República Federativa do Brasil
Ministério do Desenvolvimento, Indústria e do Comércio Exterior
Instituto Nacional da Propriedade Industrial.

(21) **PI 1006646-2 A2**

BRPI1006646A2

(22) Data de Depósito: 13/08/2010
(43) Data da Publicação: 17/04/2012
(RPI 2154)

(51) Int.Cl.:
A61K 39/008
G01N 33/569

(54) Título: COMPOSIÇÃO IMUNOGÊNICA PARA VACINA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL

(73) Titular(es): Universidade Federal de Minas Gerais

(72) Inventor(es): Angélica Rosa Faria, Hélida Monteiro de Andrade, Míriam Maria Silva Costa Franco, Ricardo Tostes Gazzinelli

(57) Resumo: COMPOSIÇÃO IMUNOGÊNICA PARA VACINA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL. A presente invenção descreve uma composição imunogênica para vacina e teste imunodiagnóstico de Leishmaniose Visceral. Mais especificamente, a invenção compreende uma vacina e um kit para teste imunodiagnóstico de Leishmaniose, desenvolvidos através da identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática, síntese de peptídeos e imunoensaio. A alta especificidade desses antígenos possibilita a realização de uma vacina eficaz contra Leishmaniose e de um teste imunodiagnóstico mais eficiente para a Leishmaniose Visceral canina ou humana.

COMPOSIÇÃO IMUNOGÊNICA PARA VACINA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL

A presente invenção descreve uma composição imunogênica para vacina e teste imunodiagnóstico de Leishmaniose Visceral. Mais especificamente, a invenção compreende uma vacina e um kit para teste imunodiagnóstico de Leishmaniose, desenvolvidos através da identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática, síntese de peptídeos e imunoensaio. A alta especificidade desses antígenos possibilita a realização de uma vacina eficaz contra Leishmaniose e de um teste imunodiagnóstico mais eficiente para a Leishmaniose Visceral canina ou humana.

As Leishmanioses são doenças causadas por protozoários do gênero *Leishmania* (Ross R 1903. (1) Note on the bodies recently described by Leishman and Donovan and (2) Further notes on Leishman's bodies. *Brit Med J* 2:1261-1401), ordem *Kinetoplastida*. Agrupam-se no gênero cerca de 30 espécies, sendo aceito que aproximadamente 21 tenham a capacidade de infectar humanos (Herwaldt B 1999. Leishmaniasis. *Lancet* v.354, n.9185, p.1191-1199; Ashford R 2000.The leishmaniasis as emerging and reemerging zoonoses. *Int J Parasitol* 30: 1269–1281). A doença ocorre em 88 países com aproximadamente 12 milhões de infectados e 350 milhões de pessoas sob risco de contrair a infecção (http://www.who.int/leishmaniasis/burden/en/).

Nos últimos 10 anos, regiões endêmicas têm se expandido e um considerável aumento no número de casos registrados da doença tem ocorrido em todo o mundo. Como a notificação da doença é compulsória em apenas 32 dos 88 países afetados pelas Leishmanioses, um grande número de casos não é contabilizado. De fato, considera-se que 2 milhões de novos casos (1,5 milhão para Leishmaniose Tegumentar e 500.000 para Leishmaniose Visceral) ocorram anualmente (http://www.who.int/leishmaniasis/burden/en/).

Dependendo da espécie de *Leishmania* e da resposta imune do hospedeiro, há várias manifestações clínicas da doença: Leishmaniose Cutânea, Mucocutânea, Difusa e Visceral. A Leishmaniose Visceral (LV), forma mais severa da doença, é uma antroponose na Índia e África Central e uma

zoonose na região do Mediterrâneo e nas Américas. A forma zoonótica da Leishmaniose Visceral (LV), causada por *L. infantum* (também denominada *L. chagasi*), representa 20% da leishmaniose visceral humana mundial (100.000 casos anualmente) e sua incidência é crescente nas áreas urbanas e peri-urbanas dos trópicos (Dye C 1996.The logic of visceral leishmaniasis control. *Am J Trop Med Hyg* 55 (2):125–30).

O cão desempenha um papel importante dentro da epidemiologia da leishmaniose visceral zoonótica, já que este tem sido considerado como o principal reservatório doméstico para a doença humana (Ashford D, David J, Freire M, David R, Sherlock I, Eulálio M, Sampaio, Badaro R 1998. Studies on control of visceral leishmaniasis: impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. *Am J Trop Med Hyg* v.59, n.1: 53-7; Alvar J, Cañavate C, Molina R, Moreno J, Nieto J 2004. Canine leishmaniasis. *Adv Parasitol* 57:1-88). A importância dos cães como reservatório é decorrente do contato frequente e da proximidade com humanos, além do fato de o animal poder apresentar infecção assintomática, apesar da alta parasitemia na pele e nas vísceras (Madeira M, Schubach A, Schubach T, Leal C, Marzochi M 2004. Identification of *Leishmania chagasi* isolated from healthy skin symptomatic and asymptomatic dogs seropositive for leishmaniasis in the Municipality of Rio de Janeiro, Brazil. *Braz J Infect Dis* 8:440-444).

O cão constitui um excelente modelo para estudo da LV. Esta espécie é alvo das medidas de controle e apresenta semelhança das alterações clínicas e anatomopatológicas com a LV humana. O modelo experimental canino pode ser utilizado para testes de drogas, identificação de moléculas marcadoras da infecção e ainda identificação de antígenos para uso diagnóstico, prognóstico e/ou vacinal em humanos. Em suma, o progresso do conhecimento da LV canina (LVC) pode ajudar a prevenir e tratar a doença humana (Moreno J, Alvar J 2002. Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol*, 18 (9): 399-05).

Nas áreas de ocorrência de LV, as medidas de controle incluem diagnóstico de caninos e humanos, para eliminação dos reservatórios e tratamento dos pacientes. Portanto, a precocidade do diagnóstico influencia no

sucesso do tratamento e no controle da transmissão. Entretanto, o diagnóstico canino enfrenta uma série de dificuldades tendo em vista os métodos disponíveis.

Atualmente, existe grande variedade de testes para diagnóstico da LVC. Entretanto, cada um apresenta algum ponto negativo. A Reação de Fixação do Complemento e a Reação de Imunofluorescência Indireta, por exemplo, podem resultar em reações cruzadas com Doença de Chagas (DC) e Leishmaniose Tegumentar Americana (LTA). Já a reação de PCR tem uso limitado pelo alto custo e pouca adaptabilidade ao campo. O ensaio imunoenzimático (ELISA), com os antígenos atualmente utilizados, também demonstra reações cruzadas.

Para o imunodiagnóstico de casos humanos, a disponibilidade de testes é pequena. No mercado brasileiro, praticamente a única opção para uso em humanos, com boa sensibilidade e especificidade, é a RIFI (Reação de Imunofluorescência Indireta), que ainda não é ideal por apresentar possibilidade de reações cruzadas devido ao uso de antígeno bruto. Além disso, é um teste que exige treinamento técnico apurado, uma vez que a interpretação do resultado é subjetiva nos casos de baixos títulos de anticorpos anti-Leishmania. A execução da RIFI é mais laboriosa do que outros métodos sorológicos, não há possibilidade de automação e sempre irá requerer a aquisição de equipamento de alto custo (microcópio de imunofluorescência).

Considerando que a maioria dos métodos disponíveis possui desvantagens e que a ocorrência de reações cruzadas com outras infecções inviabiliza um diagnóstico correto, faz-se necessária a pesquisa de novos antígenos que permitam que a doença seja diagnosticada com maior segurança, sensibilidade e especificidade, tanto em cães quanto em humanos. Entre os diferentes métodos para o diagnóstico, ELISA é o mais utilizado devido ao seu baixo custo e facilidade de execução.

Com o objetivo de melhorar os testes imunodiagnósticos disponíveis, algumas metodologias vêm sendo desenvolvidas, como as descritas nos seguintes documentos de patentes: US 2010092938 - Novel and Practical Serological Assay for the Clinical Diagnosis of Leishmaniasis, US 2007134671 - Oligonucleotides for detection of leishmaniasis and methods thereof, US

5965142 - Polypeptides and methods for the detection of L. tropica infection, WO9633414 - Compounds and methods for diagnosis of leishmaniasis, US 5942403 - Compounds and methods for the detection of t. cruzi infection, PI9610679-4 – Antígenos de Leishmania para uso na terapia e diagnose da Leishmaniose, PI9300775-2 - Processo de diagnóstico sorológico da leishmaniose visceral canina e/ou humana, WO 2005/063803 – Polypeptides for the diagnosis and therapy of leishmaniasis, WO 1996/033414 - Compounds and methods for diagnosis of leishmaniasis, WO 1989/001045 - Leishmania-specific antigens, process for preparing them, antigenic profiles containing these antigens and their application to the diagnosis of visceral leishmaniasis.

Estes documentos apresentam compostos que incluem polipeptídeos, contendo pelo menos uma parte imunogênica de pelo menos um antígeno de Leishmania, ou de uma variante deste, para serem usados em imunodiagnóstico de Leishmaniose. Os polipeptídeos neles descritos, porém, são diferentes dos utilizados na presente invenção e foram isolados por abordagens diferentes da utilizada na presente invenção. A presente invenção descreve a identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática, síntese de peptídeos e imunoensaio, permitindo a realização de um teste imunodiagnóstico mais específico e mais eficiente para a leishmaniose visceral canina ou humana.

Uma vez que a eficácia das medidas de controle da LV tem se mostrado inconsistente, a vacinação canina pode ser uma maneira efetiva de reduzir a transmissão (Gradoni L. 2001. An update on antileishmanial vaccine candidates and prospects for a canine Leishmania vaccine. Vet Parasitol 100 (1–2):87–103). Os diferentes imunógenos candidatos para o desenvolvimento de vacinas incluem parasitos mortos, frações purificadas, antígenos recombinantes de *Leishmania*, além de DNA codificante de antígeno (Melby PC. 2002. Vaccination against cutaneous leishmaniasis: current status. Am J Clin Dermatol 3(8):557–70). No modelo canino, diferentes estratégias de vacina contra LV têm sido utilizadas. A vacinação com promastigotas mortas ou misturas indefinidas de antígenos induzem resposta imune celular e proteção parcial contra a infecção (Gradoni, 2001). O ligante de fucose manose (FML)

purificado de *L. donovani* (Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante). Vaccine) e vários diferentes antígenos recombinantes, obtidos de proteínas purificadas, também conferem pelo menos proteção parcial contra infecção por *L. infantum* em cães (Saldarriaga OA, Travi BL, Park W, Perez LE, Melby PC. 2006. Immunogenicity of a multicomponent DNA vaccine against visceral leishmaniasis in dogs Vaccine 24: 1928–1940.).

O desenvolvimento de vacinas contra este parasita é a principal meta da Organização Mundial de Saúde. Uns grandes números de estudos têm demonstrado que as diferentes formulações da vacina oferecem significante proteção contra *Leishmania* em uma grande variedade de modelos animais (Kedzierski L, Zhu Y, Handman E. Leishmania vaccines: progress and problems. *Parasitology.* 2006, *133*, S87-112.; Drummelsmith J, Brochu V, Girard I, Messier N, Ouellette M. Proteome mapping of the protozoan parasite Leishmania and application to the study of drug targets and resistance mechanisms. *Mol. Cell Proteomics.* 2003, *2*:146-55, de Oliveira CI, Nascimento IP, Barral A, Soto M, Barral-Netto M. Challenges and perspectives in vaccination against leishmaniasis. *Parasitol. Int.* 2009, 19.). Atualmente, somente quatro vacinas estão licenciadas para uso: uma utilizada para humanos no Uzbesquistão, outra utilizada como imunoterapia no Brasil e duas vacinas para cães de uso profilático também no Brasil (Palatnik-de-Sousa CB. *Vaccines for leishmaniasis in the fore coming 25 years.* Vaccine. *2008 Mar 25;26(14):1709-24*; Fernandes AP, Costa MMS, Coelho EA, Michalick MS, de Freitas E, Melo MN, Luiz Tafuri W, Resende Dde M, Hermont V, Abrantes Cde F, Gazzinelli RT. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. *Vaccine.* 2008, *26*, 5888-5895.).

Entretanto, a eficácia das vacinas permanece parcial, e, portanto é necessário o desenvolvimento de vacinas de grande eficácia. O desenvolvimento de vacinas para *Leishmania* é um grande desafio devido ao

insuficiente conhecimento da patogenicidade e da complexidade da resposta imunológica necessária para proteção. Além disso, um pequeno número de antígenos de *Leishmania* tem sido testado como vacina, sugerindo que há ainda muitos antígenos potenciais para serem avaliados. (Matlashewski, G. Leishmania infection and virulence. Microbiol Immunol. 2001, 190, 37-42 El Fakhry Y, Ouellette M, Papadopoulou B. A proteomic approach to identify developmentally regulated proteins in Leishmania infantum. Proteomics. 2002, 2, 1007-1117; Dea-Ayuela MA, Rama-Iñiguez S, Bolás-Fernández F. Proteomic analysis of antigens from Leishmania infantum promastigotes. Proteomics. 2006, 6:4187-4194; Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat. Rev. Microbiol. 2007, 5, 873-882; de Oliveira CI, Nascimento IP, Barral A, Soto M, Barral-Netto M. Challenges and perspectives in vaccination against leishmaniasis. *Parasitol. Int.* 2009, 19; Herrera-Najera C, Piña-Aguilar R, Xacur-Garcia F, Ramirez-Sierra MJ, Dumonteil E. Mining the Leishmania genome for novel antigens and vaccine candidates. Proteomics. 2009 , 9:1293-301.).

Estudos proteômicos com *Leishmania* demonstraram a diferença de expressão protéica entre amastigotas e promastigotas e a possibilidade de utilização da "sorologia proteômica" como uma abordagem adequada para mapeamento da antigenicidade na leishmaniose. Recentemente, um estudo utilizando 2-DE de extrato de promastigotas de *L. infantum*, seguido de Western Blot com soro de coelhos imunizados e análise por EM, permitiu a identificação de várias proteínas antigênicas relevantes (Dea-Ayuela M, Rama-Iñiguez S, Bolás-Fernández F 2006. Proteomic analysis of antigens from *Leishmania infantum* promastigotes. *Proteomics* 6: 4187–4194). Numa abordagem semelhante, foram identificados vários antígenos de *L. donovani* utilizando soros e parasitos isolados de pacientes indianos, permitindo concluir que a abordagem proteoma-sorologia produz uma compreensiva e altamente resolvida representação da antigenicidade de *L. donovani* e da especificidade da resposta imune anti-leishmania em pacientes com LV (Forgber M, Basu R, Roychoudhury K, Theinert S, Roy S, Sundar S, Walden P 2006. Mapping the

antigenicity of the parasites in *Leishmania donovani* infection by proteome serology. *PLoS ONE* 1:e40). Todos esses estudos utilizaram amastigotas de cultura axênica. A presente invenção demonstra a utilização do extrato protéico de promastigotas para identificação de antígenos que participarão da composição imunogênica para vacina contra Leishmaniose.

Vários documentos de patentes que demonstram o uso de polipeptídeos de *Leishmania* em composição imunogênica para vacina foram encontrados. Como exemplo, citam-se os seguintes: WO 2009143006 - Recombinant polyprotein vaccines for the treatment and diagnosis of leishmaniasis, WO 2007121184 - Compounds and methods for diagnosis and treatment of leishmaniasis, WO 2006110915 - Vaccine formulations for leishmania, US 2006073170 - Vaccine complex for preventing or treating leishmaniases, CA 2503932 - Polypeptides of leishmania major and polynucleotides encoding same and vaccinal, therapeutical and diagnostic applications thereof, WO 02098359 - Leishmania antigens for use in the therapy and diagnosis of leishmaniasis. Nenhum deles, porém, utiliza as mesmas proteínas e/ou peptídeos demonstrados na presente invenção, além de não demonstrarem o isolamento destes por análise proteômica. A presente invenção descreve a identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática e imunoensaio, permitindo a produção de uma vacina mais específica e mais eficiente para a leishmaniose visceral canina ou humana.

BREVE DESCRIÇÃO DAS FIGURAS

Figura 1 - Extratos protéicos de formas promastigotas de *L. chagasi* foram fracioandos por 2-DE usando tiras de 18 cm, pH 4-7 e SDS-PAGE a 12%. Os géis foram corados com comassie (A) ou transferidos para membranes de nitrocellulose (B, C e D) e incubados com soros de cães naturalmente infectados ou não infectados (E) e revelados com anti-IgG total (B e E) anti-IgG1 (C) anti-IgG2 (D) conjugado com peroxidase. Os "spots" reconhecidos somente por animais infectados e identificados por EM (Espectrometria de Massa) estão destacados.

Figura 2 – Para avaliar a reatividade de anticorpos aos peptídeos sintetizados nas membranas, foi calculada a intensidade relativa (IR) entre soros infectados e não infectados para o mesmo peptídeo. Foram considerados reativos os peptídeos com IR>2,0. As proteínas e as seqüências dos peptídeos estão mostrados na Tabela 2.

Figura 3 - Para avaliar a reatividade de anticorpos aos peptídeos sintetizados nas membranas, foi calculada a intensidade relativa (IR) entre soros infectados e não infectados para o mesmo peptídeo. Foram considerados reativos, peptídeos com IR>2,0. As proteínas e as seqüências dos peptídeos estão mostrados na Tabela 3.

DESCRIÇÃO DETALHADA DA TECNOLOGIA

Obtenção das Proteínas

Promastigotas de *Leishmania chagasi* (MCAN/BR/2000/BH400) foram cultivadas a 23°C em meio Schneider (Gibco BRL) suplementado com 10% de soro fetal bovino (Sigma), 200U de penicilina (Sigma), 100µg de estreptomicina (Sigma). Essas formas foram utilizadas na fase exponencial de crescimento para extração de proteínas.

Para obtenção das proteínas os parasitas foram lavados 3 vezes no meio Schneider (Gibco BRL) com centrifugação a 200g por 5 minutos a 4 °C. O sedimento celular foi homogeneizado em um tampão de lise (8M uréia, 2 M tiouréia, 4% CHAPS, 65 mM dithiothreitol - DTT, 40 mM Tris-base e coquetel de inibidor de protease da GE Healhtcere) na proporção de 500 μL de tampão de lise para 10^9 parasitas. As amostras foram incubadas por 1 hora à temperatura ambiente e centrifugadas a 20.000g por 15 minutos à temperatura ambiente. O sobrenadante (extrato protéico) foi mantido a -70°C. A proteína foi quantificada por 2D-Quant kit (GE Healthcare, USA) de acordo com as instruções do fabricante.

Eletroforese Bidimensional (2-DE)

Foi empregado o protocolo descrito anteriormente (Paba J, Santana JM, Teixeira AR, Fontes W, Sousa MV, Ricart CA. 2004. Proteomic analysis of the human pathogen Trypanosoma cruzi. Proteomics 4(4):1052-9.), com

que não foi transferido, mas corado por Coomassie colloidal- CBB G-250 (Neuhoff V, Arold N, Taube D, Ehrhardt W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis. 1988 Jun;9(6):255-62.).

Géis corados e membranas provenientes do WB (**Figura 1**) foram digitalizados e a imagem analisada por programa de computador para serem normalizados e para identificar a posição das proteínas no gel reveladas no WB, utilizando como referência os marcadores de ponto isoelétrico (pI) e de massa molecular (MM).

As imagens das membranas foram analisadas para seleção dos "spots" que são reconhecidos por soros de animais infectados, descartando-se aqueles que são reconhecidas por soros de animais não infectados (reações inespecíficas). Os "spots" selecionados como imunogênicos e sem reações inespecíficas nas fases aguda e crônica da infecção canina foram retirados dos géis para identificação por espectrometria de massa (EM = MS).

