a avaliação parasitológica mesmo com a elevada dose de infecção realizada em nosso trabalho, 71.5% dos cães vacinados e infectados não apresentaram nenhum sintoma clínico, no entanto, no grupo de cães infectados (controle), apenas 28.5% dos cães permaneceram assintomáticos, sendo que os sintomas clínicos encontrados foram perda de peso intensa, diarréia profusa e sanguinolenta, caquexia, hepatoesplenomegalia, onicogrifose, lesões de pele e secreção ocular purulenta. Estes sintomas apareceram mais precocemente nos cães do grupo controle, ou seja, cães não vacinados e infectados (Fernandes et al., 2008).

Algumas pesquisas foram conduzidos no Brasil visando o desenvolvimento de vacinas para os cães. A vacina Leishmune® (Fort Dodge Saúde Animal), composta pelo complexo glicoprotéico FML (fucose-manose ligante) de *L. donovani*, em estudo de fase III, induziu proteção em 92% dos cães expostos naturalmente em áreas endêmicas de leishmaniose visceral, sendo que esses permaneceram por quase 1 ano sem o desenvolvimento de sintomas clínicos e apresentaram-se negativos no teste de PCR para *Leishmania* em amostras de sangue, pele e linfonodos. Entretanto, em todos os cães vacinados com o complexo FML, houve produção significativa de anticorpos específicos ao parasita *Leishmania* (Da Silva et al., 2001; Nogueira et al., 2005). Devido a este aspecto, esta vacina não foi recomendada pelo Ministério da Saúde, que por meio de uma portaria, impediu que a mesma fosse adquirida por prefeituras para campanha de vacinação publica contra a LV canina. Os resultados obtidos nesses estudos indicam, portanto, a necessidade de desenvolvimento de outras vacinas adequadas ao controle da LV canina em nosso meio. Isso, associado `a proteção parcial também induzida pela vacina Leish-tec® (que representam as duas únicas vacinas presentes no mercado brasileiro) nos levou a busca por novos antígenos para posteriores testes vacinais e futuras associações com o antígeno A2.

Com relação ao uso da A2 em diagnóstico, observamos que peptídeos provenientes de análises *in silico* da proteína A2, associados a peptídeos de outras proteínas como NH, LACK e K39, apresentaram elevada sensibilidade e especificidade em testes com soros humanos e caninos, principalmente nas associações dos antígenos (Costa et al., 2012). Portanto, com o intuito de otimizar ainda mais os testes diagnósticos e vacinais, optamos por uma abordagem proteômica associada a análises *in silico* para identificar potenciais antígenos de *L. (L.) chagasi*.

O proteoma é uma nova ferramenta muito importante na identificação de proteínas principalmente por integrar técnicas como eletroforese bidimensional, bioinformática, espectrometria de massa, entre outras técnicas. Seu recentes avanço têm possibilitado o estudo de processos fisiológicos intricados e dinâmicos (Leifso et al., 2007; Kumari et al., 2008; Petrak et al., 2008). Neste trabalho realizamos uma análise proteômica das formas amastigotas e promastigotas de *L. (L.) chagasi* com o objetivo de

identificar proteínas diferencialmente expressas, além de imuno-ensaios e análises *in silico* que nos permitiram identificar proteínas imunogênicas e seus respectivos peptídeos para futuro desenvolvimento de potenciais testes diagnósticos e vacinas para leishmaniose canina.

Em nossas análises foi utilizada uma cepa de *L. (L.) chagasi* muito virulenta, originalmente isolada de um cão da cidade de Belo Horizonte, MG/Brasil (Fernandes et al., 2008). Adicionalmente, este foi o primeiro trabalho com uma abordagem proteômica designado a identificar antígenos imunogênicos para cães e o primeiro a utilizar amastigotas provenientes de baço de hamster como parâmetro para comparação com as formas promastigotas das proteínas diferencialmente expressas de *L. (L.) chagasi* .

