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(54) Título: PEPTÍDEOS RECOMBINANTES, MÉTODO E KIT PARA TESTE IMUNODIAGNÓSTICO DE LEISHMANIOSE VISCERAL

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(57) Resumo: Peptídeos Recombinantes, Método e Kit para Teste Imunodiagnástico de Leishmaniose Visceral. A presente invenção descreve um método e kit para diagnosticar Leishmaniose visceral. Mais particularmente, a invenção trata da associação dos antígenos recombinantes definidos rA2, rNH e rLACK ou seus epítopos, peptídeos 47, 17, 18 e 19, permitindo o desenvolvimento de um método diagnóstico com maior especificidade, otimizando, assim, a reatividade dos testes sorológicos.

Peptídeos Recombinantes, Método e Kit para Teste Imunodiagnóstico de Leishmaniose Visceral

A presente invenção descreve um método e kit para diagnosticar Leishmaniose visceral. Mais particularmente, a invenção trata da associação dos antígenos recombinantes definidos rA2, rNH e rLACK ou seus epítopos, peptídeos 47, 17, 18 e 19, permitindo o desenvolvimento de um método diagnóstico com maior especificidade, otimizando, assim, a reatividade dos testes sorológicos.

Espécies do gênero *Leishmania* são agentes etiológicos das diferentes formas clínicas da leishmaniose (visceral, cutânea, mucocutânea e difusa) que, por sua vez, apresentam distribuição geográfica e prevalências distintas. Dentre os 88 países em que é registrada a ocorrência de leishmaniose, 72 são países em desenvolvimento e 13 estão entre os menos desenvolvidos do mundo. Cerca de 90% dos casos de leishmaniose visceral (LV) ocorrem no Brasil, Bangladesh, Índia, Nepal e Sudão e 90% dos casos de leishmaniose cutânea (LC) ocorrem no Brasil, Afeganistão, Argélia, Iran, Peru, Arábia Saudita e Síria (World Health Organization. 2002. Leishmaniasis Strategic Direction. "http://www.who.int/tdr/diseases/leish/lifecycle.htm"; Murray, H. W. J. D. Berman. 2005. "Advances in leishmaniasis." Lancet 366(9496): 1561-77). Cabe aqui ressaltar que a LV, quando não tratada, é normalmente fatal.

Em diferentes regiões geográficas, o ciclo de transmissão da doença se estabelece quando se encontram próximos, em um ambiente favorável, os vetores (fêmeas de flebotomíneos dos gêneros *Lutzomiya* e *Phlebotomus*) infectados por formas promastigotas de alguma das várias espécies de *Leishmania* e o hospedeiro definitivo (mamíferos, sendo o homem hospedeiro acidental). Formas promastigotas, após terem sido introduzidas na pele dos hospedeiros definitivos, são fagocitadas e se transformam em formas amastigotas, que permanecem em contato com o sistema imune do hospedeiro durante a infecção ativa. O cão é considerado o principal reservatório doméstico da LV em nosso meio (Matlashewski, G. 2001. Leishmania infection and virulence: Med Microbiol. Immunol., 190: 37-42).

O controle da leishmaniose visceral (LV) no Brasil se faz por meio da eliminação dos reservatórios domésticos da doença (cães infectados), controle das populações de vetor e, quando possível, pela prevenção do contato com vetores infectados. Cães infectados, sintomáticos ou não, podem transmitir os parasitas ao inseto vetor (Courtenay, O., R. J. Quinnell, L. M. Garcez, J. J. Shaw, and C. Dye. 2002. infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J. Infect. Dis. 186:1314–1320). Assim, a prevalência e incidência da infecção canina são parâmetros fundamentais para o controle da transmissão. No entanto, a estimativa das mesmas depende de métodos confiáveis de identificação dos cães infectados (Dye, C., E. Vidor, and J. Deneure. 1993. Serological diagnosis of leishmaniasis: on detecting infection as poço as disease. Epidemiol. Infect. 103:647–656; Dye, C., R. Killick-Kendrick, M. M. Vitutia, R. Walton, M. Killick-Kendrick, A. E. Harith, M. W. Guy, M.-C. Canãvate and G. Hasibeder. 1992. Epidemiology of canine leishmaniasis: prevalence, incidence and basic reproduction number calculated from a cross-sectional serological survey on the island of Gozo, Malta. Parasitology 105:35–41; Quinnell, R. J., O. Courtenay, L. Garcez, and C. Dye. 1997. The epidemiology of canine leishmaniasis: transmission rates estimated from a cohort study in Amazonian Brazil. Parasitology 115:143–156). Estudos baseados em modelagem matemática indicam, porém, que medidas de controle baseadas na detecção sorológica dos cães falham devido à alta incidência da infecção em cães, à alta infectividade dos parasitas, à falta de sensibilidade dos testes de diagnóstico sorológico e à demora entre diagnóstico e eliminação dos cães positivos, entre outros aspectos (Courtenay et al. 2002).

A aplicação das medidas de controle da LV no Brasil é extremamente complexa e onerosa, devido ao complexo ciclo de transmissão da doença, que envolve reservatórios domésticos,silvestres e insetos vetores, à precária condição sócio-econômica da população em muitas regiões do país e à urbanização e expansão da doença para áreas onde, até recentemente, ela não era registrada. Outros aspectos referem-se às diferenças ecoepidemiológicas da doença em diferentes regiões do país, à baixa

sensibilidade dos testes de diagnóstico sorológicos convencionais, especialmente para a detecção de cães que se encontram nos estágios iniciais da doença, e à demora entre o diagnóstico e a eliminação dos cães infectados. Recentemente, a introdução de uma vacina no mercado contra a LV canina (Leishmune® - FordDodge), constituída de complexo antigênico FML purificado de formas promastigotas, tem dificultado a adoção dessas medidas de controle, uma vez que cães vacinados se tornam soropositivos nos testes convencionais, que empregam antígenos de formas promastigotas dos parasitas. Esse aspecto relaciona-se à presença dos mesmos antígenos na vacina e nos testes de diagnóstico sorológico que empregam formas promastigotas do parasita. Essa dificuldade poderia ser contornada empregando-se vacinas que não induzem soroconversão dos cães nos testes de diagnóstico empregando antígenos de formas promastigotas ou empregando-se nos testes sorológicos antígenos distintos daquelas presentes nas vacinas. Independentemente das características específicas de cada vacina, em um cenário complexo como esse, a introdução de novas ferramentas de controle pressupõe a não interferência com as demais medidas de controle vigentes.

O diagnóstico da Leishmaniose Visceral Canina (LVC) é conduzido por meio de testes sorológicos e parasitológicos em aspirados de baço, medula óssea, biópsia das lesões e linfonodos (WHO, 2002; Tavares CA, Fernandes AP, Melo MN. 2003. Molecular diagnosis of leishmaniasis. Expert Rev Mol Diagn. 3(5):657-67). Devido às limitações dos métodos de diagnóstico direto, tais como microscopia, cultura, inoculação em hamsters de amostras de biopsia, a presença de anticorpos contra os parasitas por meio de reação de imunofluorescência ou ELISA empregando antígenos de formas promastigotas de *Leishmania sp.* é pesquisada rotineiramente como marcador de infecção (Dye et al., 1993; Grimaldi, G., Jr., and R. B. Tesh. 1993. Leishmaniasis of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. 6:230–250; Tavares et al., 2003).

Vários tipos de testes sorológicos e antígenos têm sido avaliados, mas a sorologia é, em geral, menos específica do que a detecção de parasitas em

amostras de biópsia, devido à reatividade cruzada com outros agentes como *Erlichia canis* e riquetsias. Os testes sorológicos disponíveis para o diagnóstico indireto incluem a reação de imunofluorêscencia (IFAT), ELISAs, dot-ELISA, teste de aglutinação direta, Western blotting, e teste imunocromatográfico (Mancianti, F. and N. Meciani. 1988. Specific serodiagnosis of canine leishmaniasis by indirect immunofluorescence, indirect hemagglutination, and counter-immunoelectrophoresis. Am. J. Vet. Res. 49:1409–1411; Badaró, R., D. Benson, M. C. Eulálio, M. Freire, S. Cunha, E. M. Neto, D. Pedral-Sampaio, C. Madureira, J. M. Burns, R. L. Houghton, J. R. David, and S. G. Reed. 1996. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. J. Infect. Dis. 173:758–761; Vercammen, F., D. Berkvens, J. Brandt, and W. Vansteenkiste. 1998. A sensitive and specific 30-min Dot-ELISA for the detection of anti-leishmania antibodies in the dog. Vet. Parasitol. 79:221–228; Harith, A., A. H. Kolk, P. A. Kager, J. Leeuwenburg, R. Muigai, S. Kiugu, and J. J. Laarman. 1986. A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis.Trans. R. Soc. Trop. Med. Hyg. 80:583–587; Aisa, M. J., S. Castillejo, M. Gallego, R. Fisa, M. C. Riera, M. De Colmenares, S. Torras, X. Roura, J. Sentis, and M. Portus. 1998. 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.; 58:154–159; Teodoro da Costa, R., J. C. Franc¸a, W. Mayrink, E. Nascimento, O. Genaro, and A. Campos-Neto. 2003. Standardization of a rapid immunochromatographic test with the recombinant antigens K39 and K26 for the diagnosis of canine visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 97:678–682). A avaliação destes testes indica que, em geral, eles apresentam sensibilidade adequada para o sorodiagnóstico da LVC, em cães sintomáticos. Além dos testes sorológicos, testes imunocitoquímicos e moleculares, baseados em reações de PCR, também foram desenvolvidos para detecção de DNA de *Leishmania* em amostras humanas e de cães (Quinnell, R. J., O. Courtenay, S. Davidson, L. Garcez, B. Lambson, P. Ramos, J. J. Shaw, M. A. Shaw, and C. Dye. 2001. Detection of *Leishmania infantum* by PCR, serology and immune response in a cohort study

of Brazilian dogs. Parasitology 122:253–261; Reithinger, R., R. J. Quinnell, B. Alexander, and C. R. Davies. 2002. Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbentassay, and PCR. J. Clin. Microbiol. 40:2352–2356). Entretanto, estes testes são laboriosos e de custo elevado, pois requerem equipamentos especializados e corpo técnico treinado, o que inviabiliza a sua aplicação em muitos casos. Estudos sugerem que a PCR é mais útil para detectar infecção ativa enquanto a sorologia pode ser mais sensível para detecção de todos os animais infectados (Quinnell et al., 2001).