Digestão das Proteínas

Os "spots" selecionados foram retirados do gel manualmente. Cada spot foi colocado em um tubo de 1,5mL e lavado 3 vezes em 100µl de bicarbonato de amônio a 25mM e 50% v/v de acetonitrila. Após secarem, os fragmentos do gel foram reidratados com 10µl de tripsina (Promega), a 20 ng/µl em 25mM de bicarbonato de amônio por 30 minutos a 4°C. O excesso da solução foi retirado e os pedaços de géis foram lavados com 10 µl da mesma solução sem adição de tripsina e deixados a 37°C por 16h. Os peptídeos extraídos foram lavados duas vezes, por 15 minutos, com 30µl de 50% acetonitrila/5% ácido fórmico. Em seguida, o material digerido por tripsina foi concentrado em SpeedVac (Savant) a um volume de aproximadamente 10µl e dessalinizados pela Zip-Tip® (C18 resina; P10, Millipore Corporation, Bedford, MA). Os peptídeos foram eluídos da coluna em 50% acetonitrila/5% ácido fórmico e congelados a -20°C até o momento do uso.

Espectrometria de Massa - MALDI-TOF-TOF

As amostras foram misturadas à matriz [10mg/mL de R-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) em acetonitrila 50%/ácido trifluoracético 0,1%] e aplicadas na placa para análise no equipamento 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Ambos MS e MS/MS foram adquiridos com o laser neodymium-doped nyttrium aluminum garnet (Nd:YAG) com 200-Hz. Os espectros foram editados usando os programas FlexAnalysis e Biotools.

Os "spots" foram identificados através de busca em banco de dados, realizada através do programa MASCOT (http//:www.matrixscience.com) e usando o banco de dados do National Center for Biotechnology (NCBI). Os parâmetros de busca foram: tolerância de desvio de massa molecular entre 100-200ppm, carbamidometilação, máximo de um sítio tríptico de corte não clivado e oxidação da metionina. Esse ensaio permitiu identificar 45 proteínas candidatas para uso em diagnóstico da LVC, inclusive na fase aguda da infecção. A **Tabela 1** mostra apenas as 10, destas 45 proteínas, que foram utilizadas na composição imunogênica para vacina e kit para teste imunodiagnóstico de leishmaniose visceral.

Tabela 1 – Proteínas de formas promastigotas de *L. chagasi* reconhecidas por soros caninos em WB com gel 2-DE e identificadas por espectrometria de massa (MS/MS), selecionadas para vacina e kit para teste imunodiagnóstico de leishmaniose visceral.

NOME DA PROTEÍNA ***(L. infantum)***	***GI*** ***(Identificador)***	***SEQ ID No***
Proteína hipotética conservada	gi 68127339	**54**
Proteína hipotética conservada	gi 134072971	**55**
Proteína hipotética, conservada	gi 68124247	**56**
Proteína hipotética, conservada	gi 70799658	**57**
Proteína hipotética, conservada	gi 134060528	**58**
Proteína hipotética, conservada	gi 134070112	**59**
Proteína hipotética	gi 146094146	**60**
Elongase de ácido graxo, putative	gi 68125282	**61**
Peroxidoxina 1	gi 11761380	**62**
Proteína do choque térmico 83-1	gi 146097493	**63**

Predição de epitopos

As seqüências de aminoácidos das 45 proteínas identificadas foram processadas usando duas abordagens distintas:

1) A localização de epitopos lineares de células B através de predição pelos softwares "BCPreds" (disponível em http://*ailab.cs.iastate.edu/bcpreds/*) e "ABCPred" (disponível em *http://www.imtech.res.in/raghava/abcpred/*), a partir de similaridade e alinhamento de seqüências, utilizando modelos matemáticos

(Yang X, Yu X 2009. An introduction to epitope prediction methods and software. *Rev Med Virol* 19: 77-96). Após mapeamento *in silico*, os epitopos gerados por ambos os softwares foram comparados, para selecionar aqueles resultantes da sobreposição de predições dos dois softwares simultaneamente. Além disso, foi estabelecido que o tamanho desses peptídeos deveria estar entre 9 e 15 aminoácidos.

2) A localização de epitopos lineares de células B através de predição pelo Programa BEPIPRED, através de uma combinação de modelos matemáticos (Improved method for predicting linear B-cell epitopes. Jens Erik Pontoppidan Larsen, Ole Lund and Morten Nielsen. Immunome Research 2:2, 2006). Após o mapeamento *in silico*, os peptídeos com "score" maior que 2,0, foram selecionados para síntese.

Dessa forma, foram selecionados 180 peptídeos resultantes da sobreposição dos programas de predição "BCPreds" e "ABCPred" (dado não mostrado) e coincidentemente também 180 diferentes peptídeos resultantes da análise pelo programa BEPIPRED (dado não mostrado).

As sequências identificadas no mapeamento indicam quais os peptídeos de cada proteína têm maior probabilidade de interagir com anticorpos. Deve ser ressaltado que diferentes peptídeos foram selecionados de uma mesma proteína. Estes foram sintetizados em duplicata, pelo método de Spot síntese descrito a seguir, e testados com pool de soros de cães infectados e não infectados por *L. chagasi*.

Síntese de Peptídeos em membrana de celulose: método de Spot síntese

Os aminoácidos são depositados em volume mínimo (0,6 μl) com auxílio de um micropipetador automático, permitindo obter aproximadamente 50 nanomoles de peptídeo por ponto. A síntese múltipla é realizada em sintetizador (Abimed Spot Synthesis–ASP222) e o plano de distribuição dos aminoácidos bem como a determinação dos protocolos dos diversos peptídeos são definidos em programa de computação Multipeps (Molina F, Laune D, Gougat C, Pau B, Granier C 1996. Improved performances of Spot multiple peptide synthesis. *Pept Res* 9: 151-155).

Os grupamentos hidroxilas livres sobre a membrana de celulose são utilizados como pontos de ancoragem para a síntese do peptídeo. Esses grupamentos são conjugados através de ligação estável com 8 a 10 unidades de polietilenoglicol (PEG), objetivando afastar o peptídeo do suporte e conferir maior estabilidade na ligação do peptídeo à membrana.

A síntese do peptídeo inicia-se pelo C-terminal do último aminoácido da seqüência determinada. Com a desproteção do grupo ligado ao 9-fluorenilmetiloxicarbonila protetor (Fmoc), pela adição de piperidina (20% em Dimetilformamida - DMF), as funções aminas são recuperadas e podem ser visualizadas pela coloração azul com bromofenol.

Os aminoácidos são, em seguida, ativados por diisopropilcarbodiimida/1-hidroxibenzotriazol (DIC/HOBT) (150 μl para cada aminoácido) e depositados para reinício de outro ciclo, sendo que os ativadores propiciam um rendimento de ligação variando de 74 a 87% por ciclo. A reposição de aminoácidos sempre começa com a arginina, por ser o aminoácido mais lábil, e cada aminoácido é depositado duas vezes por ciclo. As reações de ligação são monitoradas por mudança de coloração dos "spots', passando da cor azul ao verde-amarelado.

As funções NH_2 livres, ou que não reagiram, são acetiladas (anidrido acético 10% em DMF) para evitar a formação de peptídeos irregulares ou outras ligações indesejáveis. O grupo protetor Fmoc do próximo aminoácido é novamente clivado, em meio básico pela piperidina, verificando a ligação pela coloração com bromofenol. Efetuam-se lavagens da membrana com metanol e secagem da mesma ao ar fresco. Em seguida, a membrana é realocada no sintetizador para outro ciclo.

Pelo método de Spot síntese, o tamanho do peptídeo construído é limitado a aproximadamente 15 aminoácidos (Laune L, Molina F, Ferrières G, Villard S, Bès C, Rieunier F, Chardes T, Granier C 2002. Application of the Spot method to the identification of peptides and amino acids from the antibodie paratope that contribute to antigen binding. *J Immunol Method* 267, 53-70), pois persistem dúvidas quanto à qualidade da ligação de peptídeos muito alongados ou grandes. Ao final da síntese, os grupos laterais inicialmente protegidos dos

aminoácidos são desprotegidos pela adição de ácido trifluoracético (TFA) associado a diclorometano e trietilsilano. Finalmente, os peptídeos encontram-se fixados de maneira covalente à membrana.

A membrana construída contendo os diversos peptídeos pode ser analisada por imunoensaios de colorimetria. A capacidade dos peptídeos sintéticos de se ligarem com anticorpos é avaliada por ensaios imunológicos e estes podem ser reproduzidos várias vezes utilizando-se a mesma membrana, após regeneração da mesma (Frank R 1992. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* 48: 9217-9232).

Imunoensaio

Para os ensaios sorológicos foram utilizados soros de cães com infecção por *L. chagasi* nas fases aguda e crônica, soros de animais livres dessa parasitose e ainda soros de cães portadores de *Trypanosoma cruzi*.

Os soros de cães em fase aguda da infecção por *L. chagasi* foram provenientes de animais com infecção experimental e foram coletados até 60 dias após a inoculação. Os soros de animais com LV, *T. cruzi* (fase crônica) e de cães saudáveis foram obtidos a partir de soroteca.

Imunoensaio com peptídeos ligados à membrana

As membranas contendo os peptídeos sintéticos foram incubadas por aproximadamente 16 a 18 horas com solução de bloqueio [PBS pH7,4, Tween-20 a 0,2%, sacarose a 4% e BSA a 5%] em temperatura ambiente. Após bloqueio, as membranas foram lavadas e incubadas com os soros testes por 120 min a temperatura ambiente. Após novas lavagens, o conjugado (anticorpos anti-IgG total, anti-IgG1, anti-IgG2 ou anti-IgM caninos conjugados com fosfatase alcalina) foi adicionado e mantido sob agitação por 120 minutos. Após três lavagens com PBS contendo Tween-20 a 0,2% e outras três subseqüentes com PBS sob agitação à temperatura ambiente, foi adicionado o substrato para fosfatase alcalina (5-bromo-4-cloro-3-indolilfosfato p-toluidina) e o cromógeno (cloreto de nitroblue tetrazólio) BCIP/ NBT, Promega.

A reação, interrompida após três lavagens da membrana com água destilada, pode ser visualizada pela presença de um precipitado azulado sobre os peptídeos (spots) mais reativos (Frank, 1992).

A intensidade da cor foi calculada utilizando o software Image Master Platinum® (GE), comparando o perfil de reconhecimento de uma membrana incubada com pool de soros de cães não infectados, perante outra membrana, contendo os mesmos peptídeos, incubada com pool de soros de cães infectados. Desse modo, foi possível comparar a intensidade da cor, obtendo assim a razão entre soros infectados e não infectados. Definiu-se que apenas os "spots" com razão igual ou superior a 2 são considerados válidos. Esses "spots" foram produzidos novamente em Spot síntese para avaliação com soros individuais, tanto positivos quanto negativos.

Ao final dos ensaios imunológicos, as membranas foram documentadas e, em seguida, submetidas a um tratamento de regeneração, para sua reutilização. As membranas foram tratadas com DMF, reagente A (uréia 8M, 1% de SDS) e reagente B (ácido acético/etanol/água na proporção 60:30:10) para remover os complexos moleculares precipitados sobre os peptídeos.

EXEMPLO 1: SELEÇÃO DOS PEPTÍDEOS RECONHECIDOS ESPECIFICAMENTE ATRAVÉS DA COMPARAÇÃO DE SOROS POSITIVOS COM OS NEGATIVOS PARA LV

Após imunoensaio das membranas contendo os peptídeos sintetizados, foi realizada uma análise da intensidade relativa dos peptídeos, calculada pela razão entre a intensidade da cor da reação com soros positivos e negativos utilizando o programa de computador (Platinum 7.0). Dessa maneira, foi possível selecionar os peptídeos reconhecidos especificamente através da comparação de soros positivos com os negativos para LV (**Tabelas 2 e 3**).

Os 24 peptídeos descritos na **Tabela 2** não apresentaram reação inespecífica (com soros não infectados) e nem reação cruzada com soro de cães infectados com *T.cruzi.* Esses peptídeos são correspondentes a 16 proteínas diferentes. A intensidade relativa dos peptídeos selecionados está demonstrada na **Figura 2**.

Desse mesmo modo, outros 29 peptídeos (que reconheceram soro de cães infectados com *L. chagasi,* mas não soros de animais controle e nem infectados com *T. cruzi*) foram selecionados e estão descritos na **Tabela 3**. Esses peptídeos correspondem a 9 proteínas diferentes. A intensidade relativa dos peptídeos selecionados está demonstrada na **Figura 3**.

Esses resultados foram resumidos na **Tabela 4**.

Tabela 2: Proteínas mapeadas para epitopos B e peptídeos sintetizados utilizando sobreposição dos programas de predição "BCPreds" e "ABCPred". São mostrados apenas os peptídeos selecionados após imunoensaio.

PROTEÍNAS	GI	PEPTÍDEOS SELECIONADOS	SEQ ID No
Beta-tubulina	13569565	***NNIKSSICDIPPKG***	5
Elongase de ácido graxo, putative	68125282	***WNNNIFYDGPVGAF***	6
		FTNRRCDSNATNAR	7
Fator 1-beta de elongação	146104117	***KAKDAEKKKAKTD***	8
Peroxidoxina 1	11761380	***TVNDMPVGRNV***	9
Polipeptídeo nascente associado a complexo homólogo, cadeia alfa	8671200	***SKRYAKAMAKMGLK***	10
Proteína do choque térmico 83- 1	146097493	***ASDACDKIRYQSLT***	11
		RKNIVKKCLEMFDE	12
Proteína do choque térmico 83	123669	***KDVTKEEYAAFYKA***	13
		NDWEDPMATKHFSV	14
Proteína hipotética	146094146	***NGDRYDGEWKDDKR***	16
		EGEWQDGKMHGKGT	17
		QGDRYTGEWYQGKK	18
Proteína hipotética	146088184	***TGNSWDKVSSVVGQ***	19
Proteina hipotética conservada e Proteína	134070112 e 146088184	***KLDITGKPMV***	20

hipotética			
Proteína hipotética, conservada	134060528	***QRELKMVVAQS***	23
Proteína hipotética, conservada	70799658	***GSAASPPPSGTRT*** ***SQQLLGQRLYGLWK***	35 36
Proteína hipotética, conservada	68124247	***TMESSKSHEK*** ***FRAISTPRTGTMP*** ***AAAPVVFAAKT***	43 44 45
Proteína hipotética e Proteína hipotética conservada	146099283 e 134072971	***NGEDGKTRNDT***	50
Subunidade do fator 3 de iniciação em eucariotos	146103712	***GQVEGRTYDAAMVA*** ***IQVYRSDVPVN***	52 53

Tabela 3: Proteínas mapeadas para epitopos B e peptídeos sintetizados por apresentarem "score" maior que 2,0 utilizando o programa de predição "BEPIPRED". São mostrados apenas os peptídeos selecionados após imunoensaio.

PROTEÍNAS	**GI**	**PEPTÍDEOS SELECIONADOS**	**SEQ ID No**
3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	146095259	**FQSQPPPGVPQG** **RHQDTNAAPAGS**	1 2
Aldose 1-epimerase	72547339	**GYPKNPEEAYAD** **LPASGGPGQRYA**	3 4

Heat shock protein 83-1	146097493	VTKEYEVQNK	15
Hypothetical protein, conserved	134060528	TRGVKSSSKLPA RDDPHKVTPSDM	21 22
Hypothetical protein, conserved	70799658	PHRAGETSAAGL SQQAPAVPPLPQ TEKSPSSPQPRV QGMMSPGRSEEK VPKGDKAVSSPP GERRRGDAEDGR SSAGAAGARCST PSAQQQQQPTNA QQQQQPTNAGCT SSRPSPPSKVSS AAAAASSPSIAP	24 25 26 27 28 29 30 31 32 33 34
Hypothetical protein, conserved	68124247	ANIKGVPTRAET DSDDTEEGEDEG EGTAGEPKPPAM MRTSTDMPSQHI TRQTSQEPTPVS PLATQSYGFGSD	37 38 39 40 41 42
Hypothetical protein, conserved	68124249	GVAPPGWYDPPVQ	46
Hypothetical protein, conserved	68127339	TERPEGANFATP LTMNTNQPRMPQ	47 48
Hypothetical protein, conserved	68127339	VEGERVETT	49
Ribonucleoprotein p18, mitochondrial precursor	146081834	EAPSKQDKPVEN	51

Tabela 4 – Levantamento numérico das proteínas e peptídeos investigados até a seleção

Moléculas estudadas	***Número***
Proteínas mapeadas	45
Peptídeos sintetizados	360
Peptídeos com alta especificidade	53

Em síntese, foram selecionados 53 peptídeos sintéticos com potencial para diagnóstico sensível e específico da leishmaniose Visceral. Dentre esses 53 peptídeos, 35 pertencem a 9 diferentes proteínas hipotéticas, o que significa que são bastante promissores para novos testes diagnósticos, uma vez que essas proteínas hipotéticas ainda não haviam sido exploradas quanto a esse potencial.

EXEMPLO 2: EFICIÊNCIA DO TESTE IMUNODIAGNÓSTICO

Com o intuito de testar a eficiência do teste imunodiagnóstico, foi realizada nova síntese dos 24 peptídeos selecionados (**Tabela 2**) em membranas de celulose. Cada um desses peptídeos foi testado com diferentes soros caninos individuais, que possuíam variados títulos de anticorpos anti-Leishmania. Com as novas membranas também foi realizado mais uma vez o teste com soros caninos infectados por *T. cruzi*, para investigação de reação cruzada (**Tabela 5**).

Tabela 5: Soros caninos individuais, com diferentes títulos de anticorpos, testados em imunoensaios envolvendo membranas de celulose contendo os 24 peptídeos selecionados.

	Peptídeos SEQ ID Nº																							
Soros/Título de Ac anti-Leishmania (IgG)	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	23	35	36	43	44	45	50	52	53
Soro 01→ 1/20480						x	x	x			x					x			x		x		x	
Soro 02 → 1/20480					x	x	x			x	x	x	x	x	x	x	x	x	x					
Soro 03 → 1/40960						x	x	x		x	x		x		x	x	x	x	x				x	x
Soro 04 → 1/40960						x	x	x		x				x		x		x	x		x			x
Soro 05 → 1/40960						x	x			x			x					x	x					x
Soro 06 → 1/320	x	x		x		x	x	x			x	x	x		x		x		x		x	x		x
Soro 07 → 1/ 320	x	x		x		x	x	x		x	x	x	x		x		x		x		x	x		x
Soro 08 → 1/ 640	x	x			x	x	x			x	x	x	x		x		x			x	x	x		x
Soro 09 → 1/ 640	x					x		x	x	x	x		x	x	x		x			x	x	x		x
Soro 10 → 1/640						x	x		x		x		x		x	x	x		x	x	x	x		x
Soro 11 → 1/640	x							x			x		x			x	x				x	x		x
Soro 12 → 1/640							x						x				x				x			x
Soro 13 → 1/2560																	x				x			
Soro 14 → 1/2560						x	x												x		x	x		x
Soro 15 → 1/2560							x										x			x				
Soro 16 → 1/2560						x	x		x		x								x		x	x		
Soro 17 → 1/5120																								
Soro 18 → 1/5120											x													
Soro 19 → 1/5120						x	x				x								x	x	x			
Soro 20 → 1/5120						x	x	x			x								x		x			x
Sem reação cruzada	x		x			x	x			x	x	x			x		x		x	x	x	x		x

Foi possível observar que o perfil de reconhecimento de peptídeos foi variável entre os diferentes soros. Entretanto, houve alguns peptídeos que foram reconhecidos pela maior parte dos soros testados. Todos os 24 peptídeos testados foram reconhecidos por pelo menos um soro individual (**Tabela 5**). Pode-se verificar, portanto, que estes peptídeos possuem um grande potencial imunogênico.