Muitos trabalhos têm sido realizados utilizando as amastigotas axênicas para comparação das proteínas diferencialmente expressas com as formas promastigotas de *Leishmania* (El Fakhry et al., 2002; Nugente et al., 2004; Walker et al., 2006; McNicoll et al., 2006; Leifso et al., 2007; Brotherton et al., 2010). Aguns trabalhos enfatizaram as espécies causadoras da leishmaniose visceral (El Fakhry et al., 2002; McNicoll et al., 2006; Brotherton et al., 2010) e outros, as espécies causadoras da forma cutânea (Nugente et al., 2004; Walker et al., 2006). Adicionalmente, estudos com *L. major* comparando o perfil de proteínas entre as formas amastigotas provenientes de lesões e promastigotas também foram realizados e confrontados com a expressão gênica e com a expressão em *L. infantum* utilizando amastigotas axênicas (Leifso et al., 2007). Outros trabalhos, com fosfoproteoma, nesta mesma abordagem de comparação entre as formas amastigotas axênicas e promastigotas, utilizando a espécie *L. donovani* também foram descritos (Morales et al., 2008; Morales et al., 2010). Interessantemente, um recente trabalho do proteoma comparativo de *L. donovani* entre as formas amastigotas axênicas e amastigotas provenientes de baço de hamster também foi realizado afim de identificar os fatores de virulência necessários para o estabelecimento da infecção no hospedeiro e, além de observarem diferenças no tamanho e na infectividade entre essas amastigotas, também observaram que 6.7% das proteínas são mais abundantes em amastigotas provenientes do baço, quando comparadas com as amastigotas axênicas (Pescher et al., 2011).

Com relação às proteínas imunogênicas, demais estudos compararam formas amastigotas axênicas e promastigotas de *L. donovani* por western blot 2-D utilizando soro de pacientes humanos infectados (Forgber et al., 2006) e de *L. infantum* utilizando soro hiper-imune de coelho (Dea-Ayuela et al., 2006). Em nosso trabalho, a antigenicidade das proteínas de *Leishmania* foi avaliada utilizando uma mistura de soro de cães infectados para garantir uma melhor representatividade da especificidade dos anticorpos frente ao extrato protéico de *Leishmania*.

A obtenção da lista de proteínas imunogênicas contribui para seleção de antígenos para fase crônica e fase aguda da doença (neste caso, representa um importante passo para o desenvolvimento de testes diagnósticos para a fase inicial da doença) além de auxiliar no melhor entendimento da resposta imune na LV (Rouf et al., 2009). Em nosso estudo três proteínas (manose-1-fosfato-guaniltransferase, HSP 83-1 e alfa-tubulina) foram reconhecidas por IgM, utilizando soro de cães de fase aguda. Interessantemente, a HSP 83-1, reconhecidas por IgM, possui sítios de glicosilação e carboidratos o que é importante no reconhecimento por IgM, e, portanto, representa um dos importantes alvos para posteriores estudos de testes diagnósticos para fase aguda da doença. Utilizando soro de cães de fase crônica e peptídeos sintéticos idenficados por mapeamento de epitopos nas proteínas selecionadas por WB utilizando o software BepriPed, foi possível selecionar 25 potenciais peptídeos para futuros testes diagnósticos da doença: 1 peptídeo da proteína HSP 83-1, 2 peptídeos da proteína 3,2-trans-enoyl CoAisomerase, 1 peptídeo da proteína ribonucloptroteina, 2 peptídeos da aldose 1-epimerase e 19 peptídeos provenientes de 4 proteínas hipotéticas. Futuramente, a imunogenicidade, especificidade e sensibilidade destes peptídeos poderão ser avaliadas em testes imuno-enzimáticos (ELISA) e/ou em testes cromatográficos, que tem grande importância principalmente em testes em campo, onde é complicado o acesso a equipamentos laboratoriais (Rouf et al., 2009; Porrozzi et al., 2007).