O diagnóstico sorológico da LVC por ELISA empregando extrato de promastigotas ou Reação de Imunofluorescência ainda possui limitações relacionadas ao preparo e padronização dos antígenos de formas promastigotas e também pelo fato de requerer pessoal treinado e equipamentos especializados, dificultando sua aplicação rápida. Testes mais rápidos, como a imunocromatografia utilizando antígenos de proteínas recombinantes de *L. infantum* como o K39 (rK39) e rK26, têm auxiliado no diagnóstico de casos de LVC sintomáticos. Notam-se, no entanto, limitações para diferenciar cães assintomáticos de sintomáticos. Outro teste foi introduzido no mercado recentemente pela empresa Biogene, contendo o antígeno recombinante HSP70, denominado S7. No entanto, detecta-se uma escassez de dados da literatura em relação à avaliação de sensibilidade e especificidade e validação desse teste. Resultados preliminares obtidos pelo nosso grupo indicam elevada discordância em relação ao testes convencionais no diagnóstico de cães assintomáticos e sintomáticos.

Outro aspecto que deve ser ressaltado refere-se ao fato de que o Ministério da Saúde investiu na implantação e capacitação de laboratórios em todo o país para o diagnóstico sorológico da LVC e que esse esforço não deve ser desperdiçado, mas sim aprimorado. Testes de diagnóstico moleculares se apresentam como alternativas devido a sua alta sensibilidade, mas são ainda laboriosos e onerosos. Desta forma, o aprimoramento de testes de diagnóstico sorológico que possam se valer da infra-estrutura e capacitação instaladas tem óbvias vantagens para a aplicação no SUS.

O antígeno A2, expresso pelas formas amastigotas de espécies viscerotrópicas, pertence a uma família de proteínas que possui um número variável de repetições de uma unidade de 10 aminoácidos, sendo considerado o primeiro fator de virulência amastigota específico descrito (Charest, H. and G. Matlashewski. 1994. "Developmental gene expression in Leishmania donovani: differential cloning and analysis of an amastigote-stage-specific gene." Mol Cell Biol 14(5): 2975-84; Zhang, W. W. and G. Matlashewski. 1997. Loss of virulence in Leishmania donovani deficient in an amastigote-specific protein, A2. Proc Natl Acad Sci U S A 94(16): 8807-11). A2 se mostrou reativo em 60 e 82% dos pacientes soropositivos com o kala-azar da Índia e do Sudão, respectivamente, no teste de ELISA (Ghedin, E., W. W. Zhang, H. Charest, S. Sundar, R. T. Kenney, and G. Matlashewski. 1997. Antibody response against *Leishmania donovani* amastigote-stage-specific protein in patients with visceral leishmaniasis. Clin. Diagn.Lab. Immunol. 4:530–535). No Brasil, os anticorpos anti-A2 foram detectados por ELISA em 77% dos soros dos pacientes com LV sintomático e em 87% dos soros dos cães positivos pela IFAT para *Leishmania* ou na avaliação parasitológica (Carvalho, F. A. A., H. Charest, C. A. P. Tavares, G. Matlashewski, E. P. Valente, A. Rabello, R. T. Gazinelli, and A. P. Fernandes. 2002. Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. Diagn. Microbiol. Infect. Dis. 43:289–295). Em um estudo recente, foram avaliados comparativamente antígenos recombinantes parasita-específicos rK39, rK26, e rA2 e o antígeno solúvel (CSA) em testes de ELISA (Porrozzi, R., M. V. S. Costa, A. Teva, A., Campos-Neto, A., Fernandes, A.P., Gazzinelli, RT., Grimaldi, G. Jr. 2007. Comparative evaluation of enzime-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clinical and Vaccine Immunology 14:544-548). Os resultados mostraram que estes marcadores apresentam sensibilidade elevada para o diagnóstico da LVC em caes sintomáticos, que apresentam em geral elevados títulos de anticorpos. No entanto, quando foi calculada a razão entre casos sintomáticos e assintomáticos que foram identificados por cada antígeno, A2 foi o antígeno

que apresentou o mais alto índice, seguido por K39, K26 e CSA, sugerindo associação entre resposta a A2 e imunidade protetora. Além disso, a especificidade do teste empregando A2 foi de 96%, enquanto para os outros antígenos variou entre 85 a 90% (Porrozzi et al. 2007). Esses dados sugerem que esses marcadores podem se complementar aumentando a sensibilidade total dos testes de detecção de anticorpos em cães sintomáticos e assintomáticos infectados por espécies de leishmania que causam a LVC.

Embora os dados apresentados por Porrozzi et al. (2007) demonstrem uma considerável melhoria na sensibilidade e especificidade de testes com antígenos recombinantes em comparação com testes empregando extrato antigênico, observa-se que ainda existem limitações em relação a uma menor sensibilidade dos testes empregando os antígenos K39, K26 e extrato antigênico em relação ao diagnóstico de cães assintomáticos. Esses animais tendem em geral a apresentar títulos de anticorpos mais baixos, menores que 1:640. No entanto, animais assintomáticos podem apresentar os parasitos na pele e representar uma importante fonte de transmissão dos mesmos para os vetores, resultando na manutenção da transmissão da infecção e sendo um dos fatores importantes para a falência das medidas de controle. Assim, testes mais sensíveis e específicos, capazes de detectar cães assintomáticos e/ou com baixos títulos de anticorpos, se fazem ainda necessários. Considerando que a detecção e o sacrifício de cães com sorologia positiva ainda é adotado no Brasil como medida de controle e que animais com baixos títulos de anticorpos e assintomáticos podem transmitir a infecção, o emprego de testes de diagnóstico com maior sensibilidade para esse tipo de animal pode apresentar impacto favorável sobre as medidas de controle da leishmaniose visceral no Brasil.

A associação de antígenos definidos é uma estratégia importante para o aprimoramento dos métodos de diagnóstico sorológico, uma vez que antígenos recombinantes, tais como rK39, rK26, e rA2, apesar de apresentarem elevada especificidade, quando empregados isoladamente podem apresentar menor sensibilidade (Carvalho et al. 2002; Porrozi et al., 2007). No entanto, estes marcadores apresentam o potencial de se complementarem, aumentando a

sensibilidade total nos testes sorológicos de cães sintomáticos (Porrozzi et al., 2007). Assim, combinações de antígenos podem ser mais sensíveis do que uma única proteína ou epítopo, considerando a heterogeneidade genética das populações caninas. Antígenos recombinantes também podem apresentar especificidade reduzida se epítopos presentes em sua seqüência são também compartilhados por moléculas ortólogas presentes em outros organismos. Usando métodos moleculares, esses problemas podem ser contornados com a remoção destas seqüências, resultando em moléculas otimizadas tanto para sensibilidade quanto especificidade. Instrumentos disponíveis *on line* podem ser úteis para predição de epítopos de células B. A partir de um antígeno com sequência conhecida e que tenha apresentado reatividade com soros de cães e pacientes com leishmaniose visceral, pode-se identificar as porções da molécula com maior afinidade por anticorpos e sintetizar os peptídeos correspondentes, otimizando em cada molécula a reatividade em testes sorológicos.

Tal abordagem foi empregada no presente estudo, selecionando-se, a partir das sequências dos antígenos A2, NH e LACK, peptídeos correspondentes a regiões com características ótimas para reatividade como epítopos para células B. Assim, a presente invenção descreve um método e Kit para diagnosticar Leishmaniose através da associação dos antígenos definidos rA2, rNH e rLACK ou seus epítopos, permitindo o desenvolvimento de um método diagnóstico com maior sensibilidade, otimizando, dessa forma, a reatividade dos testes sorológicos.

Já se encontram patentes que descrevem o uso de antígenos de leishmania e a combinação destes no diagnóstico da Leishmaniose. Dentre os quais podemos citar:

O documento US20100136046- RECOMBINANT POLYPROTEIN VACCINES FOR THE TREATMENT AND DIAGNOSIS OF LEISHMANIASIS- descreve composições e métodos para prevenir, tratar e detectar leishmaniose. As composições geralmente incluem polipeptídeos de fusão compreendendo múltiplos antígenos de Leishmania, em particular, A2 KMPII, SMT, e /ou CBP,

ou porções imunogênicas ou variantes das mesmas, bem como polinucleotídeos codificando tais polipeptídeos de fusão.