EXEMPLO 3: AVALIAÇÃO DAS PROTEÍNAS MAIS PROMISSORAS PARA USO EM VACINA

Para avaliar, dentre as proteínas identificadas, quais seriam as mais promissoras para uso em vacina, todas foram mapeadas utilizando o programa

NetCTL (www.cbs.dtu.dk/services/NetCTL). Esse programa é um algoritmo combinado para fazer predição de ligação a HLA classe I, clivagem pelo proteassoma e eficiência de transporte pelo TAP (transporter associated with antigen processing) simultaneamente nas seqüências de aminoácidos de uma dada proteína (Peters, B., Bulik S., Tampe, R., Endert, P.M.V. and Holzhutter, H.G. Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. J. Immunol. 2003, 171, 1741-1749.). Também foi avaliada a capacidade de cada proteína ligar a 10 diferentes supertipos de HLA (A1, A2, A3, A24, B7, B8, B27, B44, B58 e B62) por bioinformática. Dessa forma, foram selecionadas para serem utilizadas como antígenos em composições vacinais as proteínas que apresentam maior percentual de epitopos para HLA I e/ou maior capacidade de se ligarem a diferentes supertipos de HLA (**Tabela 6**).

Tabela 6 – Proteínas que, segundo mapeamento pelo programa NetCTL, apresentam maior percentual de epitopos para HLA I.

PROTEÍNAS	SEQ ID Nº	PERCENTUAL DE EPITOPOS PARA HLA I	NÚMERO DE SUPERTIPOS DE HLA
GI - **68124247**	56	24.9	5
GI - **70799658**	57	17.2	4
GI - **134060528**	58	<15.0	5
GI - **134070112**	59	23.7	2
GI - **146094146**	60	24.3	4
GI - **68125282**	61	33.0	4
GI - **11761380**	62	29.1	4
GI - **146097493**	63	26.3	2

REIVINDICAÇÕES

1- **COMPOSTOS IMUNOGÊNICOS PARA LEISHMANIOSE VISCERAL,** caracterizados por serem selecionados de um grupo que compreende ao menos uma das proteínas ou peptídeos representados pelas Seq ID Nº 1 a 63.

2- **COMPOSTOS IMUNOGÊNICOS PARA LEISHMANIOSE VISCERAL,** de acordo com a reivindicação 1, caracterizados por gerarem resposta imune celular e/ou humoral.

3- **KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL**, caracterizado por compreender pelo menos uma das proteínas ou peptídeos representados pelas Seq ID Nº 1 a 63, ligados a um suporte.

4- **KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL**, de acordo com a reivindicação 2, caracterizado pelo suporte ser preferencialmente uma placa de microtitulação para ELISA ou uma membrana.

5- **KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL**, de acordo com as reivindicações 2 e 3, caracterizado por utilizar técnicas de imunodiagnóstico selecionadas do grupo compreendendo ELISA, Western blot, Dot blot e Imunocromatografia.

6- **VACINA CONTRA LEISHMANIOSE,** caracterizada por compreender pelo menos uma das proteínas representadas pelas Seq ID Nº 56 a 63, ou parte destas, em combinação com pelo menos um adjuvante.

7- **VACINA CONTRA LEISHMANIOSE**, de acordo com a reivindicação 6, caracterizada pelo adjuvante ser selecionado de um grupo que compreende oligonucleotídeos cotendo CpG, agonistas de TLR, saponinas, AGPs ou uma combinação destes.

8- **VACINA CONTRA LEISHMANIOSE**, de acordo com as reivindicações 6 e 7, caracterizada por ser administrada em quantidade suficiente para induzir resposta imune protetora em mamífero.

9- VACINA CONTRA LEISHMANIOSE, de acordo com as reivindicações 6 a 8, caracterizada por ser administrada pelas vias intramuscular, subcutânea, oral ou parenteral.

10- VACINA CONTRA LEISHMANIOSE, de acordo com as reivindicações 6 a 9, caracterizada por ser para imunização, profilaxia ou tratamento de mamíferos com risco de contrair ou que sofrem de Leishmaniose.

FIGURAS

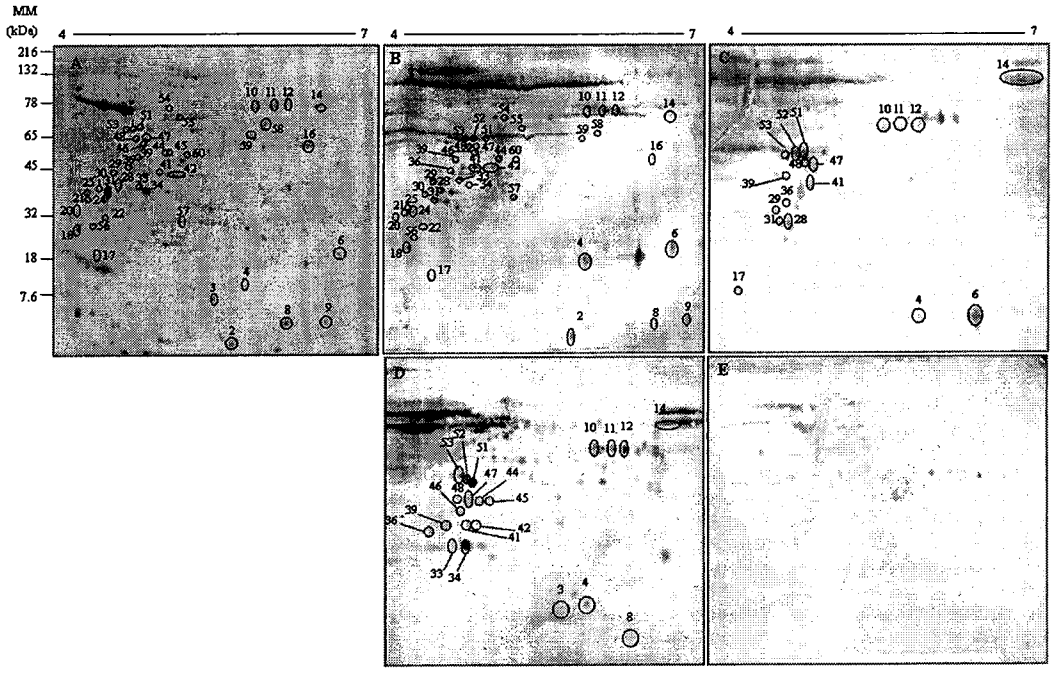


Figura 1

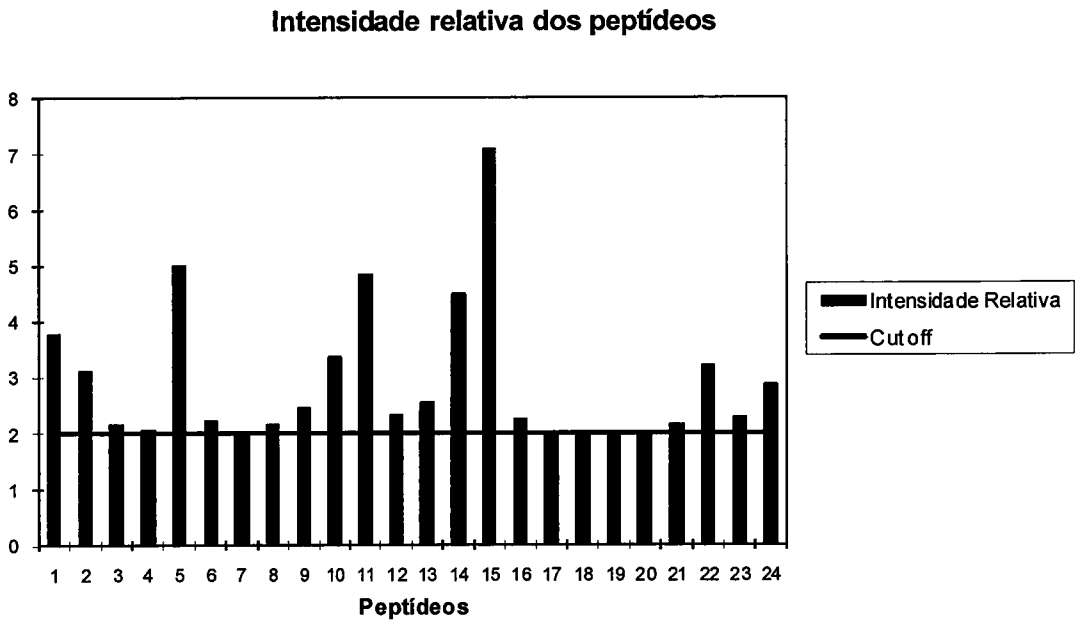


Figura 2

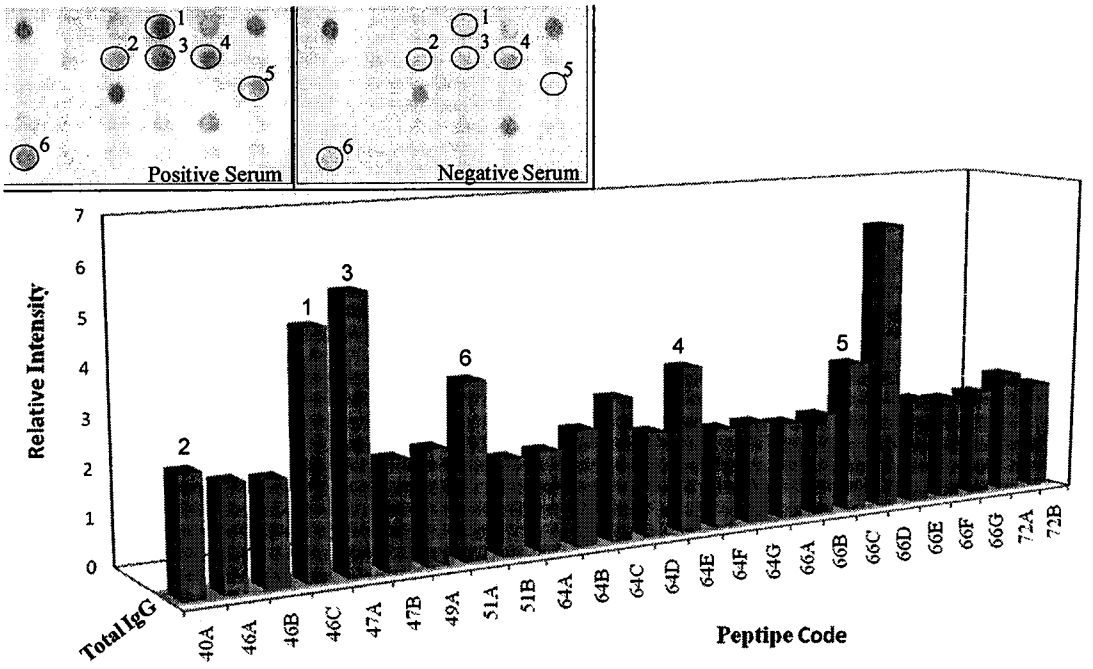

Positive Serum
Negative Serum
Relative Intensity
Total IgG
Peptipe Code
40A
46A
46B
46C
47A
47B
49A
51A
51B
64A
64B
64C
64D
64E
64F
64G
66A
66B
66C
66D
66E
66F
66G
72A
72B

Figura 3

RESUMO

COMPOSIÇÃO IMUNOGÊNICA PARA VACINA E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL

A presente invenção descreve uma composição imunogênica para vacina e teste imunodiagnóstico de Leishmaniose Visceral. Mais especificamente, a invenção compreende uma vacina e um kit para teste imunodiagnóstico de Leishmaniose, desenvolvidos através da identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática, síntese de peptídeos e imunoensaio. A alta especificidade desses antígenos possibilita a realização de uma vacina eficaz contra Leishmaniose e de um teste imunodiagnóstico mais eficiente para a Leishmaniose Visceral canina ou humana.

Journal of proteome research

ARTICLE

pubs.acs.org/jpr

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

Míriam M. Costa,[†,#] Hélida M. Andrade,[‡,#] Daniella C. Bartholomeu,[‡] Leandro M. Freitas,[‡] Simone F. Pires,[‡] Alexander D. Chapeaurouge,[§] Jonas Perales,[§] André T. Ferreira,[§] Mário S. Giusta,[†] Maria N. Melo,[‡] and Ricardo T. Gazzinelli*[,†,⊥,¶]

[†]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Bioquímica e Imunologia, 31270-910 Belo Horizonte, Minas Gerais, Brasil

[‡]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Parasitologia, 31279-910 Belo Horizonte, Minas Gerais, Brasil

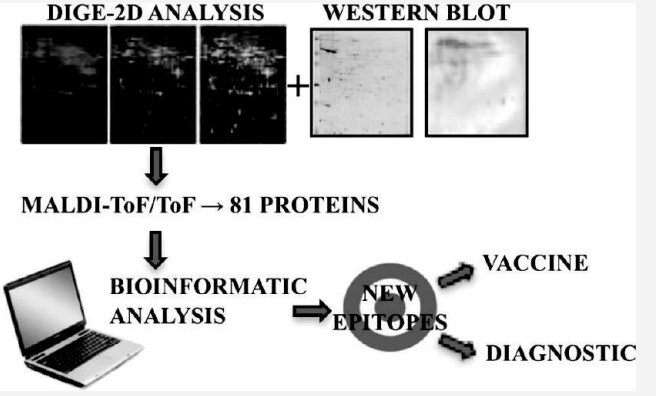
[§]Fundação Oswaldo Cruz, Instituto Oswaldo cruz, Laboratório de Toxinologia, 21040360 Rio de Janeiro, Rio de Janeiro, Brasil

[⊥]Centro de Pesquisas René Rachou—Fundação Oswaldo Cruz, 30190-002 Belo Horizonte, Minas Gerais, Brasil

[¶]University of Massachusetts Medical School, Division of Infectious Diseases and Immunology, Worcester 01605-2324, Massachusetts, United States

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.

Received: August 17, 2010
Published: February 28, 2011

Downloaded via INST NATL DE PROPRIEDADE INDUSTRIAL on August 21, 2023 at 17:21:11 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

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.

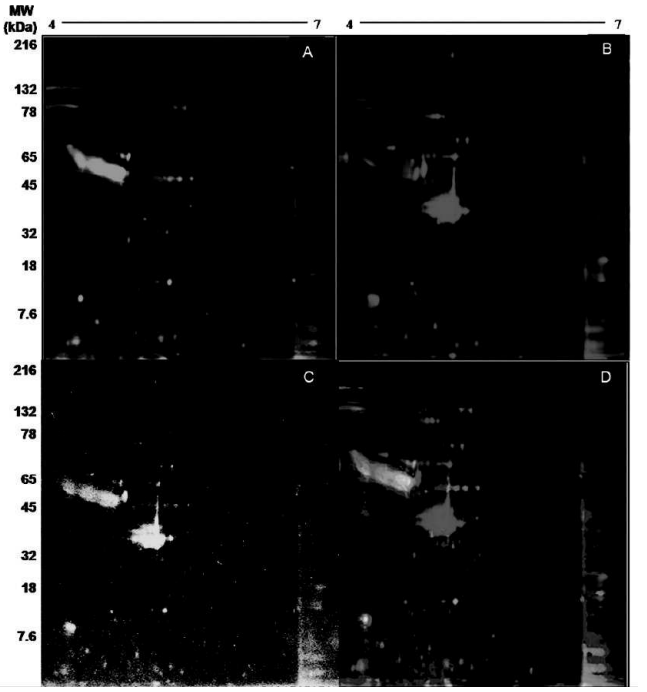


Figure 1. 2D-DIGE analysis of promastigote and amastigotes extracts from *L. chagasi*. Proteins of promastigotes and amastigotes forms, and a pooled internal standard were labeled with CyDyes Cy3, Cy5 and Cy2, respectively, mixed and separated on a 2D gel using 18 cm pH 4−7 (left to right) strips in 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)

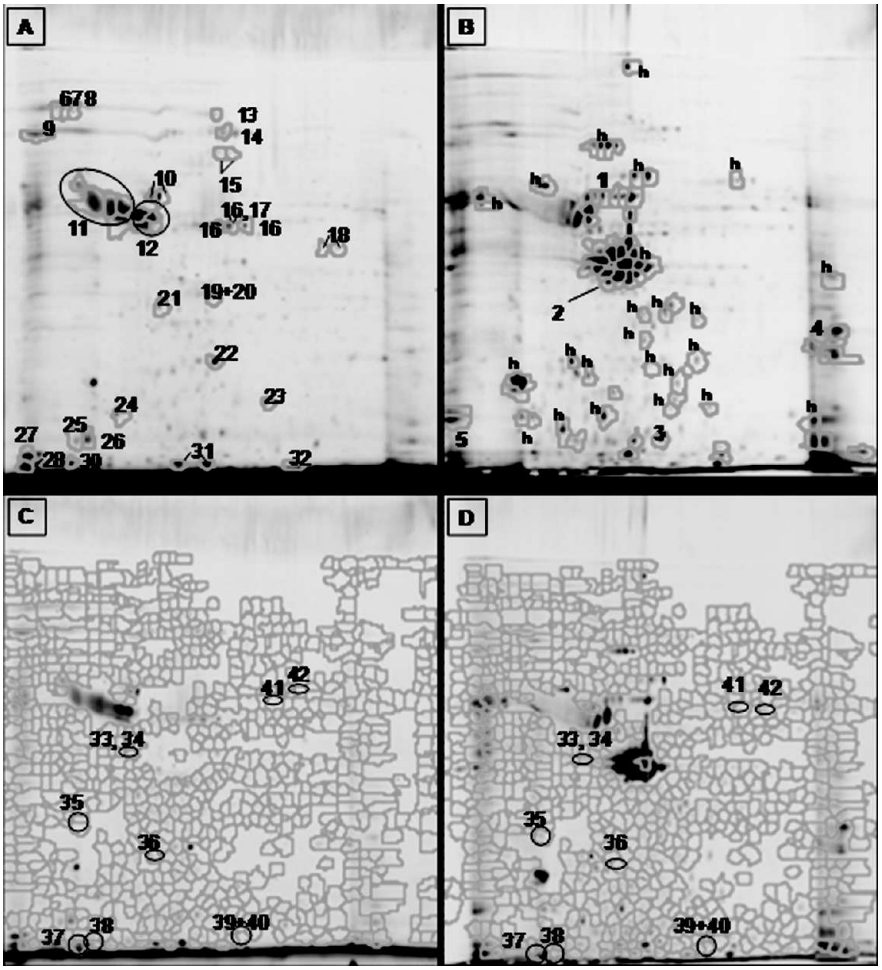


Figure 2. Proteins highly expressed by promastigotes (A) and amastigotes (B) forms derived from *L. chagasi*. The level of protein expression was determined using ImageMaster 2D Platinum 6.0 software, please see details in the Materials and Methods section. The "h" letter (in panel B) represents hamster-derived 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.