Simultaneamente, foi realizada uma análise *in silico* para todas as proteínas identificadas tanto em DIGE quanto em western blot na busca de epitopos reconhecidos por linfócitos TCD8+, os quais são muito importantes elementos na defesa do hospedeiro na LV (Barbiéri et al., 2006; Chappuis et al., 2007; de Oliveira et al., 2009; Herrera-

Najera et al., 2009). O programa NetCTL, que prediz epitopos para linfócitos TCD8+ e ligação a 10 supertipos de HLA, além de considerar na análise clivagem pelo proteassoma e eficiência de transporte por TAP (transporter associated with antigen processing), foi utilizado para seleção de potenciais antígenos vacinais, (Larsen et al., 2010) uma vez que no mercado, as vacinas disponíveis representam uma proteção parcial da doença.

Na análise por NetCTL foi utilizado um banco de dados de HLA humano pela inexistência de um banco de dados caninos, e pelo o fato de existir maior proximidade entre os HLA canino e humanos (Wagner et al., 2003). Dentre 5 proteínas de amastigotas identificadas por DIGE (fosfomanomutase, prostaglandina f2-alfa sintase, fator 1 de elongação, alfa-tubulina e uma proteína hipotética) 4 apresentaram a maior quantidade de epitopos de células T e 3 delas apresentaram epitopos com potencial de se ligarem a 4 diferentes supertipos de HLA, sendo portanto importantes alvos para estudo de antígenos vacinais.

Paralelamente, a fim de complementar a análise de predição para células B, as mesmas proteínas imunogênicas identificadas por WB-2D geraram novos 23 peptídeos como resultado da análise adicional com outros dois programas de predição de epítopos, o ABCPred (http://www.imtech.res.in/raghava/abcpred) e o BCPred (http://ailab.cs.iastate.edu/bcpreds) associado `a imuno-ensaios. Esses novos peptídeos, juntamente com os 25 previamente identificados, foram submetidos a ensaios imuno-enzimáticos que resultaram em 10 antígenos com elevada sensibilidade e especificidade (88.7% e 95%, respectivamente), inclusive maior do que a encontrada pelo kit EIE-LVC, que é o recomendado pelo Ministério da Saúde, que também falhou em detectar cães assintomáticos (Faria et al., 2011).

Portanto, observamos que as análises proteômicas, *in silico* e imuno-ensaios foram importantes em identificar novas proteínas como importantes alvos para resposta humoral e de linfócitos T contra *L. (L.) chagasi*. Estudos futuros empregando algumas dessas proteínas podem ser realizados na busca de testes diagnósticos acurados, inclusive para fase inicial da doença e também, para posteriores testes vacinais.

5. CONCLUSÕES

- Imunizações com o antígenos A2 na forma de vacina de DNA garantiu a proteção contra a infecção por *L. (L.) chagasi* e *L. (L.) amazonenis* em modelos murinos indicando potencial vacinal do antígeno A2;

- Imunizações com a proteína A2, associada ao adjuvante saponina, constituindo a vacina Leish-tec®, garantiu a proteção contra a infecção por *L. (L.) chagasi* em cães. Os resultados observados indicam o potencial da proteína A2 como antígeno vacinal e, estudos adicionais, como o de fase III com a vacina Leish-tec®, devem ser conduzidos para avaliar de forma mais precisa e completa os aspectos relacionados à resposta imune, clínica e parasitológica induzidos pela mesma;

- Nos testes diagnósticos, observamos que peptídeos provenientes de análises *in silico* da proteína A2, associados a peptídeos de outras proteínas como NH, LACK e K39 apresentaram elevada sensibilidade e especificidade em testes com soros humanos e caninos, inclusive para soros com títulos baixos;

- A abordagem proteômica associada a análises in silico permitiu a identificação de novos antígenos com potencial uso para diagnóstico e vacina na LVC;

- Peptídeos sintéticos identificados na abordagem proteômica quando testados em imunodiagnóstico da LVC, apresentaram elevada sensibilidade e especificidade, inclusive maior do que a encontrada pelo kit EIE-LVC, que é o recomendado pelo Ministério da Saúde, que também falhou em detectar cães assintomáticos.

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