O documento US20060211056- LEISHMANIA ANTIGENS SUITABLE FOR A DIAGNOSTIC KIT OF LEISHMANIA– descreve a obtenção de polipeptídeos de 10 aminoácidos consecutivos purificados a partir de *Leishmania infantum*. E, ainda, o uso desses polipeptídeos para diagnosticar LV Mediterrânea..

O documento US7740859- POLYPEPTIDES FOR THE DIAGNOSIS AND THERAPY OF LEISHANANIASIS- descreve compostos e métodos para a detecção de anticorpos anti-leishmania em indivíduos suspeitos de infecção com o parasita protozoário do gênero Leishmania, onde o agente infeccioso é uma cepa indiana, semelhante ou muito relacionada à cepa de *Leishmania indiana*.

O documento PI9405713-3- DIAGNOSE DA LEISHMANIOSE- descreve um método para o diagnóstico da leishmaniose que compreende: (a) obter uma amostra a partir de um paciente sob suspeita de estar infectado com o parasita Leishmania contendo anticorpos do paciente e (b) determinar a presença de anticorpos que se ligam a uma unidade repetitiva do antígeno K39.

O documento WO2010020028- PEPTÍDEOS SINTÉTICOS E POLÍMERO PARA IMUNIZAÇÃO CONTRA A LEISHMANIOSE- descreve dois peptídeos antigênicos sintéticos (epítopos ou mimotopos) selecionados e o método de conjugação desses peptídeos para formar um polímero. O polímero antigênico formado por esses peptídeos produz imunização contra leishmaniose visceral e será usado em método e kit imunodiagnóstico específico para detectar a doença.

Breve Descrição das Figuras

Figura 1 - Reatividade do peptídeo 47 (1µg/poço) frente a soros de cães que apresentam altos títulos de anticorpos (>1:640). Observa-se que nessas condições o peptídeo foi capaz de detectar 100% dos cães com altos títulos de anticorpos, apresentando alta sensibilidade e especificidade do peptídeo 47 com soros de cães com altos títulos de anticorpos.

Figura 2 - Reatividade do peptídeo 47 (1µg/poço) frente a soros de cães sintomáticos e assintomáticos. Observa-se que, nessas condições, o peptídeo 47 foi capaz de detectar de forma comparável soros de cães sintomáticos e assintomáticos, representando, portanto, uma otimização da sensiblidade apresentada quando a proteína A2 recombinante foi testada frente aos mesmos soros, como descrito por Porrozzi et. al. (2007).

Figura 3 - Reatividade do peptídeo 17 (4µg/poço) frente a soros de cães que apresentam altos títulos de anticorpos (>1:640).

Figura 4 - Reatividade do peptídeo 17 (250 ng/poço) frente a soros de cães sintomáticos e assintomáticos.

Figura 5 - Reatividade do peptídeo 18 (4µg/poço) frente a soros de cães que apresentam altos títulos de anticorpos (>1:640).

Figura 6 - Reatividade do peptídeo 18 (250 ng/poço) frente a soros de cães sintomáticos e assintomáticos.

Figura 7 - Reatividade do peptídeo 19 (4µg/poço) frente a soros de cães que apresentam altos títulos de anticorpos (>1:640).

Figura 8 - Reatividade do peptídeo 19 (250 ng/poço) frente a soros de cães sintomáticos e assintomáticos.

Figura 9 - Reatividade de combinações dos peptídeos 47 e 17 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 10 - Reatividade de combinações dos peptídeos 47 e 18 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 11 - Reatividade de combinações dos peptídeos 47 e 19 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 12 - Reatividade de combinações dos peptídeos 17 e 18 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 13 - Reatividade de combinações dos peptídeos 17 e 19 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 14 - Reatividade de combinações dos peptídeos 18 e 19 (500 ng/poço), frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos.

Figura 15 - Dados das reações de ELISA, utilizando-se soro humano, em que os peptídeos foram testados isoladamente na quantidade de 4µg/poço.

Figura 16 - Dados das reações de ELISA, utilizando-se soro humano, em que os peptídeos foram testados em associação.

Descrição Detalhada da Invenção

Na presente invenção foi feita uma combinação de peptídeos correspondentes a regiões com características ótimas para reatividade como epítopos para células B, selecionados a partir das sequências dos antígenos A2, NH e LACK de leishmania, empregando os critérios descritos na tabela 1. Tais peptídeos foram então testados, isoladamente ou em combinação, frente a soros de cães sintomáticos, assintomáticos e com baixos (< 1:320) e médios (1:320 > 1:640) títulos de anticorpos. Cabe ressaltar que foram empregados nessa análise os mesmos soros categorizados como provenientes de cães sintomáticos e assintomáticos testados previamente por Porrozzi et al. (2007), permitindo a comparação com os mesmos antígenos sob a forma de proteínas recombinantes. Os peptídeos foram testados também frente a soros humanos de pacientes diagnosticados com leishmaniose visceral e soros controle de indivíduos hígidos.

As sequências das proteínas A2 e LACK e NH foram submetidas a análise com programas disponíveis online no site http://www.expasy.org/tools/protscale.html. Os programas aos quais as sequencias foram submetidas geram gráficos e valores de *scores* que são considerados de acordo com as características ideais para a presença de epítopos de células B. A seleção das sequências sintetizadas como peptídeos com características de epítopos de células B obedeceu os seguintes critérios:

Tabela 1- Critérios utilizados para seleção das sequências sintetizadas

Característica	**Referência**	**Valores de Scores**
Regiões com estrutura Folha beta	de acordo com Chou & Fasman	deve ser baixo
Regiões com estrutura Alfa-hélice	/ Deleage & Roux	deve ser alto
Hidrofobicidade	/ Hopp & Woods	deve ser alta
Regiões com estrutura Alfa-hélice	Chou & Fasman	deve ser alto
Regiões com estrutura Beta-turn	Chou & Fasman	deve ser alto
Coil	Deleage & Roux	deve ser baixo
Porcentagem de resíduos acessíveis		deve ser alta

Com base nesses critérios, foram selecionadas as seguintes sequências: SVGPQSVGPLSVGPQSVGPLSC, correspondendo ao Peptídeo 47(SEQ ID N°1), derivado do antígeno A2, STPAVQKRVKEVGTKPC e STTVVGNQTLEKVTC, correspondendo ao Peptídeo 17 e 18 (SEQ ID N°2 e N°3), respectivamente, derivados do antígeno nucleosídeo hidrolase e SVVSTSRDGTAISWKC, correspondendo ao peptídeo 19(SEQ ID N°4), derivado do antígeno LACK.

A presente invenção será melhor descrita através dos exemplos a seguir. Ressalta-se que esses exemplos não possuem a intenção de limitar a natureza da invenção, mas, ao contrário, ilustram e ajudam na compreensão da tecnologia.

Ainda, o teste para imunodiagnóstico de leishmaniose pode ser selecionado do grupo compreedendo ELISA, Western blot, dot blot, imunodifusão e imunocromatografia. Nos exemplos que se seguem foi utilizado o teste de ELISA.

Exemplo 1

Para testar a reatividade do peptídeo 47 como antígeno em testes de diagnóstico da leishmaniose visceral canina, o mesmo foi utilizado para sensibilizar placas de ELISA, de acordo com o protocolo descrito a seguir.

Placas de 96 poços (Flexible PVC Microplates, flat-bottom, BD Biosciences) foram sensibilizadas com o peptídeo sintético 47, derivado do antígeno A2, diluído em água. Foram testadas variações nas quantidades de peptídeo por poço, dependendo do ensaio (4 ou 1µg/poço ou ainda 0,5 ou 0,250 µg/poço). As placas foram mantidas em estufa até a secagem e depois na geladeira por 18 horas a 4 °C. As placas foram bloqueadas com PBS-2% caseína a 37 °C por 1 hora e incubadas com soros de cães na diluição de 1:100 por 1 hora a 37 °C. Anticorpos marcados com Peroxidase específicos para IgG canina (Sigma, USA) foram diluídos 1:5000 e adicionados aos poços por 1 hora a 37 °C. Após a lavagem das placas, foi adicionado o substrato cromogênico 3,3',5,5'-tetramethylbenzidine (TMB) em tampão citrato contendo peróxido de hidrogênio. As reações foram interrompidas pela adição de H_2SO_4 2N. A densidade ótica foi determinada a 450 nm em leitor de ELISA (BioRad, Model 2550, CA, USA).

Através da figura 1 pode-se observar que o peptídeo foi capaz de detectar 100% dos cães com altos títulos de anticorpos (>1:640), apresentando a alta sensibilidade e especificidade do peptídeo 47 frente soros de cães com altos títulos de anticorpos.

Exemplo 2

Observada a elevada reatividade do peptídeo 47 (4 µg/poço) com soros de cães com títulos altos de anticorpos (> 1:640), verificou-se também a possibilidade do mesmo reagir com soros de cães sintomáticos e

assintomáticos, mas empregando-se uma menor quantidade de antígeno (250 ng/poço). Ressalta-se que foram empregados os mesmos soros utilizados por Porrozzi et. al. (2007).

Na figura 2 observa-se que o peptídeo 47 foi capaz de detectar de forma comparável soros de cães sintomáticos e assintomáticos, representando, portanto, uma otimização da sensibilidade apresentada quando a proteína A2 recombinante foi testada frente aos mesmos soros, como descrito por Porrozzi et. al. (2007).

Exemplo 3

Para testar a reatividade do peptídeo 17, derivado do antígeno nucleosídeo hidrolase (NH), como antígeno em testes de diagnóstico da leishmaniose visceral canina, o mesmo foi utilizado para sensibilizar placas de ELISA, na quantidade de 4µg/poço, de acordo com o protocolo de reações de ELISA descrito no exemplo 1. Inicialmente o peptídeo 17 foi utilizado na quantidade de 4µg/poço frente a soros de cães com títulos altos (>1:640) de anticorpos.