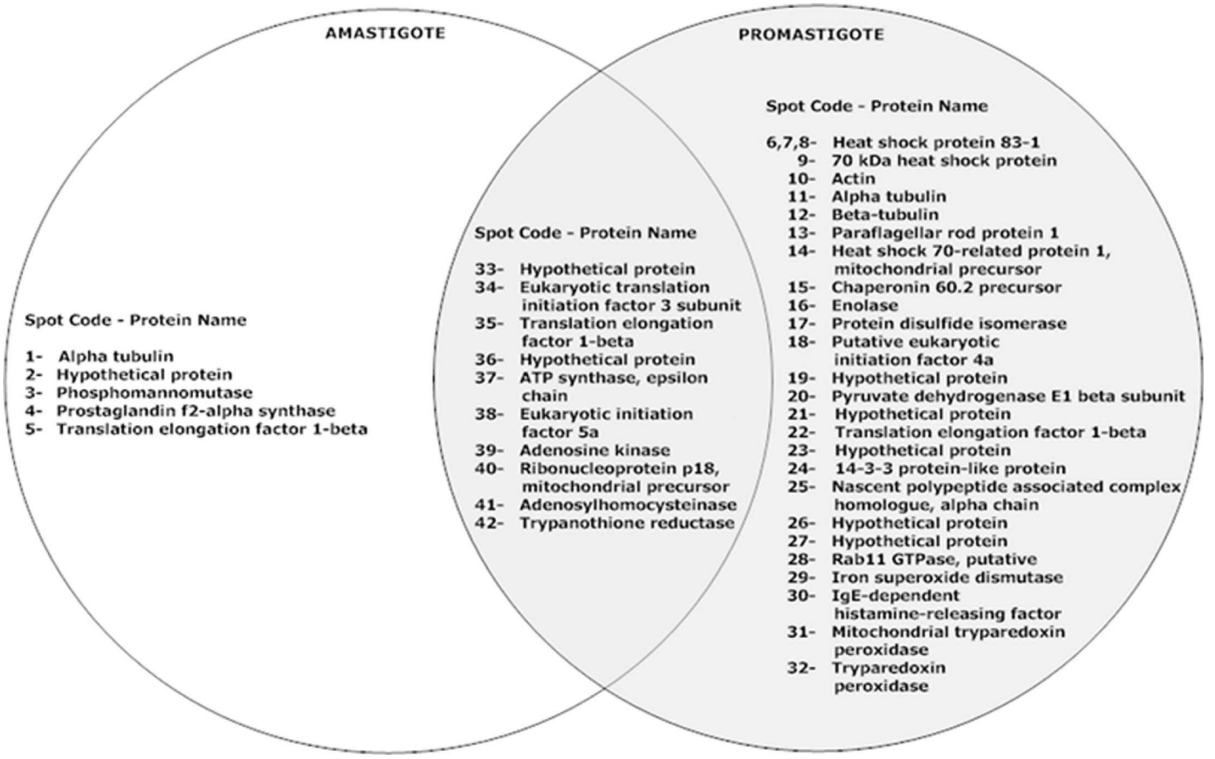


Figure 3. Venn diagram showing proteins expressed in promastigote and amastigote forms from *L. chagasi*.

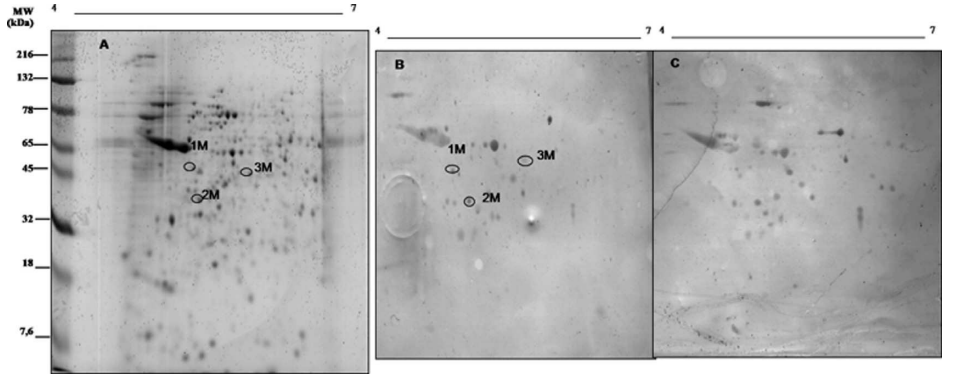


Figure 4. Proteins from promastigotes forms of *L. chagasi* recognized by IgM from sera of acutely infected dogs. Promastigote extract were fractionated using 18 cm pH 4−7 (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second dimension. (A) Gel stained with Coomassie Blue; (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

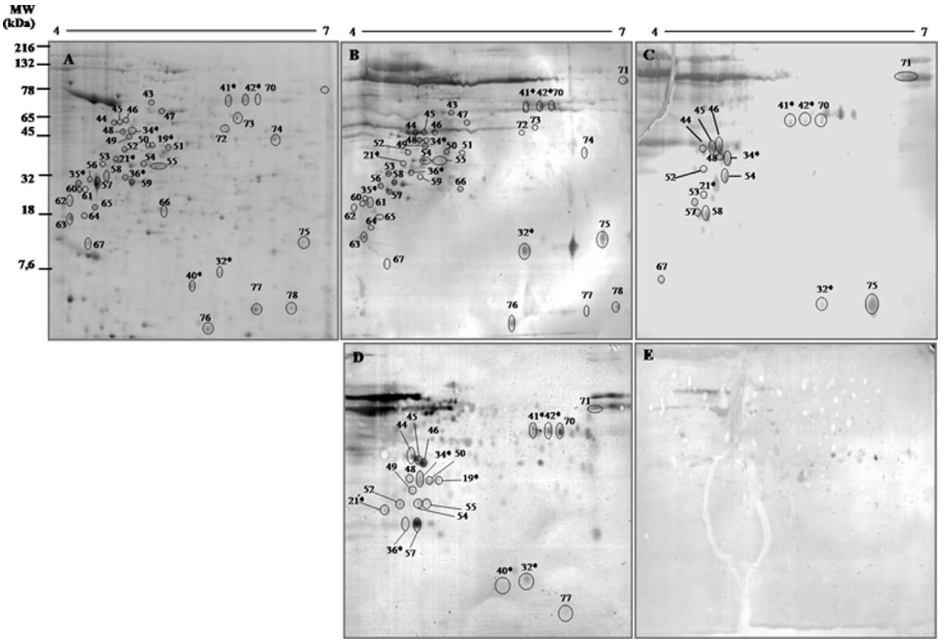


Figure 5. Proteins from promastigotes forms of *L. chagasi* recognized by IgG, IgG1 and IgG2 from sera of chronically infected dogs. Extracts from *L. chagasi* promastigotes were fractionated using 18 cm pH 4−7 (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second dimension. (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.

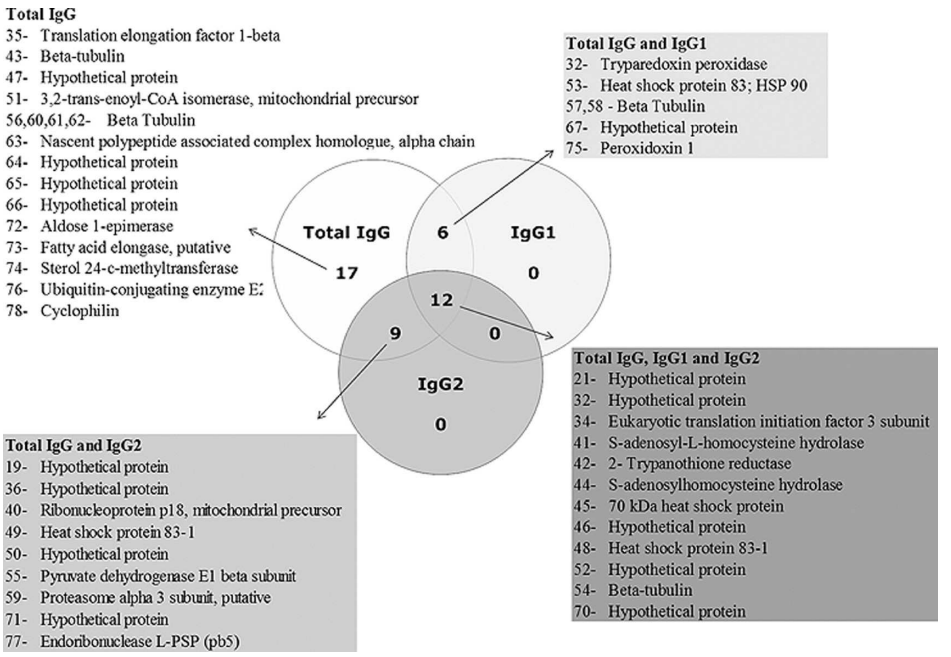


Figure 6. Venn diagram showing intersections of subclasses of immunoglobulins that recognize spots identified by MS after Western blot analysis with sera from infected dogs.

positive and negative sera, as indicated by analysis employing ImageMaster 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

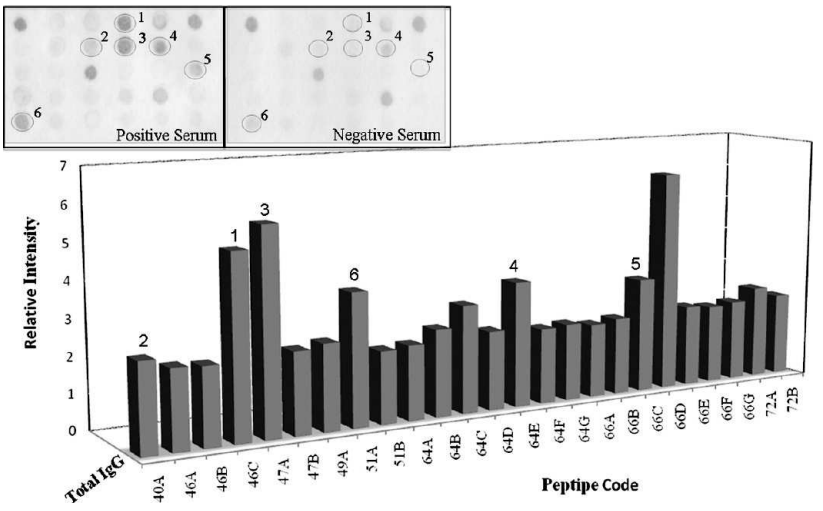


Figure 7. Peptides recognized by sera from chronically infected dogs. To evaluate the antibody reactivity in SPOT synthesis membranes, the relative intensity of the signal was estimated based on comparison of reactivity in immunoblots with sera from chronically infected dogs to the background levels, 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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ritoga@cpqrr.fiocruz.br. Phone: 55 (31) 3349-7774.

Author Contributions

#These authors have equally contributed to the work.

■ ACKNOWLEDGMENT

This study was funded by Rede Mineira de Biomoléculas—Fundação de Amparo as Pesquisa do Estado de Minas Gerais (FAPEMIG), and Instituto Nacional de Ciência e Tecnologia de Vacinas (INCTV) - Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

■ REFERENCES

(1) Dye, C. The logic of visceral leishmaniasis control. *Am. J. Trop. Med. and Hyg.* **1996**, *55*, 125–130.

(2) Chappuis, F.; Sundar, S.; Hailu, A.; Ghalib, H.; Rijal, S.; Peeling, R. W.; Alvar, J.; Boelaert, M. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?. *Nat. Rev. Microbiol.* **2007**, *5*, 873–882.

(3) Desjeux, P. Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* **2004**, *27*, 305–318.

(4) Ashford, R. W. The leishmaniases as emerging and reemerging zoonoses. *Int. J. Parasitol.* **2000**, *30*, 1269–1281.

(5) de Oliveira, C. I.; Nascimento, I. P.; Barral, A.; Soto, M.; Barral-Netto, M. Challenges and perspectives in vaccination against leishmaniasis. *Parasitol. Int.* **2009**, *58*, 319–324.

(6) Matlashewski, G. Leishmania infection and virulence. *Microbiol. Immunol.* **2001**, *190*, 37–42.

(7) Dea-Ayuela, M. A.; Rama-Iñiguez, S.; Bolás-Fernández, F. Proteomic analysis of antigens from Leishmania infantum promastigotes. *Proteomics* **2006**, *6*, 4187–4194.

(8) Herrera-Najera, C.; Piña-Aguilar, R.; Xacur-Garcia, F.; Ramirez-Sierra, M. J.; Dumonteil, E. Mining the Leishmania genome for novel antigens and vaccine candidates. *Proteomics* **2009**, *9*, 1293–1301.

(9) El Fakhry, Y.; Ouellette, M.; Papadopoulou, B. A proteomic approach to identify developmentally regulated proteins in Leishmania infantum. *Proteomics* **2002**, *2*, 1007–1117.

(10) Nugent, P. G.; Karsani, S. A.; Wait, R.; Tempero, J.; Smith, D. F. Proteomic analysis of Leishmania mexicana differentiation. *Mol. Biochem. Parasitol.* **2004**, *136*, 51–62.

(11) McNicoll, F.; Drummelsmith, J.; Müller, M.; Madore, E.; Boilard, N.; Ouellette, M.; Papadopoulou, B. A combined proteomic and transcriptomic approach to the study of stage differentiation in Leishmania infantum. *Proteomics* **2006**, *6*, 3567–3581.

(12) Walker, J.; Vasquez, J. J.; Gomez, M. A.; Drummelsmith, J.; Burchmore, R.; Girard, I.; Ouellette, M. Identification of developmentally-regulated proteins in Leishmania panamensis by proteome profiling of promastigotes and axenic amastigotes. *Mol. Biochem. Parasitol.* **2006**, *147*, 64–73.

(13) Leifso, K.; Cohen-Freue, G.; Dogra, N.; Murray, A.; McMaster, W. R. Genomic and proteomic expression analysis of Leishmania promastigote and amastigote life stages: the Leishmania genome is constitutively expressed. *Mol. Biochem. Parasitol.* **2007**, *152*, 35–46.

(14) Morales, M. A.; Watanabe, R.; Laurent, C.; Lenormand, P.; Rousselle, J. C.; Namane, A.; Späth, G. F. Phosphoproteomic analysis of Leishmania donovani pro- and amastigote stages. *Proteomics* **2008**, *8*, 350–363.

(15) Fernandes, A. P.; Costa, M. M. S.; Coelho, E. A.; Michalick, M. S.; de Freitas, E.; Melo, M. N.; Tafuri, W. L.; Resende, D.; Hermont, M. V.; Abrantes, C. F.; Gazzinelli, R. T. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. *Vaccine* **2008**, *26*, 5888–5895.

(16) Mauricio, I. L.; Gaunt, M. W.; Stothard, J. R.; Miles, M. A. Genetic typing and phylogeny of the Leishmania donovani complex by restriction analysis of PCR amplified gp63 intergenic regions. *Parasitology* **2001**, *122*, 393–403.

(17) Lukes, J.; Mauricio, I. L.; Schönian, G.; Dujardin, J. C.; Soteriadou, K.; Dedet, J. P.; Kuhls, K.; Tintaya, K. W.; Jirků, M.; Chocholová, E.; Haralambous, C.; Pratlong, F.; Oborník, M.; Horák, A.; Ayala, F. J.; Miles, M. A. Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9375–9380.

(18) Romero, G. A.; Boelaert, M. Control of visceral leishmaniasis in latin america-a systematic review. *PLoS Neglected Trop. Dis.* **2010**, *4* (1), No. e584.

(19) Schönian, G.; Mauricio, I.; Cupolillo, E. Is it time to revise the nomenclature of Leishmania?. *Trends Parasitol.* **2010**, *26*, 466–469.

(20) Dujardin, J. C.; Campino, L.; Cañavate, C.; Dedet, J. P.; Gradoni, L.; Soteriadou, K.; Mazeris, A.; Ozbel, Y.; Boelaert, M. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. *Emerging Infect. Dis.* **2008**, *14*, 1013–1018.

(21) Gontijo, C. M. F.; Melo, M. N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. *Rev. Bras. Epidemiol.* **2004**, *7*, 338–349.
(22) Chang, K. P. Human cutaneous Leishmania in a mouse macrophage line: propagation and isolation of intracellular parasites. *Science* **1980**, *209*, 1240–1242.
(23) Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255–262.
(24) Towbin, H.; Gordon, J. Immunoblotting and dot immunobinding-current status and outlook. *J. Immunol. Methods* **1984**, *72*, 313–340.
(25) Medzihradszky, K. F. Peptide sequence analysis. *Methods Enzymol.* **2005**, *402*, 209–244.
(26) Larsen, M. V.; Lundegaard, C.; Lamberth, K.; Buus, S.; Brunak, S.; Lund, O.; Nielsen, M. An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur. J. Immunol.* **2005**, *35*, 2295–2303.
(27) Larsen, M. V.; Lundegaard, C.; Lamberth, K.; Buus, S.; Lund, O.; Nielsen, M. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. *BMC Bioinf.* **2007**, *8*, 424.
(28) Lv, H.; Gao, Y.; Wu, Y.; Zhai, M.; Li, L.; Zhu, Y.; Liu, W.; Wu, Z.; Chen, F.; Qi, Y. Identification of a novel cytotoxic T lymphocyte epitope from CFP21, a secreted protein of Mycobacterium tuberculosis. *Immunol. Lett.* **2010**, *30* (133), 94–98.
(29) Singh, S. P.; Mishra, B. N. Identification and characterization of merozoite surface protein 1 epitope. *Bioinformation* **2009**, *17*, 1–5.
(30) Larsen, M. V.; Lelic, A.; Parsons, R.; Nielsen, M.; Hoof, I.; Lamberth, K.; Loeb, M. B.; Buus, S.; Bramson, J.; Lund, O. Identification of CD8+ T cell epitopes in the West Nile virus polyprotein by reverse-immunology using NetCTL. *PLoS One* **2010**, *5*, e12697.
(31) Wang, M.; Larsen, M. V.; Nielsen, M.; Harndahl, M.; Justesen, S.; Dziegiel, M. H.; Buus, S.; Tang, S. T.; Lund, O.; Claesson, M. H.; HLA Class, I Binding 9mer Peptides from Influenza A Virus Induce CD4+ T Cell responses. *PLoS One* **2010**, *5*, e10533.
(32) Pérez, C. L.; Larsen, M. V.; Gustafsson, R.; Norström, M. M.; Atlas, A.; Nixon, D. F.; Nielsen, M.; Lund, O.; Karlsson, A. C. Broadly immunogenic HLA class I supertype-restricted elite CTL epitopes recognized in a diverse population infected with different HIV-1 subtypes. *Immunol.* **2008**, *180* (7), 5092–100.
(33) Tang, S. T.; Wang, M.; Lamberth, K.; Harndahl, M.; Dziegiel, M. H.; Claesson, M. H.; Buus, S.; Lund, O. MHC-I-restricted epitopes conserved among variola and other related orthopoxviruses are recognized by T cells 30 years after vaccination. *Arch. Virol.* **2008**, *153*, 1833–1844.
(34) Larsen, J. E.; Lund, O.; Nielsen, M. Improved method for predicting linear B-cell epitopes. *Immunome Res.* **2006**, *2*, 2–5.
(35) Frank, R.; Overwin, H. Spot-synthesis: epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. In *Methods in Molecular Biology. Epitope Mapping Protocols*; Morris, G. E., Ed.; Humana Press: Totowa, 1996; Vol. 66, pp 149−169.
(36) Frank, R. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **1992**, *48*, 9217–9232.
(37) Soutullo, A.; Santi, M. N.; Perin, J. C.; Beltramini, L. M.; Borel, I. M.; Frank, R.; Tonarelli, G. G. Systematic epitope analysis of the p26 EIAV core protein. *J. Mol. Recognit.* **2007**, *20*, 227–237.
(38) Sundar, S.; Rai, M. Laboratory diagnosis of visceral leishmaniasis. *Clin. Diagn. Lab. Immunol.* **2002**, *9*, 951–958.
(39) Rouf, M. A.; Rahman, M. E.; Islam, M. N.; Ferdous, N. N.; Hossain, M. A. Sensitivity, specificity and predictive values of immunochromatographic strip test in diagnosis of childhood kala-azar. *Mymensingh Med. J.* **2009**, *18* (1 Suppl.), S1–S5.
(40) Mojtahedi, Z.; Clos, J.; Kamali-Sarvestani, E. Leishmania major: identification of developmentally regulated proteins in procyclic and metacyclic promastigotes. *Exp. Parasitol.* **2008**, *119*, 422–429.
(41) Cuervo, P.; de Jesus, J. B.; Junqueira, M.; Mendonça-Lima, L.; González, L. J.; Betancourt, L.; Grimaldi, G., Jr; Domont, G. B.; Fernandes, O.; Cupolillo, E. Proteome analysis of Leishmania (Viannia) braziliensis by two-dimensional gel electrophoresis and mass spectrometry. *Mol. Biochem. Parasitol.* **2007**, *154*, 6–21.
(42) Paape, D.; Lippuner, C.; Schmid, M.; Ackermann, R.; Barrios-Llerena, M. E.; Zimny-Arndt, U.; Brinkmann, V.; Arndt, B.; Pleissner, K. P.; Jungblut, P. R.; Aebischer, T. Transgenic, fluorescent Leishmania mexicana allow direct analysis of the proteome of intracellular amastigotes. *Mol. Cell. Proteomics* **2008**, *7*, 1688–1701.
(43) Paape, D.; Barrios-Llerena, M.; Le Bihan, T.; Mackay, L.; Aebischer, T. Gel free analysis of the proteome of intracellular Leishmania mexicana. *Mol. Biochem. Parasitol.* **2010**, *169*, 108–114.
(44) Forgber, M.; Basu, R.; Roychoudhury, K.; Theinert, S.; Roy, S.; Sundar, S.; Walden, P. Mapping the antigenicity of the parasites in Leishmania donovani infection by proteome serology. *PLoS One* **2006**, *1*, e40.
(45) Gupta, S. K.; Sisodia, B. S.; Sinha, S.; Hajela, K.; Naik, S.; Shasany, A. K.; Dube, A. Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of Leishmania donovani promastigotes. *Proteomics* **2007**, *7*, 816–823.
(46) Winkler, D. F.; Campbell, W. D. The spot technique: synthesis and screening of peptide macroarrays on cellulose membranes. *Methods Mol. Biol.* **2008**, *494*, 47–70.
(47) Volkmer, R. Synthesis and application of peptide arrays: quo vadis SPOT technology. *ChemBioChem* **2009**, *10*, 1431–1442.
(48) Reineke, U.; Sabat, R. Antibody epitope mapping using SPOT peptide arrays. *Methods Mol. Biol.* **2009**, *524*, 145–167.
(49) Drummelsmith, J.; Brochu, V.; Girard, I.; Messier, N.; Ouellette, M. Proteome mapping of the protozoan parasite Leishmania and application to the study of drug targets and resistance mechanisms. *Mol. Cell, Proteomics* **2003**, *2*, 146–155.
(50) Kedzierski, L.; Zhu, Y.; Handman, E. Leishmania vaccines: progress and problems. *Parasitology* **2006**, *133*, S87–112.
(51) Palatnik-de-Sousa, C. B.; Silva-Antunes, I.; Morgado, A.; de, A.; Menz, I.; Palatnik, M.; Lavor, C. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas. *Vaccine* **2009**, *27*, 3505–3512.
(52) Collins, F. M. Vaccines and cell-mediated immunity. *Bacteriol. Rev.* **1974**, *38*, 371–402.
(53) Mohabatkar, H. Prediction of epitopes and structural properties of Iranian HPV-16 E6 by bioinformatics methods. *Asian Pac. J. Cancer Prev.* **2007**, *8*, 602–606.
(54) Belkaid, Y.; Von Stebut, E.; Mendez, S.; Lira, R.; Caler, E.; Bertholet, S.; Udey, M. C.; Sacks, D. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. *J. Immunol.* **2002**, *168*, 3992–4000.
(55) Peters, B.; Bulik, S.; Tampe, R.; Endert, P. M. V.; Holzhutter, H. G. Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. *J. Immunol.* **2003**, *171*, 1741–1749.
(56) Wagner, J. L. Molecular Organization of the Canine Major Histocompatibility Complex. *J. Hered.* **2003**, *94*, 23–26.
(57) McMahon-Pratt, D.; Kima, P. E.; Soong, L. Leishmania amastigote target antigens: the challenge of a stealthy intracellular parasite. *Parasitol. Today* **1998**, *14*, 31–34.
(58) Mary, C.; Lamouroux, D.; Dunan, S.; Quilici, M. Western blot analysis of antibodies to Leishmania infantum antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. *Am. J. Trop. Med. Hyg.* **1992**, *47*, 764–771.
(59) Mancianti, F.; Falcone, M. L.; Giannelli, C.; Poli, A. Comparison between an enzyme-linked immunosorbent assay using a detergent-soluble Leishmania infantum antigen and indirect immunofluorescence for the diagnosis of canine leishmaniosis. *Vet. Parasitol.* **1995**, *59*, 13–21.
(60) Carrera, L.; Fermin, M. L.; Tesouro, M.; Garcia, P.; Rollán, E.; González, J. L.; Méndez, S.; Cuquerella, M.; Alunda, J. M. Antibody response in dogs experimentally infected with Leishmania infantum: infection course antigen markers. *Exp. Parasitol.* **1996**, *82*, 139–146.
(61) Aisa, M. J.; Castillejo, S.; Gallego, M.; Fisa, R.; Riera, M. C.; de Colmenares, M.; Torras, S.; Roura, X.; Sentis, J.; Portus, M. Diagnostic

potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. *Am. J. Trop. Med. Hyg.* **1998**, *58*, 154–159.

(62) Basu, R.; Roy, S.; Walden, P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by Leishmania donovani-infected human macrophages. *J. Infect. Dis.* **2007**, *195*, 1373–1380.

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

Identification of Proteins in Promastigote and Amastigote-like *Leishmania* Using an Immunoproteomic Approach

Vinicio T. S. Coelho[1], Jamil S. Oliveira[1], Diogo G. Valadares[1], Miguel A. Chávez-Fumagalli[2], Mariana C. Duarte[3], Paula S. Lage[4], Manuel Soto[5], Marcelo M. Santoro[1], Carlos A. P. Tavares[1], Ana Paula Fernandes[6], Eduardo A. F. Coelho[3,4]*

1 Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **2** Programa de Pós-Graduação em Medicina Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **3** Departamento de Patologia Clínica, Coltec, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **4** Programa de Pós-Graduação em Ciências Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, Belo Horizonte, Minas Gerais, Brazil, **5** Centro de Biología Molecular Severo Ochoa, CSIC, UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain, **6** Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Abstract

Background: The present study aims to identify antigens in protein extracts of promastigote and amastigote-like *Leishmania (Leishmania) chagasi* syn. *L. (L.) infantum* recognized by antibodies present in the sera of dogs with asymptomatic and symptomatic visceral leishmaniasis (VL).

Methodology/Principal Findings: Proteins recognized by sera samples were separated by two-dimensional electrophoresis (2DE) and identified by mass spectrometry. A total of 550 spots were observed in the 2DE gels, and approximately 104 proteins were identified. Several stage-specific proteins could be identified by either or both classes of sera, including, as expected, previously known proteins identified as diagnosis, virulence factors, drug targets, or vaccine candidates. Three, seven, and five hypothetical proteins could be identified in promastigote antigenic extracts; while two, eleven, and three hypothetical proteins could be identified in amastigote-like antigenic extracts by asymptomatic and symptomatic sera, as well as a combination of both, respectively.

Conclusions/Significance: The present study represents a significant contribution not only in identifying stage-specific *L. infantum* molecules, but also in revealing the expression of a large number of hypothetical proteins. Moreover, when combined, the identified proteins constitute a significant source of information for the improvement of diagnostic tools and/or vaccine development to VL.

Citation: Coelho VTS, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, et al. (2012) Identification of Proteins in Promastigote and Amastigote-like *Leishmania* Using an Immunoproteomic Approach. PLoS Negl Trop Dis 6(1): e1430. doi:10.1371/journal.pntd.0001430

Editor: Rodrigo Correa-Oliveira, René Rachou Research Center, Brazil

Received February 23, 2011; **Accepted** October 27, 2011; **Published** January 17, 2012

Copyright: © 2012 Coelho et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by grants from Pró-Reitoria de Pesquisa (PRPq) from UFMG (Edital 07/2010), FAPEMIG (CBB-APQ-01322-08), CNPq (APQ-577483/2008-0), the National Institute of Science and Technology for Vaccines (INCTV), and the National Institute of Science and Technology in Nanobiofarmacêutica (INCT-nanoBIOFAR). V.T.S.C, A.P.F., E.A.F.C. and D.G.V. received fellowships from CNPq, and M.A.C.F. received from CAPES. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

* E-mail: eduardoferrazcoelho@yahoo.com.br

Introduction

Visceral leishmaniasis (VL) is an important parasitic disease, with a worldwide distribution in 88 countries, where a total of 350 million people may be at risk. In Brazil, the disease is an endemic zoonosis caused by the parasitic protozoa *Leishmania (Leishmania) chagasi* syn. *L. (L.) infantum* [1]. Dogs are the main parasite domestic reservoirs, and culling of seropositive dogs, as detected by means of serological tests using promastigote antigens, *i.e.* RIFI or ELISA, is a major VL control measure adopted in Brazil. Therefore, to reduce the transmission of parasites between dogs and humans, it is necessary, among other aspects, to diagnose canine visceral leishmaniasis (CVL) as early as possible, by means of sensitive and specific diagnostic tools [2,3].

Upon infection, dogs develop three different stages of the disease: symptomatic, oligosymptomatic, and asymptomatic [4]. Symptomatic infections tend to evolve into animal deaths, and their clinical manifestations include cutaneous alterations, such as alopecia, dermatitis, and onychogryphosis [5,6], as well as visceral dysfunctions in the kidneys, liver, and heart [7,8]. A high number of infected dogs remain asymptomatic and present low levels of specific antibodies; however, some dogs do in fact develop a few mild symptoms, which are classified as oligosymptomatic [4].

Routine diagnosis of leishmaniasis has been based on classic parasitological methods, where infected skin tissue and aspirates, or biopsy specimens of visceral tissues (*i.e.*, spleen, liver, and bone marrow), undergo microscopic examinations and cultures [9]. Classic serological methods are limited by low sensitivity and/or

Author Summary

Canine visceral leishmaniasis (CVL) is an important emerging zoonosis caused by *Leishmania (Leishmania) infantum* in the Mediterranean and Middle East and *L. (L.) chagasi* (syn. *L. (L.) infantum*) in Latin America. Due to their genotypic relationships, these species are considered identical. The present study focused on comparing the protein expression profiles of the promastigote and amastigote-like stages of *L. infantum*, by means of a protein separation by two-dimensional electrophoresis and identification by mass spectrometry. The present study attempted to identify proteins recognized by antibodies present in the sera of dogs with asymptomatic and symptomatic visceral leishmaniasis. A total of one hundred and four proteins were identified. Of these, several stage-specific proteins had been previously identified as diagnosis and/or vaccine candidates. In addition, antibodies from infected dogs recognized thirty-one proteins, which had been previously considered hypothetical, indicating that these proteins are expressed during active infection. Therefore, the present study reveals new potential candidates for the improvement of diagnosis of CVL.

specificity of the tests, requiring repeated tissue sampling and a trained laboratory staff [10]. The diagnosis of CVL, by means of ELISA, based on *Leishmania* antigens has shown variable values of sensitivity and/or specificity, mainly due to antigenic similarities between *Leishmania* and other protozoa [10]. As a strategy to develop a more specific test, several parasite antigens have been tested in prior studies [11–14]; however, due to frequent low specificity and sensitivity in detecting asymptomatic infections and the high variability observed in the humoral response of individual infected dogs [15], it has been postulated that an efficient diagnosis may require a mixture of antigens or the use of chimerical antigens [16–19].

Proteomic approaches applied to study *Leishmania* protein expression patterns offer the possibility to assign potential functions for proteins, including those previously identified by genomics as hypothetical, new diagnostic markers, vaccine candidates, and/or potential drug targets [20–23]. Several proteomic studies have been performed to study stage-specific expression and differentiation in *Leishmania* [24–32]. The coupling of antibodies specific to parasite antigens generated during different stages of disease progression in dogs will certainly contribute to refining this analysis, which aims to identify not only differentially expressed proteins, but also potentially new antigens identified by the immune system during active infection. Recently, the discovery of antigens through proteomics has been indicated as one of the main research priorities for further development and improvement of leishmaniasis vaccines [33].

In this work, an immunoproteomic approach, together with two-dimensional electrophoresis (2DE) and mass spectrometry, was carried out to analyze the protein expression profiles of promastigote and amastigote-like *L. infantum*. Aimed at identifying new diagnostic markers and/or vaccine candidates, antibodies present in the sera of dogs with asymptomatic and symptomatic VL were added to this analysis, allowing for the identification of several known, as well as hypothetical, *L. infantum* antigenic proteins.

Materials and Methods

Parasite culture

Experiments were carried out using the *Leishmania (Leishmania) chagasi* syn. *L. (L.) infantum* (MHOM/BR/1970/BH46) strain. The stationary phase of promastigote cells were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.2, as previously described [34]. The amastigote-like cells were obtained as described by Doyle et al. (1991) [35].

Sera samples

The present study used sera samples from 60 *L. infantum*-infected dogs (40 clinically symptomatic and 20 asymptomatic) from Belo Horizonte, Minas Gerais, Brazil. Animals were considered symptomatic when three or more of the following symptoms were present: loss of weight, hepatomegaly, alopecia, adenopathy, onychogryposis, conjunctivitis, and exfoliative dermatitis on the nose, tail, or ear tips. The asymptomatic animals were free from clinical symptoms. All sera samples from either symptomatic or asymptomatic animals were positive when tested by RIFI and ELISA, and the presence of amastigote stage of the parasite was confirmed by microscopic observation and *in vitro* culture using aspirates from popliteal and/or prescapular lymphoid nodes or bone marrow and/or tissue fragments. The control group consisted of sera from 20 dogs living in non-endemic areas from VL, with no clinical signs or suspicion of leishmaniasis, and which showed negative parasitological and serological tests. Sera samples used in this study were kindly provided by Dr. Maria Norma Melo (Departmento de Parasitologia, Instituto de Ciências Biológicas, UFMG).

Preparation of protein extracts

The protein extraction from promastigote and amastigote-like stages *L. infantum* and 2DE were performed following a modified protocol [36]. Briefly, cells from both stages (1×10^{10} cells) were washed three times in 40 mM Tris-HCl, pH 7.2, by centrifugation at $5000\times$ *g* for 10 min at 4°C. The pellets were resuspended in lyses buffer solution [7 M urea, 2 M thiourea, 4% chol-amidopropyl dimethylammonio-1-propanesulfonate (CHAPS), 40 mM dithiothreitol (DTT), 2% IPG buffer (pH 4–7), 40 mM Tris], and a protease inhibitor cocktail (GE Healthcare, Upsala, Sweden) was added. Samples were incubated for 1 h at room temperature, with occasional vortexing. Purification was carried out by protein precipitation using a 2D Clean UpKit (GE Healthcare), according to manufacturer instructions. Whole cell extracts were measured by a 2D Quant-Kit (GE Healthcare), and aliquots were immediately frozen at −80°C, until use.

Isoeletric focusing (IEF)

For the first-dimension electrophoresis, 150 µg of protein extract was added to a volume of 250 µL with a rehydration solution [7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 2% immobilized pH gradient (IPG-buffer, pH 4–7, trace bromophenol blue)]. Next, samples were applied to IPG strips (13 cm, pH 4–7; GE Healthcare) for passive rehydration overnight at room temperature. After in-gel rehydration for 12 h, isoeletric focusing was performed at 500 V for 1 h, 1.000 V for 1 h, and 8.000 V for 8 h, using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham, Piscataway, NJ, USA).

SDS-PAGE

After IEF, each strip was incubated for 15 min in a solution made up of 10 mL of a 50 mM Tris-HCl buffer pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol-blue, and 125 mM DTT, followed by a second incubation step in

the same buffer solution, excluding DTT, which was replaced by 125 mM iodacetamide. IPG strips were transferred to a 12% polyacrilamide and sealed with agarose solution (agarose and bromophenol blue in a Tris-glicine cathode buffer). The protein standard was purchased from Invitrogen (BenchMark Protein Ladder). Electrophoresis was performed in a Mini-Protean II system (BioRad) connected to a MultiTemp II cooling bath (Amersham Biosciences), in a Tris/glycine/SDS buffer. Proteins were separated at 200 V, until the dye front had reached the bottom of the gel.

Immunoblotting 2DE analysis and protein identification

To identify the reactive spots that were recognized by the antibodies present in the sera samples from asymptomatic and/or symptomatic CVL, Western blot analyses were performed. Whole cell extracts of promastigote and amastigote-like *L. infantum* were separated electrophoretically and transferred onto cellulose membranes (Schleicher & Schull, Dassel, Germany) by semi-dry blotting for 2 h at 400 mA. Membranes were blocked in 5% (w/v) low-fat dried milk in TBS 1× (pH 7.4) plus 0.05% Tween 20 for 2 h at room temperature. Next, the membranes were washed 6 times (10 min each) with the blocking solution and pre-incubated in a pool of sera of symptomatic or asymptomatic CVL (1:200 diluted) for 2 h at room temperature. Then, membranes were incubated with a peroxidase-conjugated goat anti-dog IgG secondary antibody (1:5.000 diluted) for 2 h at room temperature. After having been washed 3 times with TBS 1× plus 0.5% Tween 20, immunoblots were developed, using a solution made up of chloronaphtol, diaminobenzidine and H_2O_2. To select and identify the spots recognized by antibodies of CVL sera, three independent protein preparations, each obtained from independent parasite cultures, were performed. The 2DE gels were stained with colloidal Coomassie Brilliant Blue G-250, following procedures described by Neuhoff et al. (1988) [37]. For image analysis, the stained gels were scanned using an ImageScanner III (GE Healthcare). Reactive spots recognized by antibodies in the sera samples of asymptomatic and/or symptomatic CVL were excised manually from the gels for protein identification.

Protein digestion, peptide extraction, and spot handling

Spots were manually excised, and fragments were washed in 25 mM ammonium bicarbonate/50% acetonitrile until completely destained. After drying, gel fragments were placed on ice in a 50 µL protease solution (20 ng/mL of a sequence grade-modified trypsin in a 25 mM ammonium bicarbonate) (Promega Biosciences, CA, USA), for 30 min. Excess protease solution was removed and replaced by 25 mM ammonium bicarbonate. Digestion was performed at 37°C for 18 h. Peptide extraction was performed twice for 15 min, using 30 µL of 50% acetonitrile/5% formic acid. Trypsin (Promega) digests were concentrated in a Speed-Vac (Savant, USA) to approximately 10 µL and desalted using Zip-Tip (C18 resin; P10, Millipore Corporation, Bedford, MA, USA). Samples were mixed with a matrix (5 mg/ml recrystallized α-cyano-4-hydroxycinnamic acid) in a volume of 1 mL (1:1 ratio) and then spotted for MALDI-TOF/TOF Ultraflex III (Bruker, Daltonics, Germany).

Protein identification and database search

To determine the MS spectrum of the immunoreactive spots, the digests were spotted onto 600 µm Anchorchips (Bruker Daltonics). Spotting was achieved by pipetting, in duplicate, 1 µL of analyte onto the MALDI target plate, then adding 5 mg/mL α-cyano-4-hydroxycinnamic acid diluted in 3% TFA/50% acetonitrile, which contained 2 mM ammonium phosphate. The Bruker peptide calibration mixture was spotted down for external calibration. All samples were allowed to air dry at room temperature, and 0.1% TFA was used for on-target washing. All samples were analyzed in the positive-ion, reflection mode, through a MALDI-TOF/TOF Ultraflex III mass spectrometer (Bruker, Daltonics, Germany). Each spectrum was produced by accumulating data from 200 consecutive laser shots, with a frequency of 100 Hz, and an m/z range of 1.000–4.000. Instrument calibration was achieved by using peptide calibration standard II (Bruker Daltonics), a mixture of angiotensin I & II, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39 and somatostatin 28, as the internal standard. Peptide masses were measured as mono-isotopic masses. The MS peaks with the highest intensities were selected for MS/MS fragmentation analyses.