Na figura 3 observa-se que, nas condições testadas, o peptídeo foi capaz de detectar 80% dos cães com altos títulos de anticorpos, apresentando alta sensibilidade do peptídeo 17 com soros de cães com altos títulos de anticorpos.

Exemplo 4

Tendo sido observada elevada reatividade com soros de cães com títulos altos de anticorpos (> 1:640) empregando o peptídeo 17, utilizado como descrito no exemplo 1 para sensibilizar placas de ELISA na quantidade de 4µg/poço, verificou-se a possibilidade do mesmo reagir com soros de cães sintomáticos e assintomáticos, mas empregando uma menor quantidade de antígeno (250 ng/poço). Ressalta-se que foram empregados os mesmos soros utilizados por Porrozzi et. al. (2007).

Na figura 4 observa-se que, nas condições testadas, o peptídeo 17 foi capaz de detectar de forma comparável soros de cães sintomáticos e

assintomáticos, representando, portanto, uma otimização da sensibilidade por outros testes de diagnóstico que com frequência não detectam com a mesma sensibilidade a LV em cães sintomáticos e assintomáticos.

Exemplo 5

Para testar a reatividade do peptideo 18, derivado do antígeno nucleosídeo hidrolase (NH), como antígeno em testes de diagnóstico da leishmaniose visceral canina, o mesmo foi utilizado para sensibilizar placas de ELISA, na quantidade de 4µg/poço, de acordo com o protocolo de reações de ELISA descrito no exemplo 1. Inicialmente o peptídeo 18 foi utilizado na quantidade de 4µg/poço frente a soros de cães com títulos altos (>1:640) de anticorpos

Na figura 5 observa-se que, nas condições testadas, o peptídeo 18 foi capaz de detectar 100% dos cães com altos títulos de anticorpos, apresentando alta sensibilidade do peptídeo 18 com soros de cães com altos títulos de anticorpos.

Exemplo 6

Tendo sido observada elevada reatividade com soros de cães com títulos altos de anticorpos (> 1:640) empregando-se o peptídeo 18, utilizado como descrito no exemplo 1 para sensibilizar placas de ELISA na quantidade de 4µg/poço, verificou-se a possibilidade do mesmo reagir com soros de cães sintomáticos e assintomáticos, mas empregando-se uma menor quantidade de antígeno (250 ng/poço). Ressalta-se que foram empregados os mesmos soros utilizados por Porrozzi et. al. (2007).

Na figura 6 observa-se que, nas condições testadas, o peptídeo 18 foi capaz de detectar de forma comparável soros de cães sintomáticos e assintomáticos, representando, portanto, uma otimização da sensibilidade apresentada por outros testes de diagnóstico que, com frequência, não detectam com a mesma sensibilidade a LV em cães sintomáticos e assintomáticos.

Exemplo 7

Para testar a reatividade do peptídeo 19, derivado do antígeno LACK, como antígeno em testes de diagnóstico da leishmaniose visceral canina, o mesmo foi utilizado para sensibilizar placas de ELISA, na quantidade de 4µg/poço, de acordo com o protocolo de reações de ELISA descrito, no exemplo 1. Inicialmente o peptídeo 18 foi utilizado na quantidade de 4µg/poço frente a soros de cães com títulos altos (>1:640) de anticorpos.

Na figura 7 observa-se que, nas condições testadas, o peptídeo foi capaz de detectar 80% dos cães com altos títulos de anticorpos, apresentando alta sensibilidade do peptídeo 19 com soros de cães com altos títulos de anticorpos.

Exemplo 8

Tendo sido observada elevada reatividade com soros de cães com títulos altos de anticorpos (> 1:640) empregando-se o peptídeo 19, utilizado como descrito no exemplo 1 para sensibilizar placas de ELISA na quantidade de 4µg/poço, verificou-se a possibilidade do mesmo reagir com soros de cães sintomáticos e assintomáticos, mas empregando-se uma menor quantidade de antígeno (250 ng/poço). Ressalta-se que foram empregados os mesmos soros utilizados por Porrozzi et al. (2007).

Na figura 8 observa-se que, nas condições testadas, o peptídeo 19 foi capaz de detectar de forma comparável soros de cães sintomáticos e assintomáticos, representando, portanto, uma otimização da sensibilidade por outros testes de diagnóstico que com frequência não detectam com a mesma sensibilidade a LV em cães sintomáticos e assintomáticos.

Exemplo 9

Considerando que a detecção da infecção em cães com baixos títulos de anticorpos também é uma limitação de testes de diagnóstico que empregam os antígenos K39 e K26, como demonstrado por Porrozzi et al. (2007), os peptídeos 47 (derivado de A2), 17 e 18 (derivados de NH) e 19 (derivados de LACK) foram testados isoladamente ou em associação, com o intuito de

aumentar a sensibilidade para soros de cães com títulos de anticorpos baixos ou médios. Esses soros são provenientes de animais com exame parasitológico positivo, mas com títulos de anticorpos baixos ou médios, como detectado na reação de imunofluorescência ou em ELISA com extrato antigênico de formas promastigotas do parasita, testes considerados convencionais para o diagnóstico da leishmaniose visceral canina. Ressalta-se mais uma vez que, nessa faixa de detecção, os testes sorológicos empregando antígenos definidos como o K39 ou K26 têm limitações de sensibilidade.

Nas figuras 9, 10, 11, 12, 13 e 14 comparam-se os resultados dos testes realizados com os peptídeos 47, 17, 18 e 19 isoladamente ou associados 2 a 2. Cabe mencionar que associações desses peptídeos 3 a 3 não resultaram em dados expressivos, uma vez que quando associados 2 a 2 o limite máximo de sensibilidade do teste foi atingido.

A figura 9 compara a reatividade de combinações dos peptídeos 47 e 17 (500 ng/poço) frente a soros de cães com títulos baixos (< 1:320) e médios (>1:320<1:640) de anticorpos. Esses soros são provenientes de animais com exame parasitológico positivo, mas com títulos de anticorpos baixos ou médios, como detectado na reação de imunofluorescência ou em ELISA com extrato antigênico de formas promastigotas do parasita, testes considerados convencionais para o diagnóstico da leishmaniose visceral canina. Ressalta-se mais uma vez que, nessa faixa de detecção, os testes sorológicos empregando antígenos definidos como o K39 ou K26 têm limitações de sensibilidade. Observa-se que, nessas condições, a associação do peptídeo 47 com o peptídeo 17 foi capaz de aumentar a sensibilidade do teste, resultando, portanto, na otimização da sensibilidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Na figura 10 observa-se que a associação do peptídeo 47 com o peptídeo 18 foi capaz de aumentar a sensibilidade do teste, resultando, portanto, na otimização da sensibilidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Na figura 11 observa-se que, a associação do peptídeo 47 com o peptídeo 19 foi capaz de aumentar a sensibilidade do teste, resultando,

portanto, na otimização da sensibilidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Na figura 12 observa-se que, a associação do peptídeo 17 com o peptídeo 18 foi capaz de aumentar a sensibilidade do teste, resultando, portanto, na otimização da sensiblidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Na figura 13 observa-se que, a associação do peptídeo 17 com o peptídeo 19 foi capaz de aumentar a sensibilidade do teste, resultando, portanto, na otimização da sensiblidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Na figura 14 observa-se que, a associação do peptídeo 18 com o peptídeo 19 foi capaz de aumentar a sensibilidade do teste, resultando, portanto, na otimização da sensiblidade apresentada pelo uso dos peptídeos individualmente. Resultados similares foram obtidos com 250 ng/poço.

Exemplo 10

Os peptídeos 47, 17, 18 e 19 foram empregados também em reações de ELISA com soros humanos de pacientes com diagnóstico de leishmaniose visceral, tendo como controles soros de indivíduos hígidos. Na figura 15, são apresentados os dados das reações de ELISA em que os peptídeos foram testados isoladamente na quantidade de 4µg/poço. Isoladamente, os peptídeos 17, 18, 19 e 47 apresentaram sensibilidade de 86,6%, 96,6%, 100% e 90%, respectivamente. Esses valores são próximos ou superiores à sensibilidade relatada para testes de diagnóstico da LV humana, tais como as técnicas RIFI (imunofluorescência indireta) (88%), ELISA *L. (L.) chagasi* 92%, empregando antígenos de formas promastigotas do parasita ou a ELISA empregando o antígeno recombinante rK39 (97%) ou para o teste imunocromatográfico rápido IT-LEISH® (DiaMed IT-LEISH®) (93%) que também emprega o K39 (Machado de Assis et. al., 2008)

Na figura 16, são apresentados os dados das reações de ELISA em que os peptídeos foram testados em associação. Observa-se aumento de sensibilidade quando o peptídeo 19 foi associado aos peptídeos 47 e 18,

alcançando um valor de 100%. Embora o valor de 100% de sensibilidade tenha sido alcançado nos testes em que o peptídeo 19 foi avaliado isoladamente, a sua associação melhorou a sensibilidade do testes empregando os peptídeos 47 e 18, os quais, isoladamente, apresentaram 90 e 96,6% de sensibilidade. Para as outras associações não foram observadas alterações significativas. Considerando a heterogeneidade da condição clínica dos pacientes e da resposta imune humoral dos mesmos, a associação de peptídeos pode ser uma alternativa para o aprimoramento de testes de diagnóstico, uma vez que epítopos adicionais são acrescentados aos testes sorológicos. Esses resultados demonstram a capacidade dos peptídeos testados de otimizar a sensibilidade dos testes para detecção de leishmaniose visceral humana.

REIVINDICAÇÕES

1- **Peptídeos recombinantes de *Leishmania*** caracterizados por consistir das sequências SEQ ID N^{os} 1, 2, 3 e 4.

2- **Peptídeos recombinantes de *Leishmania*,** de acordo com a reivindicação 1, caracterizados por poderem ser modificados em suas extremidades.

3- **Método para teste imunodiagnóstico de Leishmaniose** caracterizado por compreender:

a) ligação de anticorpos leishmaniais de uma amostra a um ou mais polipeptídios, ligados a um suporte sólido ou um carreador, consistindo das sequências de aminoácidos SEQ ID N^{os} 1, 2, 3 e 4 ou consistindo das proteínas recombinantes definidas associadas rA2, rNH e rLACK, SEQ ID N^{os} 5, 6 e 7 respectivamente.

b) contactar os anticorpos do passo (a) com um anticorpo secundário ou uma proteína, conjugados a uma enzima ou a um marcador e que se ligam aos anticorpos do passo (a)

c) detectar os anticorpos antileshmaniais na amostra supracitada pela detecção do anticorpo secundário ou proteína especificamente ligados ao dito anticorpo anti-leishmanial.

4- **Método para teste imunodiagnóstico de Leishmaniose,** de acordo com a reivindicação 3, caracterizado pelos polipeptídeos SEQ ID N^{os} 1, 2, 3 e 4 poderem ser modificados em suas extremidades.

5- **Método para teste Imunodiagnóstico de Leishmaniose**, de acordo com a reivindicação 3, caracterizado pelas amostras serem selecionadas do grupo consistindo de sangue, soro, plasma e outro fluído corporal.

6- **Método para teste imunodiagnóstico de Leishmaniose**, de acordo com a reivindicação 3, caracterizado pelo suporte sólido ser selecionado de um grupo de materiais consistindo de nitrocelulose, nylon, látex, polipropileno e poliestireno.

7- Método para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pelo carreador ser uma partícula de ouro.

8- Método para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pelo anticorpo secundário ser selecionado de um grupo consistindo de IgG, IgM, IgA, IgE e subclasses deles.

9- Método para teste imunodiagnostico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pela proteína ser selecionada de um grupo consistindo de Proteína A e Proteína G.

10- Método para teste Imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pela enzima ser selecionada do grupo consistindo de fosfatase alcalina, peroxidase, β-galactosidase, urease, xantina oxidase, glicose oxidase e penicilinase.

11- Método para teste Imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pelo marcador ser selecionado do grupo consistindo de enzimas, radioisótopos, biotina, cromóforos, fluoróforos e quimioluminescentes.

12- Método para teste Imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 3, caracterizado pelo passo de detecção do anticorpo anti leshmanial ser selecionado do grupo consistindo de detecção de fluorescência, de imunoluminescência, de absorbância ou de radioisótopos.

13- Kit para teste imunodiagnóstico de Leishmaniose, caracterizado por compreender:

- os peptídeos consistindo das sequências SEQ ID N^{os} 1, 2, 3 e 4 associados ou isolados ou as proteínas recombinantes definidas rA2, rNH e rLACK associadas representadas pelas SEQ ID N^{os} 5, 6 e 7 respectivamente;

- Um anticorpo secundário ou uma proteína, onde o anticorpo secundário ou a proteína está conjugado a um enzima ou marcador, e um reagente para detectar a dita enzima ou marcador.

14- Kit para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 12, caracterizado pelas proteínas recombinantes ou os peptídeos estarem ligados a uma suporte sólido ou carreador.

15- Kit para teste imunodiagnóstico de Leishmaniose,de acordo com a reivindicação 13, caracterizado pelo suporte sólido ser selecionado do grupo de material consistindo de nitrocelulose, nylon, látex, polipropileno e polistireno.

16- Kit para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 13, caracterizado pelo carreador consistir de partículas de ouro.

17- Kit para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 13, caracterizado pelo anticorpo secundário ser selecionado do grupo consistindo de IgG, IgM, IgA, IgE e subclasses deles.

18- Kit para teste imunodiagnóstico de Leishmaniose, de acordo com a reivindicação 13, caracterizado pela proteína ser selecionada do grupo consistindo de proteína A e proteína G.

19- Kit para teste imunodiagnóstico de Leishmaniose,de acordo com a reivindicação 13, caracterizado pelo dito marcador ser selecionado do grupo consistindo de enzimas, redioisótopos, biotina, cromóforos, fluoróforos e quimioluminescentes.

20- Kit para teste imunodiagnóstico de Leishmaniose,de acordo com a reivindicação 13, caracterizado pela enzima ser selecionada do grupo compreendendo fosfatase alcalina, peroxidase, β-galactosidase, uréase, xantina oxidase, glicose oxidase e penicilinase.