The resulting spectra were processed using Flex analysis software, version 2.4 (Bruker Daltonics), with the following settings: peak detection algorithm set at SNAP (Sort Neaten Assign and Place), S/N threshold at 3, precursor and product ion tolerances were set at 0.5 Da, and quality factor threshold at 50. The trypsin autodigestion ion peaks (842.51, 1045.56, 2211.10, and 2225.12 Da) were used as internal standards to validate the external calibration procedure. Matrix, and/or autoproteolytic trypsin fragments, and known contaminants (*i.e.*, keratins) were manually removed. The resulting peptides list was used to search in the NCBI database (http://blast.ncbi.nlm.nih.gov) for the organism option of *Leishmania* (taxid:5658). According to the obtained results, and using the peptide sequences identified for each protein, the following parameters were used as selection criteria: total score, query coverage, and *E* value. Poor quality spectra were not considered for selection in the protein sequence database.

Results

2DE protein maps of promastigote and amastigote-like total extracts of *Leishmania infantum*

Electrofocusing on pH 4–7 IPG strips, approximately 350 protein spots in *L. infantum* promastigote and 200 spots in amastigote-like stages could be observed clearly (Figure 1). Promastigote stages, as compared to amastigote-like forms, presented a larger number of visible spots, and differences could be observed in the molecular weights of the band profiles obtained between them: most of the promastigote spots were found between 15 and 50 kDa (Figure 1A), while in the amastigote-like stage, these bands tended to be distributed between 25 and 70 kDa (Figure 1B). The 2DE spot profiles obtained from promastigote and amastigote-like were highly reproducible in terms of both the total number of protein spots and their relative positions and intensities in the three 2DE gels performed in this study (data not shown).

Immunoblotting analysis of 2DE maps of promastigote and amastigote-like stages of *Leishmania infantum*

To investigate the antigenicity of proteins in the *L. infantum* promastigote stage, immunoblots were performed, using a pool of symptomatic and asymptomatic VL dogs' sera. Using the profile obtained from the 2DE gel as a comparison (Figure 2A), the pool of sera from asymptomatic VL dogs reacted by presenting approximately 40 protein spots in the promastigote extract (Figure 2B), whereas when the pool of sera from symptomatic VL dogs were used, the immunoblots revealed approximately 80 protein spots (Figure 2C).

In this same manner, using the 2DE gel profile obtained of amastigote-like extract for comparison (Figure 3A), the sera of asymptomatic VL dogs reacted by presenting approximately 30 protein spots (Figure 3B), whereas when the sera from VL symptomatic

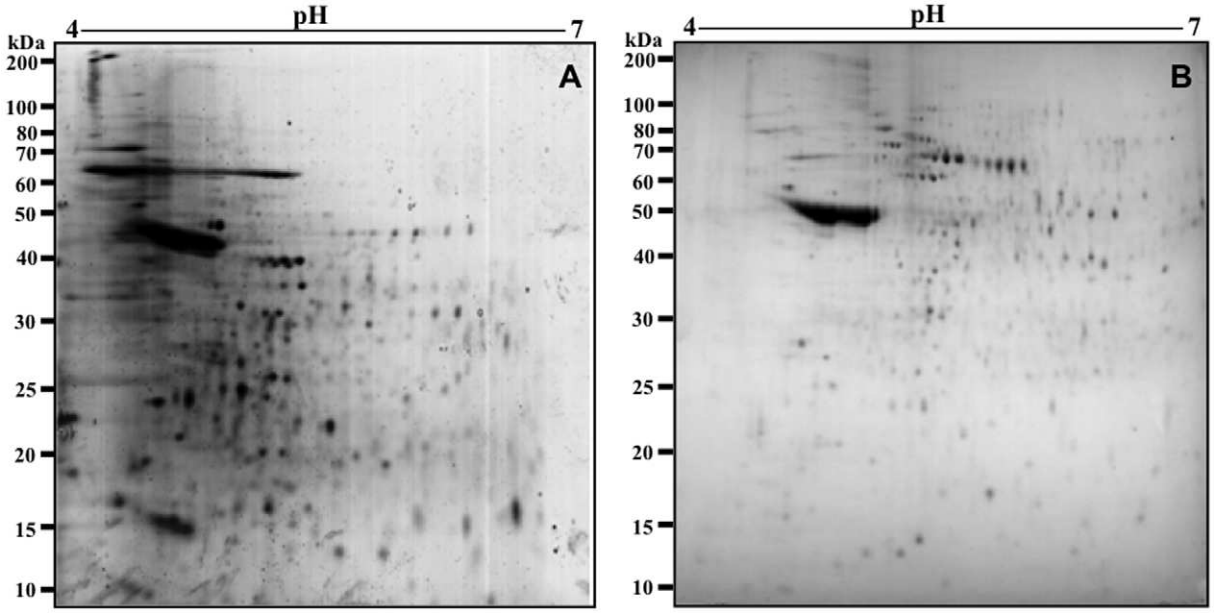


Figure 1. Two-dimensional profiles of the total extracts from *Leishmania infantum* promastigote and amastigote-like stages. 2DE gels were obtained after separation of promastigote (in A) and amastigote-like (in B) protein extracts (150 μg, each one) by 2DE (first dimension: IEF pH range 4–7, second dimension: 12% SDS-PAGE), and staining with colloidal Coomassie Brilliant Blue G-250. 2DE gels were derived from three independent protein preparations. One representative preparation of each parasite stage was used in this study.
doi:10.1371/journal.pntd.0001430.g001

dogs were used, immunoblots revealed approximately 70 protein spots (Figure 3C). It is important to note that how a pool of sera of symptomatic (n = 40) or asymptomatic (n = 20) VL dogs was used in the experiments, the individual variability in the humoral responses did not bias the reactivity observed in the immunoblotting analysis. As a control, the different 2DE gels and immunoblots applied to promastigote and amastigote-like extracts were probed with sera of control dogs presenting negative parasitological, clinical, and serological analyses, and no protein spot could be detected in either case (data not shown).

In Figure 4, the diagram shows that, from a total of 104 (100%) proteins in both promastigote and amastigote-like extracts, 64 (62%) could be identified by the sera of symptomatic CVL, while the sera of asymptomatic animals detected that 19 (18%) and 21 (20%) proteins proved to be reactive in both classes of sera, respectively. Of the proteins identified in promastigote antigenic extracts, the sera of symptomatic and asymptomatic VL dogs, as well as the combination of both sera, could identify approximately 49%, 20%, and 31% of the proteins, respectively. In amastigote-like extract, the sera of asymptomatic and symptomatic VL

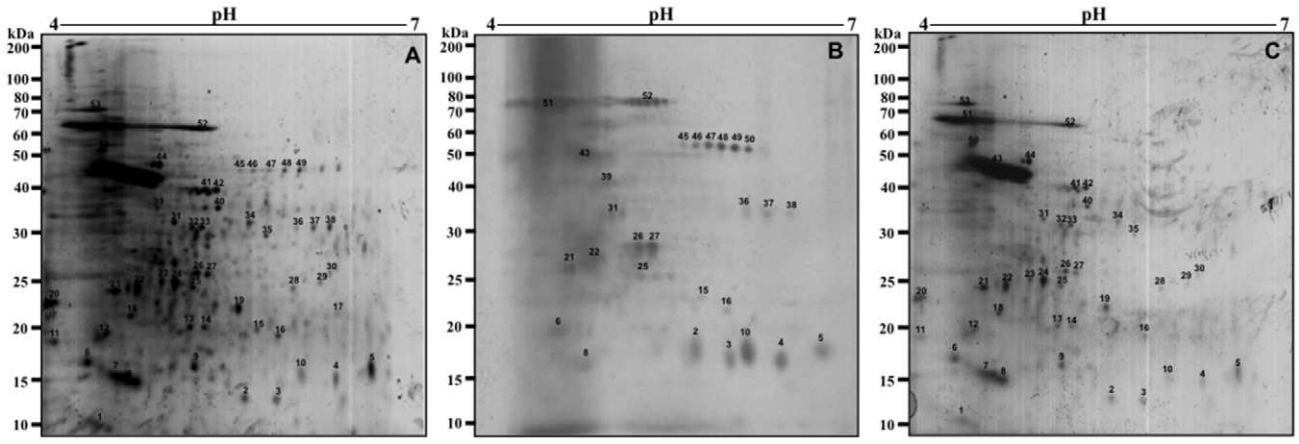


Figure 2. Immunoproteomic analyses of the protein extract from the *Leishmania infantum* promastigote stage. 2DE gels obtained after separation of total protein extract (150 μg) of promastigote stage by 2DE (first dimension: IEF pH range 4–7, second dimension: 12% SDS-PAGE), and staining with colloidal Coomassie Brilliant Blue G-250 (A, as described in Figure 1). Immunoblots of reactive spots were identified after incubation of the membrane with pools of sera of asymptomatic (B) or symptomatic (C) VL dogs. Bound antibodies were detected with goat anti-dog IgG antibodies at a 1:5.000 dilution. The x-axis represents the tentative isoeletric point (*pI*), while the y-axis represents the approximate molecular weight (kDa) as determined by a commercial 2DE gel marker (BenchMark Protein Ladder). Protein spots were numbered, and their identities are given in Figure 5. Immunoblots are a reliable representation of three independent experiments.
doi:10.1371/journal.pntd.0001430.g002

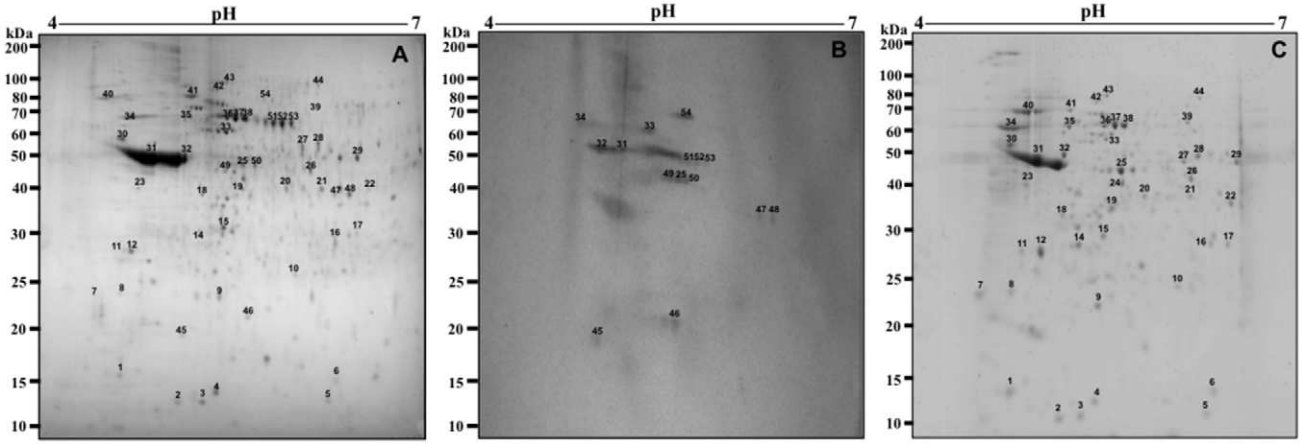


Figure 3. Immunoproteomic analyses of the protein extract from the *Leishmania infantum* amastigote-like stage. 2DE gels obtained after the separation of total protein extracts (150 μg) of amastigote-like stages by 2DE (first dimension: IEF pH range 4–7, second dimension: 12% SDS-PAGE), and staining with colloidal Coomassie Brilliant Blue G-250 (A, as described in Figure 1). Immunoblots of reactive spots were identified after incubation of the membrane with pools of sera of asymptomatic (B) or symptomatic (C) VL dogs. Bound antibodies were detected with goat anti-dog IgG antibodies at a 1:5.000 dilution. The x-axis represents the tentative isoeletric point (*pI*), while the y-axis represents the approximate molecular weight (kDa) as determined by a commercial 2DE gel marker (BenchMark Protein Ladder). Protein spots were numbered, and their identities are listed in Figure 6. Immunoblots are a reliable representation of three independent experiments.
doi:10.1371/journal.pntd.0001430.g003

animals and the combination of both sera classes identified approximately 74%, 17%, and 9% of the proteins, respectively.

Identification of *Leishmania infantum* promastigote proteins by MS/MS and the use of protein databases

In an attempt to establish a reference map of identified spots using the sera of asymptomatic and symptomatic VL dogs in immunoblots with *L. infantum* promastigote stage, 51 well-identified spots were used (25, 10, and 16 identified by symptomatic, asymptomatic, and the combination of both sera classes, respectively). Reactive spots were selected and excised from 2DE gels for analyses by mass spectrometry, as described in the Materials and Methods Section. Results are summarized in Figure 5.

Among the proteins recognized by the sera of symptomatic VL dogs, 7 hypothetical and 18 known proteins, which included cysteine proteinases, heat shock proteins (HSP70 and HSP83), and other proteins related to parasite virulence, such as disulfide isomerase [38,39], cyclophilin [40], and cytochrome *c* oxidase VII [41,42] were detected. Possible targets for therapeutic interventions, such as GTP-binding protein; proteins already characterized for diagnosis, such as KMP-11 [43,44] and calreticulin [45]; and vaccine candidates, such as KMP-11 [46] and Lcr1 protein [47], were also observed. Using the sera of asymptomatic VL dogs, 3 hypothetical and another 7 known proteins were detected, including a protein kinase, elongation factor (eIFE), and cythcrome p450, which have been considered therapeutic targets for leishmaniasis [48–51]. Five hypothetical proteins could be identified by both sera classes, whereas among the proteins with identifiable functions, some have been previously evaluated as candidates for the diagnosis and/or vaccine for leishmaniasis, such as nucleoside hydrolase [52], ribosomal proteins [53,54], peroxidoxin [55], and β-tubulins [56,57].

Identification of *Leishmania infantum* amastigote-like proteins by MS/MS and the use of protein databases

Due to the importance of the amastigote life cycle in leishmaniasis, this parasite stage cultured in axenic conditions was immunoblotted with the sera of asymptomatic and symptomatic VL dogs. The analysis of approximately 200 protein spots allowed for the identification of 53 well-defined proteins that were recognized by the sera of asymptomatic and symptomatic CVL; with 39, 9, and 5 identified by symptomatic, asymptomatic, and the combination of both sera classes, respectively. Similar to the study with promastigote stage, reactive spots were selected and excised from 2DE gels for identification. It is worth noting that A2 [13], ATP-dependent RNA helicase [58], and amastin [59] proteins were identified only in the amastigote-like extract, since these proteins are characterized as amastigote-specific in *Leishmania* (Figure 6).

When the sera of asymptomatic VL dogs were used against amastigote-like stage, 2 hypothetical and 7 known proteins, including the phosphatase 2C protein [60], virulence factors (prohibitin) [61], diagnosis markers, vaccine candidates (mitochondrial tryparedoxin peroxidase) [62], and drug targets (succinyl-coA ligase [GDP-forming] β-chain) [63] could also be identified.

A significant number of proteins (39 of 53) were found to be present in both stages and to react with sera of symptomatic VL dogs. Several of these are linked to housekeeping metabolism pathways, such as protein synthesis or cellular stress, and included ribosomal proteins, cyclophilin, Haspb, cysteine proteinases, eIFE, and heat shock proteins [30,53,54]. In addition, some proteins involved in parasite virulence, such as fructose-1,6-biphosphatealdolase (aldolase) [64,65], as well as therapeutic targets, such as ATPase β-subunit [66], cysteine peptidases [67], and methylthioadenosine phosphorylase [68], could also be identified.

Discussion

The present work applied an immunoproteomic approach in *L. infantum* promastigote and amastigote-like antigenic extracts, using pools of sera of asymptomatic and/or symptomatic VL dogs, in an attempt to compare their protein expression profiles and identify new targets for future immunological applications of VL. The use of pools of sera of both asymptomatic and symptomatic VL dogs in this study appears to have reduced the impact of individual animal immune response variations on *L. infantum* antigens.

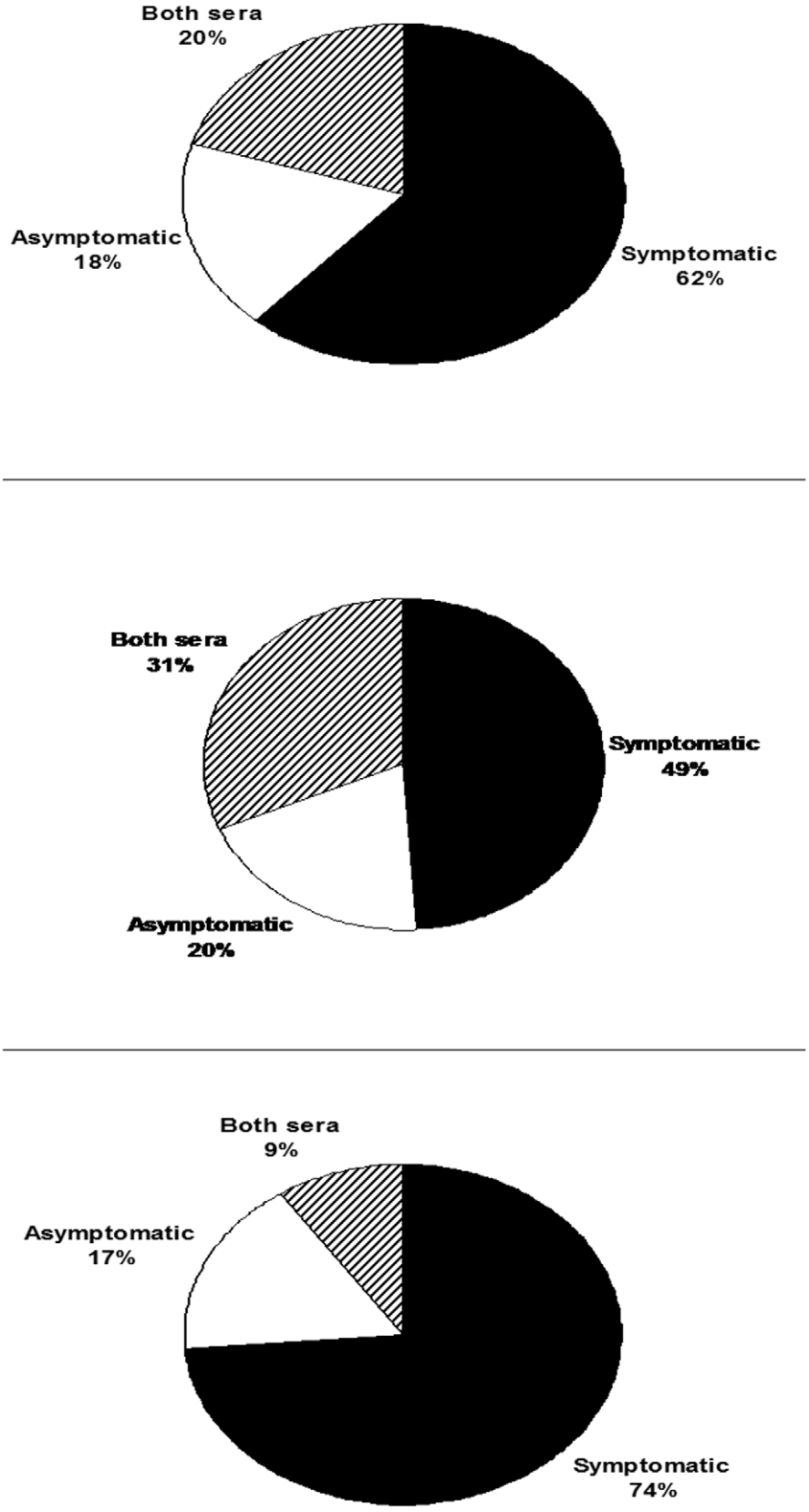


Figure 4. Comparison of spots identified in protein extracts from promastigote and amastigote-like stages of *Leishmania infantum*. Diagrams show the percentage of protein spots identified in either individual or combined parasite stages. In A, the percentage of total proteins identified by asymptomatic (19/18%), symptomatic (64/62%), and a combination of both sera classes (21/20%). In B, the percentage of proteins from the promastigote stage identified by asymptomatic (10/20%), symptomatic (25/49%), and a combination of both sera classes (16/31%). In C, the percentage of proteins from amastigote-like stage identified by asymptomatic (9/17%), symptomatic (39/74%), and a combination of both sera classes (5/9%).
doi:10.1371/journal.pntd.0001430.g004