FIGURAS

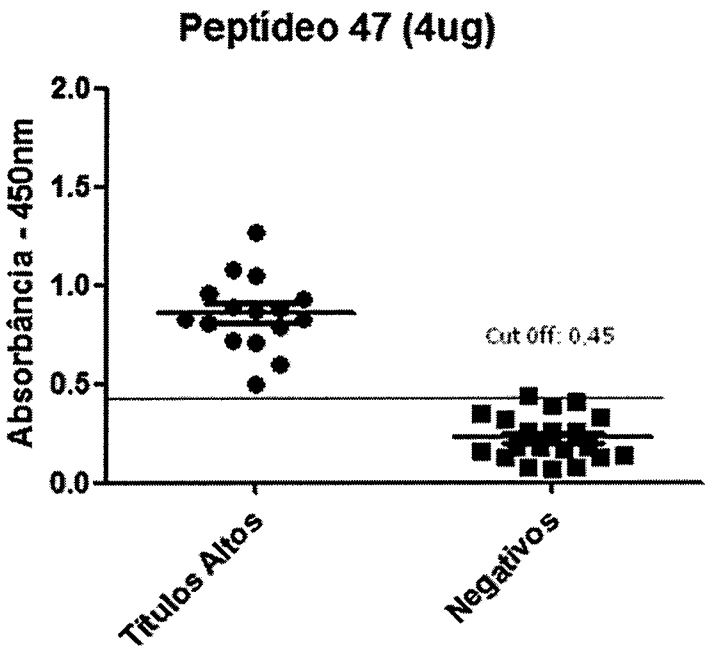


Figura 1

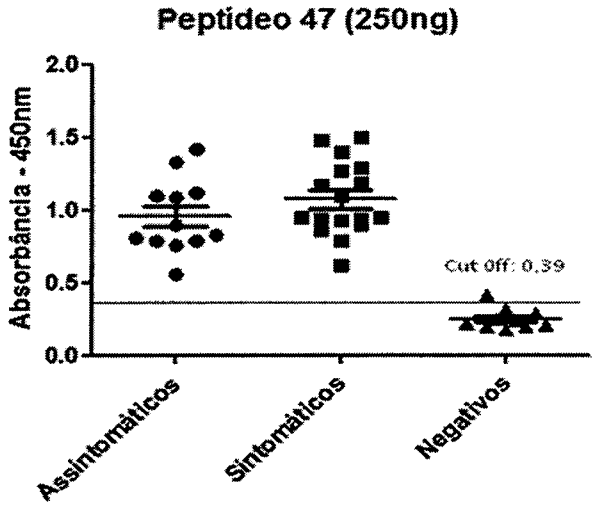


Figura 2

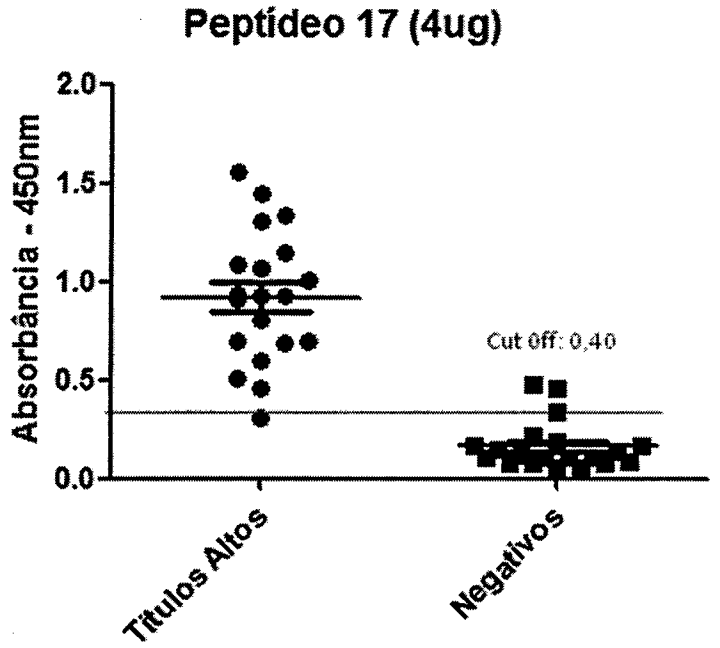


Figura 3

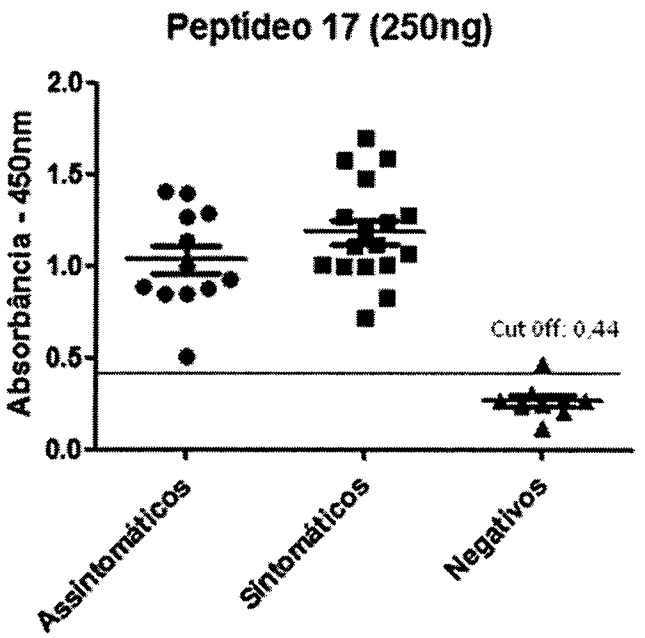


Figura 4

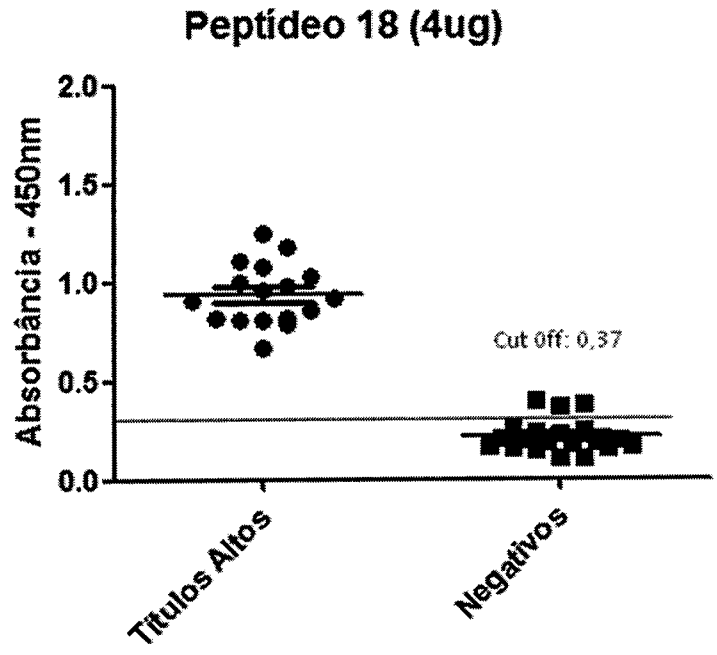


Figura 5

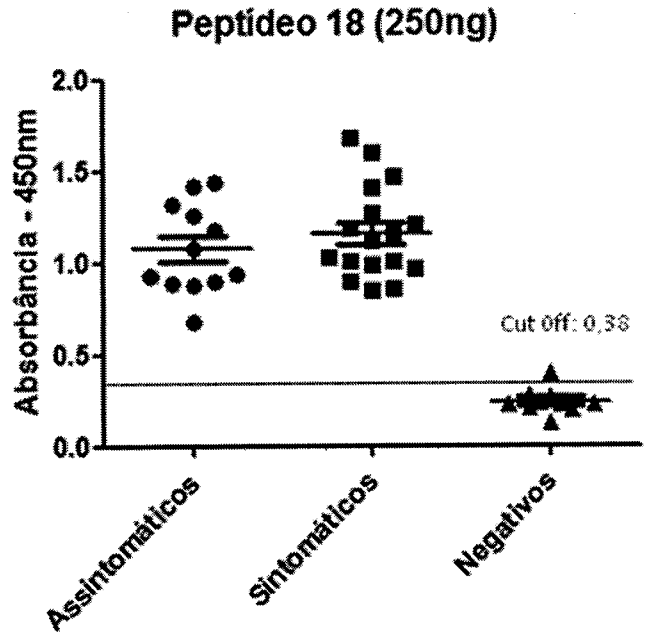


Figura 6

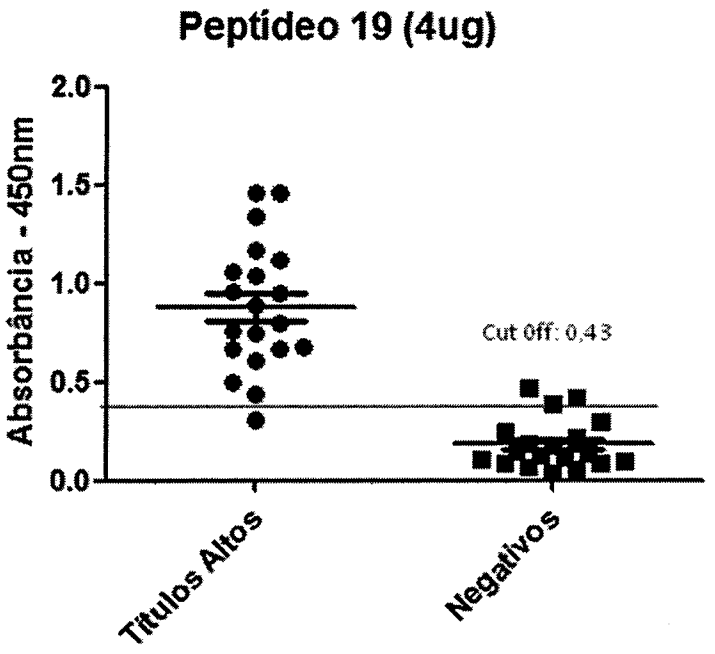


Figura 7

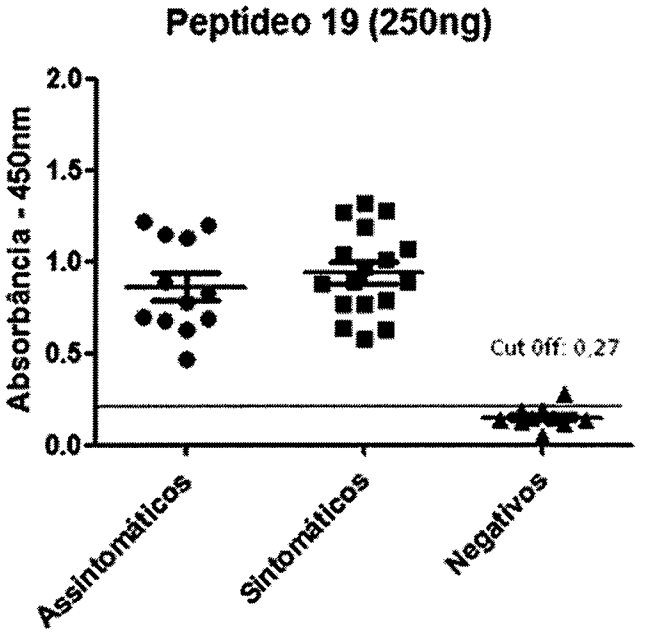


Figura 8

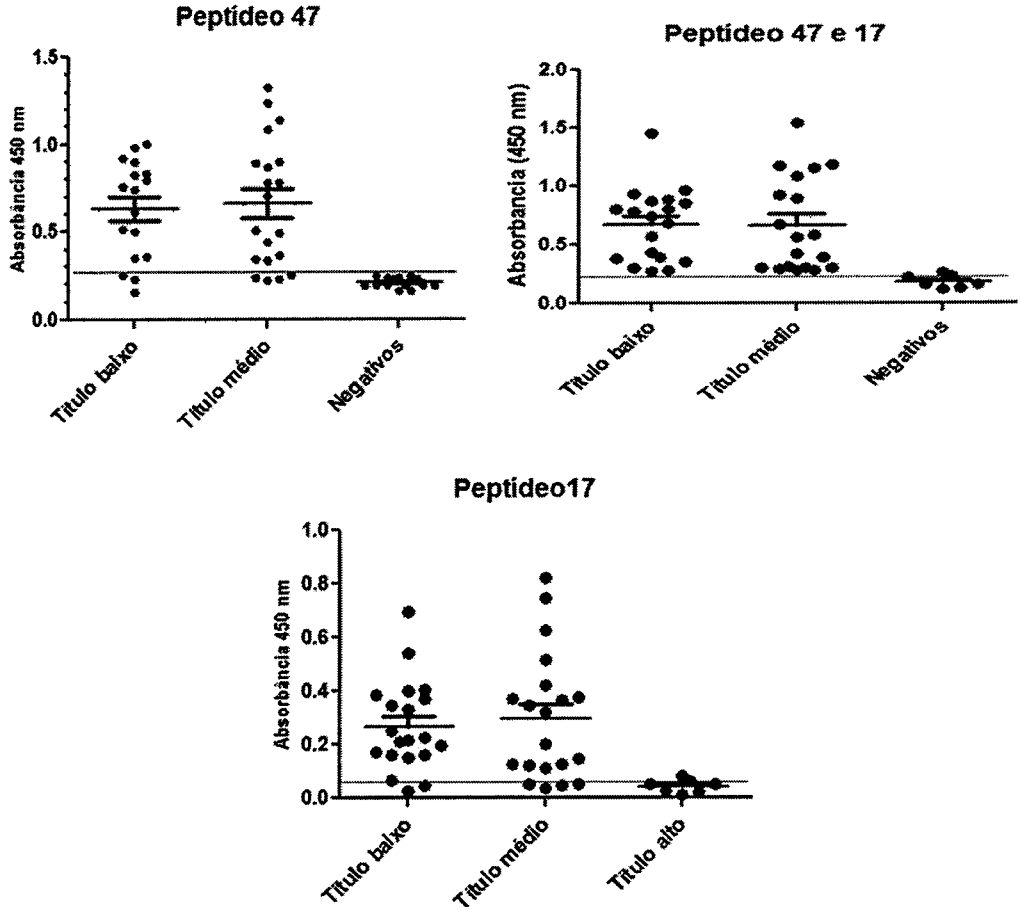

Peptídeo 47
Absorbância 450 nm
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptídeo 47 e 17
Absorbancia (450 nm)
2.0
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptídeo17
Absorbância 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
Título baixo
Título médio
Título alto