Spot No.	CVL[a]	Identified protein/Sp.[b]	Accession No.[c]	*Mr* (kDa) (exp/pred)[d]	*pI* (exp/pred)[e]
15	asymptomatic	Hypothetical protein [*Lmj*]	CAJ09012.1	23/24	5.88/6.44
36	asymptomatic	Hypothetical protein [*Lbr*]	XP_001567688.1	36/72	6.21/6.33
37	asymptomatic	Eukaryotic translation initiation factor [*Lbr*]	XP_001566296.1	37/37	6.75/8.84
38	asymptomatic	Cytochrome p450-like protein [*Lbr*]	XP_001565772.1	37/67	6.89/8.87
39	asymptomatic	Hypothetical protein [*Li*]	XP_001466647.1	42/41	5.05/4.89
46	asymptomatic	Protein kinase [*Lmj*]	XP_001682848.1	50/360	6.58/6.91
47	asymptomatic	Protein kinase [*Lmj*]	XP_001682848.1	50/360	6.61/6.91
48	asymptomatic	Protein kinase [*Lmj*]	XP_001682848.1	50/360	6.65/6.91
49	asymptomatic	Protein kinase [*Lmj*]	XP_001682848.1	50/360	6.68/6.91
50	asymptomatic	Protein kinase [*Lmj*]	XP_001682848.1	50/360	6.72/6.91
1	symptomatic	Kinetoplastid membrane protein-11 [*Ld*]	S53442	12/11	4.50/5.96
7	symptomatic	Hypothetical protein [*Li*]	XP_001463668.1	18/23	4.81/5.97
9	symptomatic	Cytochrome c oxidase VII [*Lbr*]	XP_001565615.1	19/19	5.61/9.05
11	symptomatic	Heat shock protein 83 kDa [*Li*]	ABA06419.1	20/24	4.31/5.21
12	symptomatic	Hypothetical protein [*Lbr*]	XP_001564693.1	21/24	5.55/10.64
13	symptomatic	Hypothetical protein [*Lmj*]	XP_001684884.1	21/21	6.20/6.60
14	symptomatic	Hypothetical protein [*Lbr*]	XP_001565846.1	22/35	6.45/7.68
18	symptomatic	Hypothetical protein [*Lmj*]	XP_001686061.1	24/37	4.52/9.17
19	symptomatic	Haspb [*Lmj*]	CAB39972.1	22/18	5.52/4.99
20	symptomatic	IgE-dependent histamine-releasing factor putative [*Lmj*]	CAJ05086.1	19/23	4.41/4.39
23	symptomatic	Ribosomal protein SA 40S putative [*Lmj*]	CAJ09484.1	26/27	6.25/7.76
24	symptomatic	Flagellum transition zone component [*Lbr*]	XP_001569267.1	25/159	6.20/5.36
28	symptomatic	GTP-binding protein putative [*Lmj*]	CAJ05272.1	24/24	6.06/6.11
29	symptomatic	Cyclophilin [*Li*]	XP_001468254.1	26/28	6.65/9.38
30	symptomatic	Hypothetical protein [*Lbr*]	XP_001568689.1	27/24	6.80/8.89
31	symptomatic	Lcr1 protein [*Li*]	AAA73921.1	33/30	5.10/5.09
32	symptomatic	Cysteine protease [*Li*]	AAC38833.2	35/38	6.25/6.38
33	symptomatic	Cysteine protease [*Li*]	AAC38832.2	34/47	6.35/7.05
34	symptomatic	Phosphoglycan beta 1,3 galactosyltransferase [*Lbr*]	XP_001567057.1	35/90	6.65/8.72
35	symptomatic	Calreticulin [*Ld*]	ACE74538.1	32/33	6.22/4.77
40	symptomatic	Pyruvate dehydrogenase E1 beta subunit putative [*Lbr*]	CAM39227.1	35/36	5.02/5.26
41	symptomatic	Protein disulfide isomerase [*Li*]	CAM72512.1	44/52	5.80/5.42
42	symptomatic	Protein disulfide isomerase [*Li*]	CAM72512.1	44/52	5.80/5.42
44	symptomatic	Hypothetical protein [*Lmj*]	XP_888524.1	50/54	5.35/5.20
45	symptomatic	Heat shock protein 70 kDa [*Lbr*]	AAC28558.1	55/55	5.16/5.33
2	symptomatic and asymptomatic	Hypothetical protein [*Lbr*]	XP_001568117.1	13/35	5.23/5.25
3	symptomatic and asymptomatic	Hypothetical protein [*Lmj*]	XP_001682223.1	12/59	5.85/5.64
4	symptomatic and asymptomatic	Ribosomal protein L25 [*Lbr*]	AF234162_1	15/16	6.85/10.43
5	symptomatic and asymptomatic	Ribosomal protein 60S L23a [*Lbr*]	XP_001561992.1	16/17	6.95/10.29
6	symptomatic and asymptomatic	Hypothetical protein [*Lmj*]	XP_001684096.1	18/19	4.65/9.75
8	symptomatic and asymptomatic	NADH:ubiquinone oxidoreductase 78 Kd subunit-like protein [*Li*]	XP_001464959.1	15/28	4.62/4.88
10	symptomatic and asymptomatic	p21 protein [*Lmj*]	AAZ14759.1	17/21	6.32/5.22
16	symptomatic and asymptomatic	Nucleoside hydrolase [*Li*]	AAS48353.1	23/32	6.01/6.06
21	symptomatic and asymptomatic	Peroxidoxin/tryparedoxin peroxidase [*Lmj*]	XP_001683326.1	24/25	5.75/6.43
22	symptomatic and asymptomatic	Hypothetical protein [*Li*]	XP_001468309.1	25/82	5.82/6.41
25	symptomatic and asymptomatic	Cytochrome b-domain protein putative [*Lmj*]	AAZ10014.1	26/24	6.30/5.97
31	symptomatic and asymptomatic	Surface antigen-like protein [*Lbr*]	XP_001561808.1	34/33	5.12/4.42
43	symptomatic and asymptomatic	Beta-tubulin [*Li*]	CAM71245.1	51/49	4.68/4.71
51	symptomatic and asymptomatic	Hypothetical protein [*Lbr*]	XP_001568364.1	68/71	4.85/5.89
52	symptomatic and asymptomatic	Heat shock protein 70 kDa [*Li*]	CAA69282.1	70/71	5.42/5.36
53	symptomatic and asymptomatic	Histidine secretory acid phosphatase [*Lbr*]	XP_001569291.1	75/48	4.90/5.19

Figure 5. Proteins of *Leishmania infantum* promastigotes identified by an immunoproteomic approach. a) Sera samples of dogs with VL. b) Name of the identified protein and species: Lmj, *L. major*; Lbr, *L. braziliensis*; Li, *L. infantum*; Ld, *L. donovani*. c) Accession numbers according to NCBI. d) Experimental expected and predicted molecular weights (*M*r, in KDa). e) Experimental expected and predicted isoeletric points (p*I*).
doi:10.1371/journal.pntd.0001430.g005

The life cycle and the clonal propagation of *Leishmania* represent particular problems to obtain homogeneous populations of parasites to use in comparative proteomic analyses. In addition, it is difficult to purify amastigote-like stages from host tissues and, in general, contamination with host proteins is an important drawback to be overcome. Although axenic amastigotes display many of the features of *in vivo* intracellular parasites, a constant concern among researchers has been the extent to which axenic amastigotes resemble the intracellular parasites [69,70].

The present study employed the protocol described by Doyle et al. (1991) [35] to obtain amastigote-like stages of *L. infantum*. Carvalho et al. (2002), using the same protocol in amastigote-like stage, demonstrated the expression of the amastigote-specific A2 protein in *L. chagasi* and *L. amazonensis* [13], by applying Western blot experiments using an A2-specific monoclonal antibody. In the present work, A2 and two other amastigote-specific proteins – ATP-dependent RNA helicase [58] and amastin [59] – were detected in the immunoblots. The expression of these proteins by the axenic amastigotes suggests that they are, at least in part, comparable to tissue amastigotes and their gene expression, which is in accordance with the approach used in the present study to identify amastigote-specific antigens. Conversely, some proteins that are known to be specific, or that are more highly expressed in promastigotes, such as the flagellum transition zone component and the phosphoglycan beta-1,3-galactosyltransferase, which is linked to LPG synthesis, could only be detected in immunoblots of promastigote antigenic extracts.

As expected, some of the proteins identified in the present work have been previously associated with humoral responses in VL and are candidate antigens for diagnosis. Curiously, Haspb, a protein identified in promastigote extracts, presents a high homology, together with a family of related hydrophilic, kinesin antigens of *Leishmania spp.*, which includes the K26 and K39. These antigens were largely tested and used in serological diagnosis of VL, although they have been reported to be more sensitive for the diagnosis of symptomatic dogs [10,16,71].

The evolution from an asymptomatic to a symptomatic disease is largely dependent on host immune responses. Immunopathogenesis of CVL has been associated with two major responses: a Th1 immune response is linked to the control of infection and a predominant, although not polarized, Th2 response responsible for the development of a patent disease [72]. Here, several proteins proved to be reactive when in contact with sera of asymptomatic animals, a stage of infection in which dogs developing immune responses able to control parasite replication. Although humoral responses cannot be correlated directly with protection, IgG1 and IgG2 responses are largely T-cell dependent. Moreover, IgG2 antibodies have been commonly associated with protective immune responses and IFN-γ production [73]. Therefore, parasite antigens that react with antibodies from asymptomatic animals, in addition to their potential as diagnostic antigens, may be associated with protective responses and may well represent potential vaccine candidates.

In addition, the use of pools of sera of both asymptomatic and symptomatic VL dogs in the present study implies that no immune response variations by individual animals to *L. infantum* antigens

Spot No.	CVL[a]	Identified protein/Sp.[b]	Accession No.[c]	*Mr* (kDa) (exp/pred)[d]	*pI* (exp/pred)[e]
45	asymptomatic	Hypothetical protein [*Li*]	XP_001467126.1	19/21	5.25/6.60
46	asymptomatic	Mitochondrial tryparedoxin peroxidase [*Ld*]	ABP68406.1	25	6.35/7.66
47	asymptomatic	Hypothetical protein [*Li*]	XP_001468941.1	37/36	6.53/5.72
48	asymptomatic	Prohibitin [*Lmj*]	XP_843118.1	36/32	6.68/9.18
49	asymptomatic	Enolase [*Li*]	XP_001464303.1	47/46	5.45/5.33
50	asymptomatic	Succinyl-CoA ligase [GDP-forming] beta-chain [*Li*]	XP_001469702.1	41/44	5.95/6.77
51	asymptomatic	Protein phosphatase 2C [*Lmj*]	XP_001685487.1	62/61	6.51/8.72
52	asymptomatic	Protein phosphatase 2C [*Lmj*]	XP_001685487.1	62/61	6.59/8.72
53	asymptomatic	Protein phosphatase 2C [*Lmj*]	XP_001685487.1	62/61	6.88/8.72
1	symptomatic	NADH:ubiquinone oxidoreductase 78 Kd subunit-like protein [*Li*]	XP_001464959.1	15/28	4.62/4.88
2	symptomatic	Acidic ribosomal protein 60S P2 [*Lbr*]	XP_001566999.1	11/10	5.25/4.38
3	symptomatic	Calpain-like cysteine peptidase, putative; cysteine peptidase, Clan CA family C2 putative [*Lmj*]	CAJ03089.1	13/13	5.60/4.70
4	symptomatic	Hypothetical protein [*Li*]	XP_001568117.1	13/35	5.23/5.25
5	symptomatic	Unknown protein [*Li*]	AF398369_1	14/13	6.55/5.27
6	symptomatic	Hypothetical protein [*Lmj*]	XP_001684096.1	18/19	4.65/9.75
7	symptomatic	Peroxidoxin; tryparedoxin peroxidase [*Lmj*]	XP_001683326.1	24/25	5.75/6.43
8	symptomatic	Hypothetical protein [*Li*]	XP_001468309.1	25/82	5.82/6.41
9	symptomatic	Haspb [*Lmj*]	CAB39972.1	22/18	5.52/4.99
10	symptomatic	Cyclophilin [*Li*]	XP_001468254.1	26/28	6.65/9.38
11	symptomatic	Hypothetical protein [*Lbr*]	XP_001564596.1	28794	5.02/5.58
12	symptomatic	Ama1 protein [*Lmj*]	XP_001684736.1	28/20	5.15/5.10
13	symptomatic	Amastin [*Ld*]	ABI79069.1	26/19	6.09/8.85
14	symptomatic	Modification methylase-like protein [*Li*]	XP_001466154.1	28/89	5.38/6.42
15	symptomatic	Cysteine protease [*Li*]	AAC38832.2	34/47	6.35/7.05
16	symptomatic	Hypothetical protein [*Li*]	XP_001467567.1	25	6.40/8.40
17	symptomatic	ATPase beta subunit [*Li*]	XP_001466151.1	28/28	6.45/9.28
18	symptomatic	Translation initiation factor IF-2 [*Lbr*]	XP_001565418.1	35/100	5.10/8.32
19	symptomatic	Tagatose-6-phosphate kinase-like protein [*Li*]	XP_001468814.1	34/34	5.66/5.75

Spot No.	CVL[a]	Identified protein/Sp.[b]	Accession No.[c]	*Mr* (kDa) (exp/pred)[d]	*pI* (exp/pred)[e]
20	symptomatic	Protein disulfide isomerase [*Li*]	XP_001469404.1	40/52	6.10/5.42
21	symptomatic	Hypothetical protein [*Lmj*]	XP_843545.1	41/45	6.18/6.21
22	symptomatic	Methylthioadenosine phosphorylase, putative [*Lmj*]	CAJ02003.1	37/33	6.20/6.26
23	symptomatic	Fructose-1,6-bisphosphate aldolase [*Li*]	CAM72586.1	40/41	5.80/8.83
24	symptomatic	TPR domain protein, conserved [*Lbr*]	CAM70173.1	40/45	5.68/5.19
26	symptomatic	Hypothetical protein [*Lbr*]	XP_001564657.1	46/52	6.25/10.09
27	symptomatic	Hypothetical protein [*Li*]	XP_001465588	50/45	6.09/4.04
28	symptomatic	MAP kinase-like protein [*Li*]	XP_001463669.1	52/46	6.10/6.05
29	symptomatic	Aldehyde dehydrogenase [*Lbr*]	XP_001569159.1	52/57	6.55/8.27
30	symptomatic	Heat shock protein 70 [*Lbr*]	AAC28558.1	55/55	5.16/5.33
35	symptomatic	ATPase domain protein [*Lbr*]	XP_001565285.1	63/70	5.25/7.30
36	symptomatic	Heat shock protein 70 [*Li*]	CAA69282.1	70/71	5.28/5.36
37	symptomatic	Heat shock protein 70 [*Li*]	CAA69282.1	70/71	5.32/5.36
38	symptomatic	Heat shock protein 70 [*Li*]	CAA69282.1	70/71	5.42/5.36
39	symptomatic	Hypothetical protein [*Lbr*]	XP_001564657.1	60/52	6.12/10.09
40	symptomatic	Acetyl-CoA carboxylase [*Lbr*]	XP_001567323.1	74/240	5.10/6.00
41	symptomatic	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [*Lmj*]	XP_001687258.1	70/60	5.11/5.21
42	symptomatic	Hypothetical protein [*Li*]	XP_001463134.1	75/72	5.40/8.95
43	symptomatic	ATP-dependent RNA helicase-like protein [*Li*]	XP_001468500.1	70/197	6.02/8.93
44	symptomatic	Hypothetical protein [*Li*]	XP_001466114.1	72/108	6.25/6.12
25	symptomatic and asymptomatic	Serine/threonine protein phosphatase 2b catalytic subunit A2, putative [*Li*]	CAM72901.1	40/45	6.70/7.13
31	symptomatic and asymptomatic	Beta-tubulin [*Li*]	CAM71245.1	51/49	4.68/4.71
32	symptomatic and asymptomatic	Hypothetical protein [*Lmj*]	XP_888524.1	50/54	5.35/5.20
33	symptomatic and asymptomatic	Hypothetical protein [*Li*]	XP_001469969.1	58/86	5.25/5.52
34	symptomatic and asymptomatic	Hypothetical protein [*Lbr*]	XP_001568364.1	68/71	4.85/5.89

Figure 6. Proteins of *Leishmania infantum* amastigotes-like identified by an immunoproteomic approach. a) Sera samples of dogs with VL. b) Name of the identified protein and species: Lmj, *L. major*; Lbr, *L. braziliensis*; Li, *L. infantum*; Ld, *L. donovani*. c) Accession numbers according to NCBI. d) Experimental expected and predicted molecular weights (*Mr*, in KDa). e) Experimental expected and predicted isoeletric points (*pI*).
doi:10.1371/journal.pntd.0001430.g006

could be observed. Due to the high degree of variability found in the humoral responses to different parasite antigens in CVL sera [16], the results give rise to the possibility of obtaining new recombinant antigens and analyzing their properties as tools for the diagnosis of all forms of CVL.

Predominant proteins in the *pI* 4–7 2DE gels presented a molecular mass range of between 15 and 50 kDa for promastigote stage and of 25 to 70 kDa for the amastigote-like stage. These results are in agreement with findings from Dea-Ayuela et al. (2006) [74], who identified approximately 700 spots in promastigote extracts, with molecular masses similar to those found in the present study. By contrast, Brotherton et al. (2010) reported, for the first time, several highly basic proteins in both amastigote and promastigote protein extracts, which were enriched by coupling fractionation by *pI* with free-flow electrophoresis in their proteomic analysis of stage-specific expressions of *L. infantum* [32]. Therefore, the selection of a *pI* 4–7 range may have limited our analysis.

In addition, the presence of elongation factors; heat shock proteins, such as HSP70, HSP83, and other chaperones; as well as tubulin and other housekeeping proteins, among the most abundantly detected in both antigenic extracts, were in good agreement with other studies and present a reliable validation of the immunoproteomic analysis performed herein [56,57]. Some proteins detected in *Leishmania* extracts could be found in multiple spots or as proteolytic fragments. In addition, protein degradation cannot be completely discarded, although the protein extracts were obtained in the presence of a cocktail of protease inhibitors. However, this finding may also be associated with the presence of isoforms or to the extensive post-translational modification and processing of proteins, known to occur in *Leishmania sp.*, and as previously observed in other proteomic analyses [32].

The analysis of the three available *Leishmania* species genomes (*L. braziliensis*, *L. major*, and *L. infantum*) revealed that they are highly conserved at both nucleotide (less than 1% species-specific genes) and aminoacid levels (77 to 92%), although it has been estimated that *Leishmania* species have evolved from a common ancestor as far as 15–50 million years ago [75]. Although *Leishmania* has a digenetic life cycle with significant biochemical and morphological alterations, it has been estimated that only 0.2% to 13.0% of these genes show preferential stage-specific expression [76]. Therefore, there is no consensus on the number of genes that are differentially expressed in different stages, and discrepancies are likely due to the design of the genomic and cDNA arrays used in different studies [77]. Nevertheless, protein expression levels showed a weak correlation with gene expression levels [29,75], which could be linked to post-transcriptional events. In this context, proteomic studies are crucial and may reveal how *Leishmania* uses this conserved genetic background to generate protein variability, an alternation of stages during its life cycle, and to cause rather distinct diseases.

Tests based on serological techniques to diagnose human and canine VL are facilitated by the strong humoral response that accompanies the infection by viscerotropic *Leishmania* species [78]. Nonetheless, detection of asymptomatic dogs may be critical to control epidemics and to avoid the spread of the disease among dogs, as well as between dog and human populations [4,5,79]. However, total and soluble *Leishmania* antigen-based ELISA fails to detect a great percentage of asymptomatic cases of the disease [13,80]. Similar findings have also been reported for recombinant antigens [16]. Therefore, there is still space to identify new antigens capable, whether alone or in combination, of improving the serological diagnosis of CVL. In this sense, the present study represents a step forward in the proteomic analysis of *Leishmania*

species since, in addition to known antigenic stage-specific proteins, a high number of hypothetical proteins of *L. infantum* were also identified. Altogether, these proteins warrant further investigation in an attempt to potentially improve diagnosis. The fact that antibodies present in the pools of sera of infected dogs identified hypothetical proteins indicates that these proteins are expressed during active infection. Therefore, the data obtained in the present study represent not only a contribution toward the future improvement of diagnostic tools and vaccines for CVL, but also a step towards a better understanding of the biological role of these proteins in *L. infantum* metabolism, virulence, and pathogenesis. Thus, additional studies are most certainly encouraged.

References

1. Lainson R, Rangel EF (2006) *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. Mem Inst Oswaldo Cruz 101: 117–118.
2. Alvar J, Canavate C, Molina R, Moreno J, Nieto J (2004) Canine leishmaniasis. Adv Parasitol 57: 1–88.
3. Tavares CA, Fernandes AP, Melo MN (2003) Molecular diagnosis of leishmaniasis. Expert Rev Mol Diagn 3: 657–667.
4. Barbiéri CL (2006) Immunology of canine leishmaniasis. Parasite Immunol 28: 329–337.
5. Ciaramella P, Oliva G, Luna RD, Gradoni L, Ambrosio R, et al. (1997) A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. Vet Rec 141: 539–543.
6. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L (2008) Canine leishmaniasis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol 24: 324–330.
7. Nieto CG, Navarrete I, Habela MA, Serrano F, Redondo E (1992) Pathological changes in kidneys of dogs with natural *Leishmania* infection. Vet Parasitol 45: 33–47.
8. Garcia-Alonso M, Nieto CG, Blanco A, Requena JM, Alonso C, et al. (1996) Presence of antibodies in the aqueous humour and cerebrospinal fluid during *Leishmania* infections in dogs. Pathological features at the central nervous system. Parasite Immunol 18: 539–546.
9. Reed SG (1996) Diagnosis of leishmaniasis. Clin Dermatol 14: 471–478.
10. Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, et al. (1996) rK39: a cloned antigen of *Leishmania infantum* that predicts active visceral leishmaniasis. J Infect Dis 173: 758–761.
11. Ferreira WA, Mayrink W, Mares-Guia ML, Tavares CA (2003) Detection and characterization of *Leishmania* antigens from an American cutaneous leishmaniasis vaccine for diagnosis of visceral leishmaniasis. Diagn Microbiol Infect Dis 45: 35–43.
12. Barbosa-de-Deus R, Mares-Guia ML, Nunes AZ, Costa KM, Junqueira RG, et al. (2002) *Leishmania major*-like antigen for specific and sensitive serodiagnosis of human and canine visceral leishmaniasis. Clin Diagn Lab Immunol 9: 1361–1366.
13. Carvalho FA, Charest H, Tavares CA, Matlashewski G, Valente EP, et al. (2002) Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. Diagn Microbiol Infect Dis 43: 289–295.
14. Kubar J, Fragaki K (2005) Recombinant DNA-derived *Leishmania* proteins: from the laboratory to the field. Lancet Infect Dis 5: 107–114.
15. Goto Y, Howard RF, Bhatia A, Trigo J, Nakatani M, et al. (2009) Distinct antigen recognition pattern during zoonotic visceral leishmaniasis in humans and dogs. Vet Parasitol 160: 215–220.
16. Porrozzi R, Da Costa MVS, Teva A, Falqueto A, Ferreira AL, et al. (2007) Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clin Vaccine Immunol 14: 544–548.
17. Soto M, Requena JM, Quijada L, Alonso C (1998) Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis. J Clin Microbiol 36: 58–63.
18. Boarino A, Scalone A, Gradoni L, Ferroglio E, Vitale F, et al. (2005) Development of recombinant chimeric antigen expressing immunodominant B epitopes of *Leishmania infantum* for serodiagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol 12: 647–653.
19. Coelho EA, Ramírez L, Costa MA, Coelho VT, Martins VT, et al. (2009) Specific serodiagnosis of canine visceral leishmaniasis using *Leishmania* species ribosomal protein extracts. Clin Vaccine Immunol 16: 1774–1780.
20. Gopfert U, Goehring N, Klein C, Ilg T (1999) Proteophosphoglycans of *Leishmania mexicana*. Molecular cloning and characterization of the *Leishmania mexicana* ppg2 gene encoding the proteophosphoglycans aPPG and pPPG2 that are secreted by amastigotes and promastigote. J Biochem 344: 787–795.
21. Chenik M, Lakhal S, Ben Khalef N, Zribi L, Louzir H, et al. (2006) Approaches for the identification of potential excreted/secreted proteins of *Leishmania major* parasites. Parasitology 132: 493–509.
22. Paape D, Barrios-Lerena ME, Le Bihan T, Mackay L, Aebischer T (2010) Gel free analysis of the proteome of intracellular *Leishmania mexicana*. Mol Biochem Parasitol 169: 108–114.
23. Drummelsmith J, Brochu V, Girard I, Messier N, Ouellette M (2003) Proteome mapping of the protozoan parasite *Leishmania* and application to the study of drug targets and resistance mechanisms. Mol Cell Proteomics 2: 146–155.
24. El Fakhry Y, Ouellette M, Papadopoulou B (2002) A proteomic approach to identify developmentally regulated proteins in *Leishmania infantum*. Proteomics 2: 1007–1017.
25. Bente M, Harder S, Wiesgigl M, Heukeshoven J, Gelhaus C, et al. (2003) Developmentally induced changes of the proteome in the protozoan parasite *Leishmania donovani*. Proteomics 3: 1811–1829.
26. Nugent PG, Karsani SA, Wait R, Tempero J, Smith DF (2004) Proteomic analysis of *Leishmania mexicana* differentiation. Mol Biochem Parasit 136: 51–62.
27. Mc Nicoll F, Drummelsmith J, Muller M, Madore E, Boilard N, et al. (2006) A combined proteomic and transcriptomic approach to the study of stage differentiation in *Leishmania infantum*. Proteomics 6: 3567–3581.
28. Walker J, Vasquez JJ, Gomez MA, Drummelsmith J, Burchmore R, et al. (2006) Identification of developmentally-regulated proteins in *Leishmania panamensis* by proteome profiling of promastigote and axenic amastigotes. Mol Biochem Parasitol 147: 64–73.
29. Leifso K, Cohen-Freue G, Dogra N, Murray A, Mc Master WR (2007) Genomic and proteomic expression analysis of *Leishmania* promastigote and amastigote life stages: the *Leishmania* genome is constitutively expressed. Mol Biochem Parasitol 152: 35–46.
30. Rosenzweig D, Smith D, Myler PJ, Olafson RW, Zilberstein D (2008) Post-translational modification of cellular proteins during *Leishmania donovani* differentiation. Proteomics 8: 1843–1850.
31. Morales MA, Watanabe R, Laurent C, Lenormand P, Rousselle JC, et al. (2008) Phosphoproteomic analysis of *Leishmania donovani* pro- and amastigote stages. Proteomics 8: 350–363.
32. Brotherton MC, Racine G, Foucher AL, Drummelsmith J, Papadopoulou B, et al. (2010) Analysis of stage-specific expression of basic proteins in *Leishmania infantum*. J Proteome Res 9: 3842–3853.
33. Costa CHN, Peters NC, Maruyama SR, Brito Jr. EC, Santos IKFM (2011) Vaccines for the Leishmaniases: Proposals for a Research Agenda. The Working Group on Research Priorities for Development of Leishmaniasis Vaccines. PLoS Negl Trop Dis 5: e943.
34. Coelho EA, Tavares CA, Carvalho FA, Chaves KF, Teixeira KN, et al. (2003) Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infect Immun 71: 3988–3994.
35. Doyle PS, Engel JC, Pimenta PFP, Silva PP, Dwyer DM (1991) *Leishmania donovani*: long-term culture of axenic amastigotes at 37°C. Exp Parasitol 73: 326–334.
36. Lewis TS, Hunt JB, Aveline LD, Jonscher KR, Louie DF, et al. (2000) Identification of novel MAP kinase pathway signalling targets by functional proteomics and mass spectrometry. Mol Cell 6: 1343–1354.
37. Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrilamide gels including isoeletric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255–262.
38. Hong BX, Soong L (2008) Identification and enzymatic activities of four protein disulfide isomerase (PDI) isoforms of *Leishmania amazonensis*. Parasitol Res 102: 437–446.
39. Santos CXC, Stolf BS, Takemoto PVA, Amanso AM, Lopes LR, et al. (2009) Protein disulfide isomerase (PDI) associates with NADPH oxidase and is required for phagocytosis of *Leishmania infantum* promastigote by macrophages. J Leukocyte Biol 86: 989–998.
40. Yurchenko V, Xuea Z, Sherryb B, Bukrinskyc M (2008) Functional analysis of *Leishmania major* cyclophilin. Int J Parasitol 38: 633–639.
41. Luque-Ortega JR, Rivas L (2007) Miltefosine (Hexadecylphosphocholine) inhibits cytochrome *c* oxidase in *Leishmania donovani* promastigote. Antim Agents and Chem 51: 1327–1332.

Acknowledgments

The authors would like to thank Dr Maria Norma Melo, Dr Luiz M Farias and Dr Maria AR Carvalho for providing the canine serum samples and for their assistance.

Author Contributions

Conceived and designed the experiments: EAFC CAPT MMS. Performed the experiments: VTSC JSO DGV MACF MCD PSL. Analyzed the data: EAFC APF MS MMS CAPT. Wrote the paper: EAFC APF MMS MS CAPT.

42. Dey D, Meneses C, Salotra P, Kamhawi S, Nakhasi HL, et al. (2010) Characterization of a *Leishmania* stage-specific mitochondrial membrane protein that enhances the activity of cytochrome c oxidase and its role in virulence. Mol Microbiol 77: 399–414.
43. Trujillo C, Ramírez R, Vélez ID, Berberich C (1999) The humoral immune response to the kinetoplastid membrane protein-11 in patients with American Leishmaniasis and Chagas' disease: prevalence of IgG subclasses and mapping of epitopes. Immunol Letters 70: 203–209.
44. Iniesta V, Corraliza I, Carcelén J, Gordo LG, Fernández-Cotrina J, et al. (2008) *Leishmania major* infection in susceptible and resistant mice elicit a differential humoral response against a total soluble fraction and defined recombinant antigens of the parasite. Parasitol Res 102: 887–893.
45. Marcelain K, Colombo A, Molina MC, Ferreira L, Lorca M, et al. (2000) Development of an immunoenzymatic assay for the detection of human antibodies against *Trypanosoma cruzi* calreticulin, an immunodominant antigen. Acta Tropica 75: 291–300.
46. Basu R, Bhaumik S, Basu JM, Naskar, De T, et al. (2005) Responses in visceral leishmaniasis evidence for mixed Th1- and Th2-like synthase activity and IL-4 generation: that correlates with inducible nitric oxide and resistant strains of *Leishmania donovani* against both pentavalent antimonial-sensitive vaccination induces complete protection kinetoplastid membrane protein-11 DNA. J Immunol 174: 7160–7171.
47. Streit JA, Recker TJ, Donelson JE, Wilson ME (2000) BCG expressing LCR1 of *Leishmania infantum* induces protective immunity in susceptible mice. Exp Parasit 94: 33–41.
48. Naula C, Parsons M, Mottram JC (2005) Protein kinases as drug targets in trypanosomes and *Leishmania*. Biochimica et Biophysica Acta 1754: 151–159.
49. Yoffe Y, Zuberek J, Lerer A, Lewdorowicz M, Stepinski J, et al. (2006) Binding specificities and potential roles of isoforms of eukaryotic initiation factor 4E in *Leishmania*. Eukaryotic Cell 5: 1969–1979.
50. Johnson RE, Campbell-Bright S, Ralph H, Raasch Jo, Rodgers E (2008) Proteomic analysis of miltefosine-resistant *Leishmania* reveals the possible involvement of eukaryotic initiation factor 4A (eIF4A). Int J Antimicrobial Agents 31: 581–592.
51. Siqueira-Neto JL, Song OR, Jeong-Hun HOS, Yang G, Nam J, et al. (2010) Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. PLOS Negl Trop Dis 4: e675.
52. Aguilar-Be I, Zardo RS, Paraguai-de-Souza E, Borja-Cabrera P, Rosado-Vallado M, et al. (2005) Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. Infect Immun 73: 812–819.
53. Iborra S, Parody N, Abánades DR, Bonay P, Prates D, et al. (2008) Vaccination with the *Leishmania major* ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice. Microbes and Infection 10: 1133–1141.
54. Chávez-Fumagalli MA, Costa MAF, Oliveira DM, Ramírez L, Costa LE, et al. (2010) Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania infantum* and *Leishmania amazonensis* challenge. Microbes and Infection 12: 967–977.
55. Santarém N, Tomás A, Ouaissi A, Tavares J, Ferreira N, et al. (2005) Antibodies against a *Leishmania infantum* peroxiredoxin as a possible marker for diagnosis of visceral leishmaniasis and for monitoring the efficacy of treatment. Immunol Letters 101: 18–23.
56. Pateraki E, Portocala R, Labrousse H, Guesdon JL (1983) Antiactin and antitubulin antibodies in canine visceral leishmaniasis. Infect Immun 42: 496–500.
57. Shapira M, Mc Ewen JG, Jaffe CL (1988) Temperature effects on molecular processes which lead to stage differentiation in *Leishmania*. The EMBO J 7: 2895–2901.
58. Barhoumi M, Tanner NK, Banroques J, Linder P, Guizani I (2006) *Leishmania infantum* LeIF protein is an ATP-dependent RNA helicase and an eIF4A-like factor that inhibits translation in yeast. FEBS Journal 273: 5086–5100.
59. Nasereddin A, Schweynoch C, Schonian G, Jaffe CL (2010) Characterization of *Leishmania (Leishmania) tropica* axenic amastigotes. Acta Trop 113: 72–79.
60. Nandan D, Tran T, Trinh E, Silverman JM, Lopez M (2007) Identification of *Leishmania* fructose-1,6-bisphosphate aldolase as a novel activator of host macrophage Src homology 2 domain containing protein tyrosine phosphatase SHP-1. Biochem Bioph Res Com 364: 601–607.
61. Jain R, Ghoshal A, Mandal C, Shaha C (2010) *Leishmania* cell surface prohibitin: role in host–parasite interactions. Cel Microbiol 12: 432–452.
62. Castro H, Romao S, Gadelha FR, Tomás AM (2008) *Leishmania infantum*: provision of reducing equivalents to the mitochondrial tryparedoxin/tryparedoxin peroxidase system. Exp Parasitol 120: 421–423.
63. Davis AJ, Perugini MA, Smith BJ, Stewart JD, Ilg T, et al. (2004) Properties of GDP-mannose pyrophosphorylase, a critical enzyme and drug target in *Leishmania mexicana*. J Biol Chem 279: 12462–12468.
64. Mc Carthy JS, Wieseman M, Tropea J, Kaslow D, Abraham D, et al. (2002) *Onchocerca volvulus* glycolytic enzyme fructose-1,6-bisphosphate aldolase as a target for a protective immune response in humans. Infect Immun 70: 851–858.
65. Walque S, Opperdoes FR, Michels PAM (1999) Cloning and characterization of *Leishmania mexicana* fructose-1,6-bisphosphate aldolase. Mol Biochem Parasitol 103: 279–283.
66. Sánchez-Cañete MP, Carvalho L, Pérez-Victoria FJ, Gamarro F, Castanys S (2009) Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. Antim Agents Chem 53: 1305–1313.
67. Poot J, Spreeuwenberg K, Sanderson SJ, Schijns VECJ, Mottram JC, et al. (2006) Vaccination with a preparation based on recombinant cysteine peptidases and canine IL-12 does not protect dogs from infection with *Leishmania infantum*. Vaccine 24: 2460–2468.
68. Koszalka GW, Krenitsky TA (1986) 5′-Methylthioadenosine (MTA) phosphorylase from promastigote of *Leishmania donovani*. Adv Exp Med Biol 195: 559–563.
69. Bates PA (1994) Complete developmental cycle of *Leishmania mexicana* in axenic culture. Parasitology 8: 1–9.
70. Paape D, Lippuner C, Schmid M, Ackermann R, Barrios-Llerena ME, et al. (2008) Transgenic, fluorescent *Leishmania mexicana* allow direct analysis of the proteome of intracellular amastigotes. Mol Cell Proteomics 7: 1688–1701.
71. Bhatia A, Daifalla NS, Jen S, Badaro R, Reed SG, et al. (1999) Cloning, characterization and serological evaluation of K9 and K26: two related hydrophilic antigens of *Leishmania chagasi*. Mol Biochem Parasitol 102: 249–261.
72. Ferrer L, Solano-Gallego L, Arboix M, Arberola J (2002) Evaluation of the specific immune response in dogs infected by *Leishmania infantum*. In: Thoday KL, Foil CS, Bond R, Editors. Adv Vet Dermatol, Blackwell Science Oxford 4: 92–99.
73. Reis AB, Giunchetti RC, Carrillo E, Martins-Filho OA, Moreno J (2010) Immunity to *Leishmania* and the rational search for vaccines against canine leishmaniasis. Trends Parasitol 26: 341–349.
74. Dea-Ayuela MA, Rama-Iñiguez S, Bolás-Fernández F (2006) Proteomic analysis of antigens from *Leishmania infantum* promastigote. Proteomics 6: 4187–4194.
75. Lynn MA, Mc Master WR (2008) *Leishmania*: conserved evolution – diverse diseases. Trends Parasitol 24: 103–105.
76. Almeida R, Gilmartin BJ, Mc Canna SH, Norrish A, Ivens AC, et al. (2004) Expression profiling of the *Leishmania* life cycle: cDNA arrays identify developmentally regulated genes present but not annotated in the genome. Mol Biochem Parasitol 136: 87–100.
77. Cohen-Freue G, Holzer TR, Forney JD, McMaster WR (2007) Global gene expression in *Leishmania*. Int J Parasitol 37: 1077–1086.
78. Gramiccia M, Gradoni L (2005) The current status of zoonotic leishmaniasis and approaches to disease control. Int J Parasitol 35: 1169–1180.
79. Santarém RS, Cardoso L, Schallig H, Reed SG, Cordeiro-da-Silva A (2010) Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. J Clin Microb 48: 1866–1874.
80. Almeida MAO, Jesus EEV, Sousa-Atta MLB, Alves LC, Berne MEA, et al. (2005) Antileishmanial antibody profile in dogs naturally infected with *Leishmania chagasi*. Vet Immunol Immunopathol 106: 151–158.