Figura 9

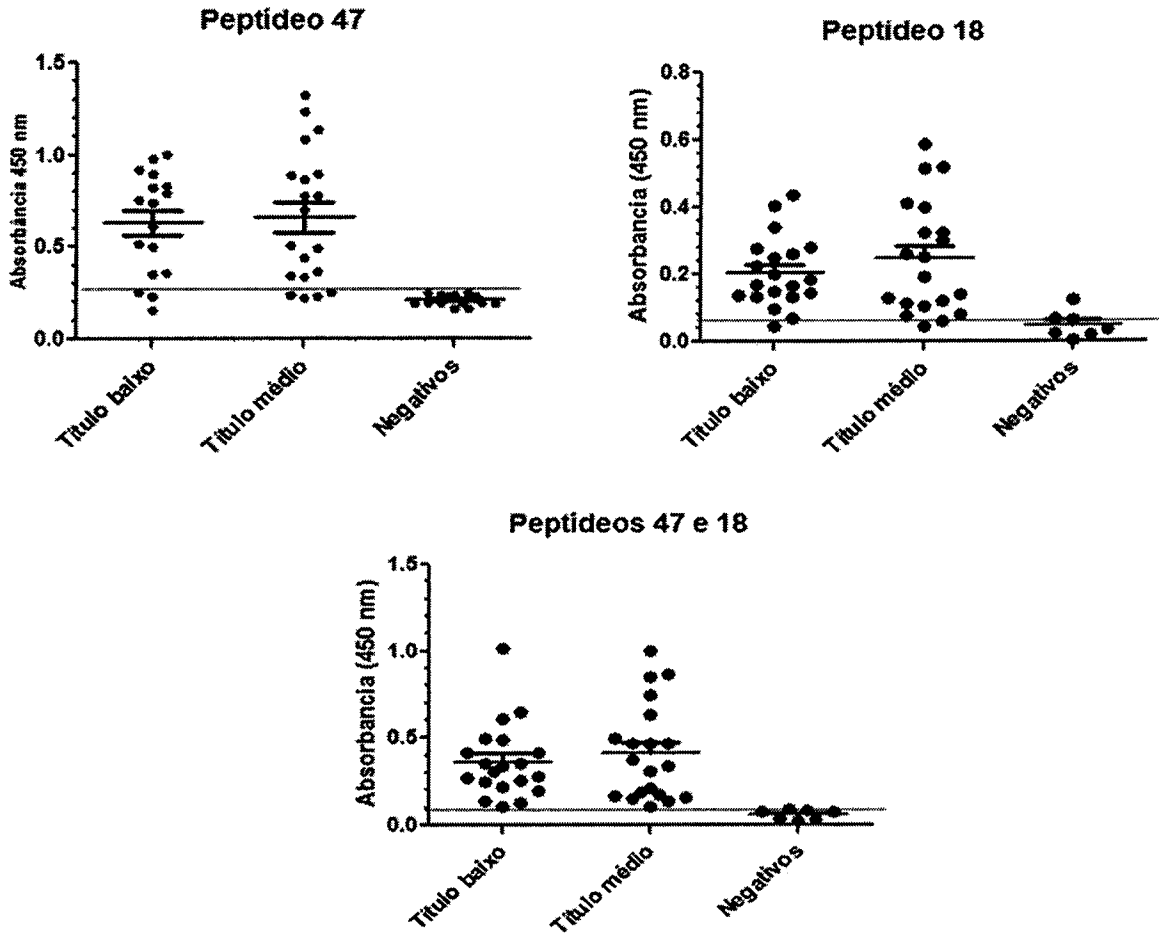

Peptideo 47
Absorbância 450 nm
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptideo 18
Absorbancia (450 nm)
0.8
0.6
0.4
0.2
0.0
Título baixo
Título médio
Negativos
Peptideos 47 e 18
Absorbancia (450 nm)
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos

Figura 10

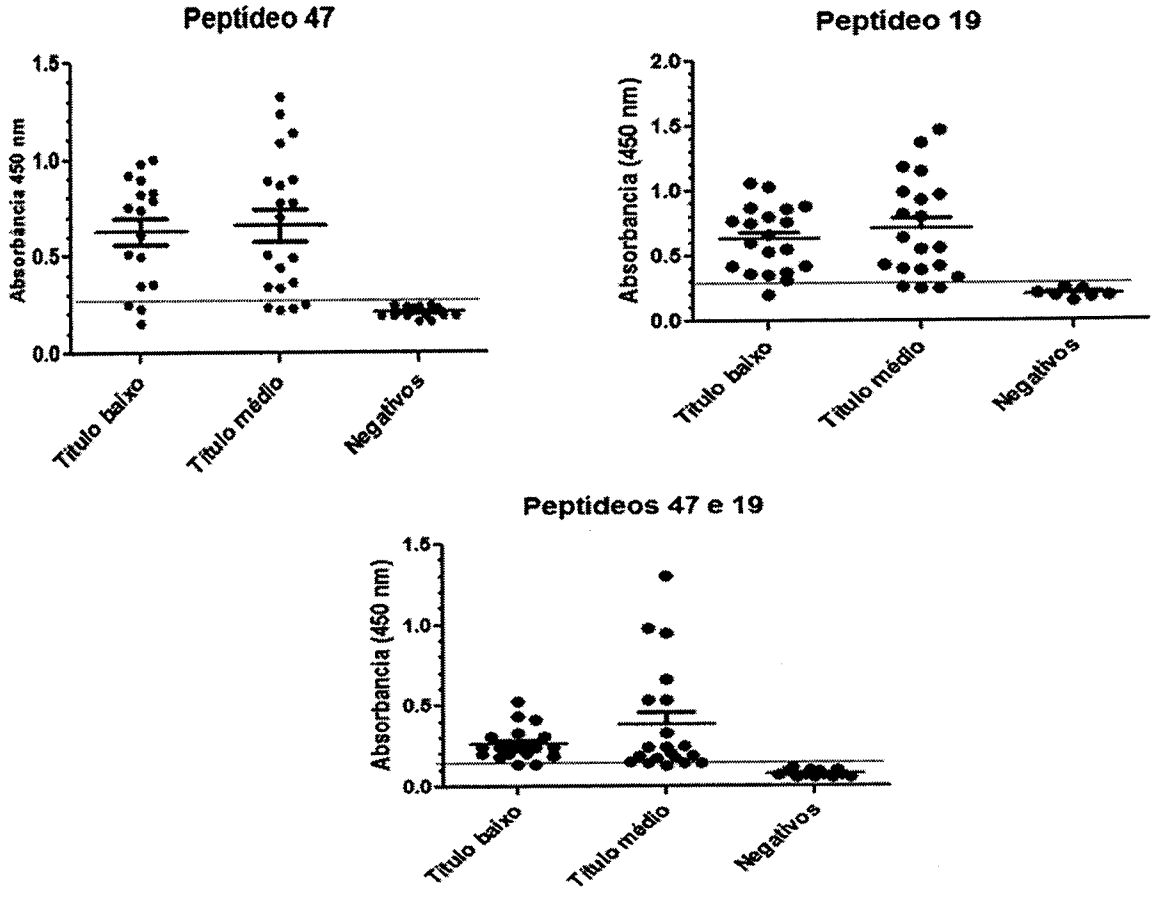

Peptídeo 47
Absorbância 450 nm
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptídeo 19
Absorbancia (450 nm)
2.0
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptídeos 47 e 19
Absorbancia (450 nm)
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos

Figura 11

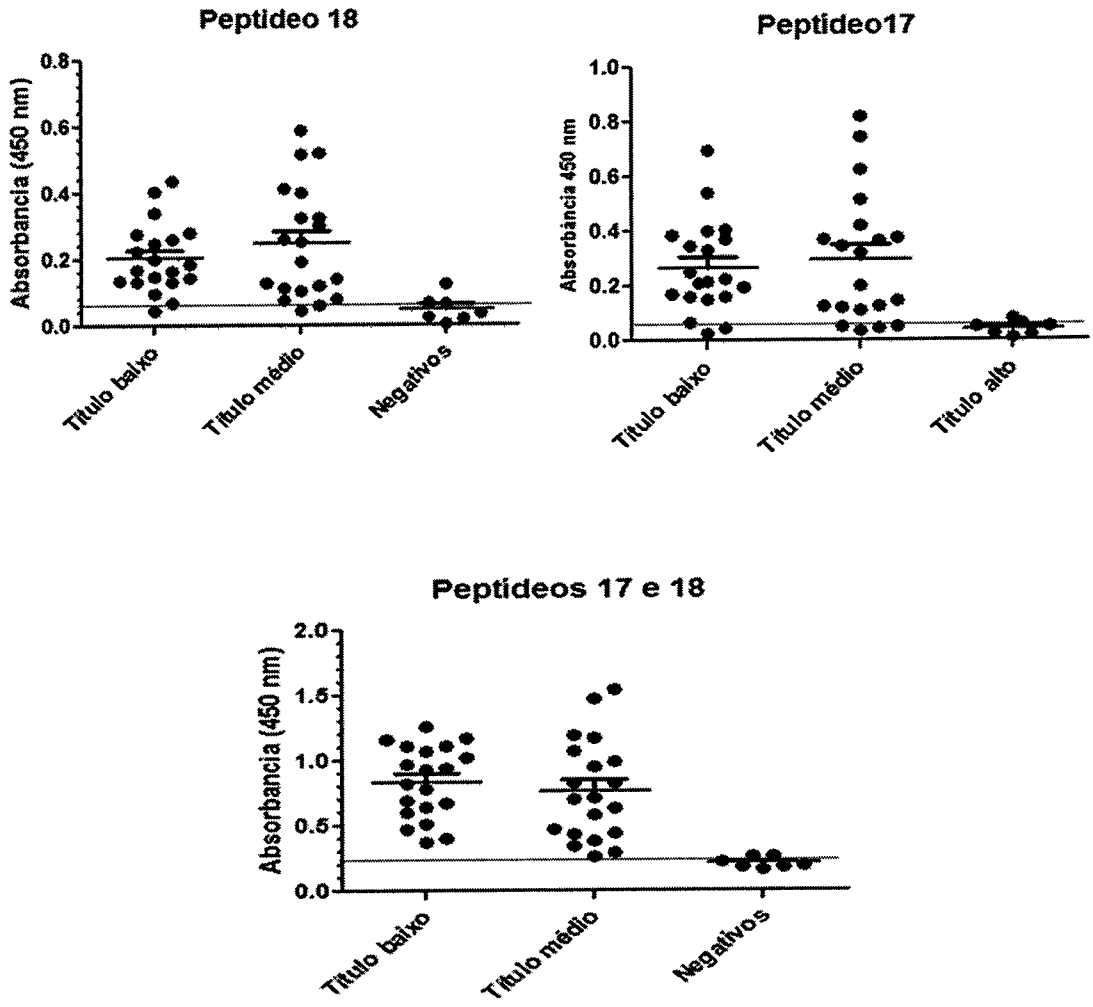

Peptideo 18
Absorbancia (450 nm)
0.0
0.2
0.4
0.6
0.8
Título baixo
Título médio
Negativos
Peptideo17
Absorbância 450 nm
0.0
0.2
0.4
0.6
0.8
1.0
Título baixo
Título médio
Título alto
Peptideos 17 e 18
Absorbancia (450 nm)
0.0
0.5
1.0
1.5
2.0
Título baixo
Título médio
Negativos

Figura 12

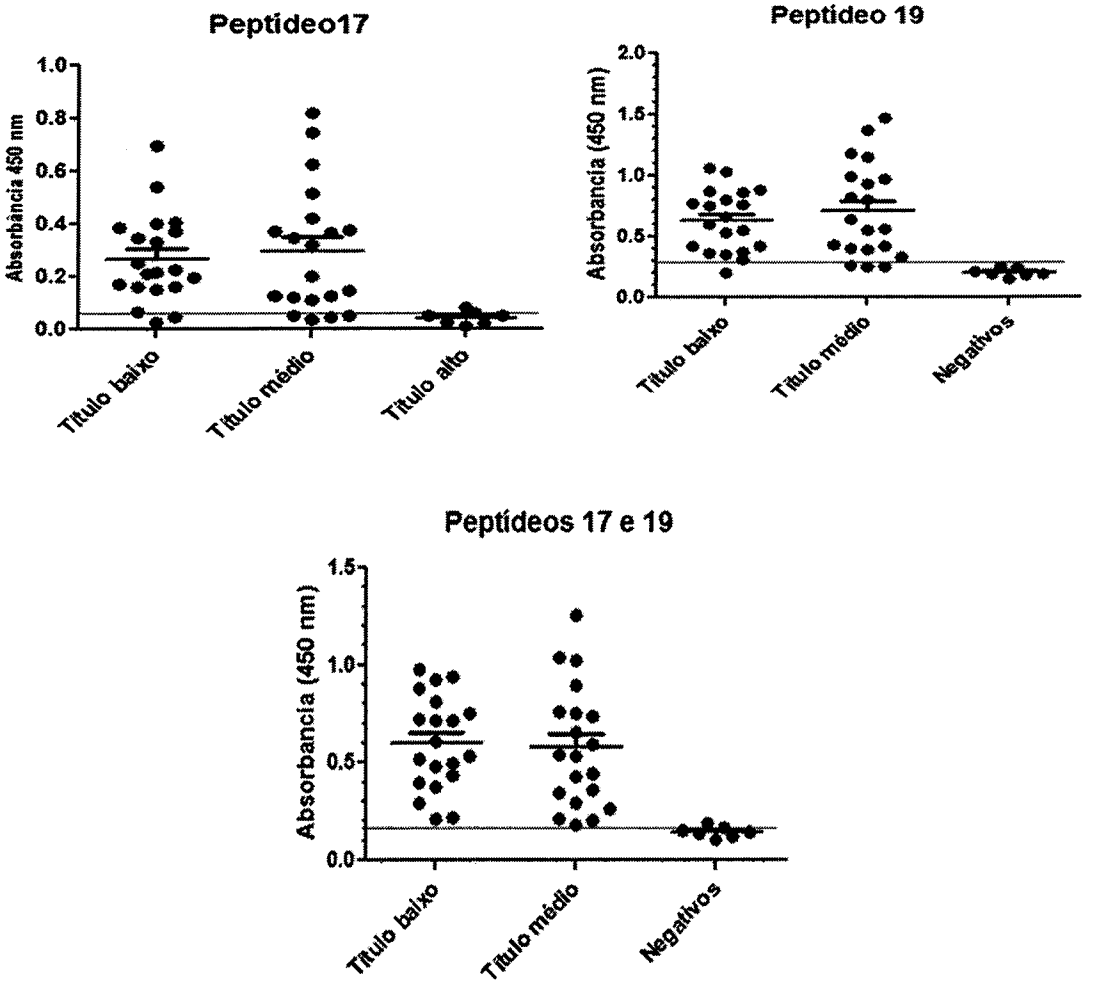
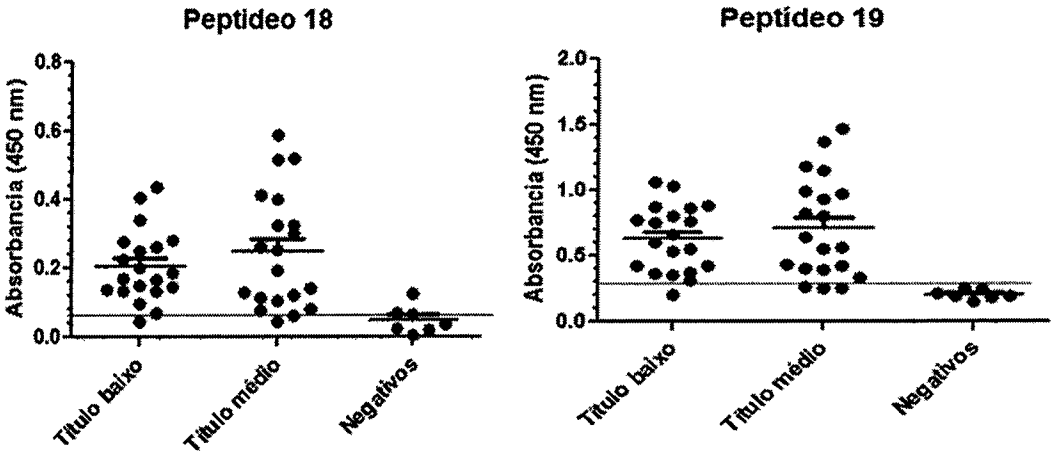

Peptideo17
Absorbância 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
Título baixo
Título médio
Título alto
Peptideo 19
Absorbancia (450 nm)
2.0
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos
Peptídeos 17 e 19
Absorbancia (450 nm)
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos

Figura 13


Peptideo 18
Absorbancia (450 nm)
0.8
0.6
0.4
0.2
0.0
Título baixo
Título médio
Negativos
Peptídeo 19
Absorbancia (450 nm)
2.0
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos

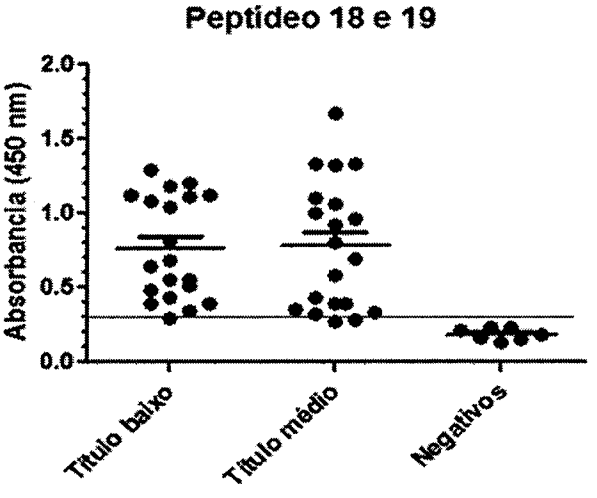

Peptídeo 18 e 19
Absorbancia (450 nm)
2.0
1.5
1.0
0.5
0.0
Título baixo
Título médio
Negativos

Figura 14

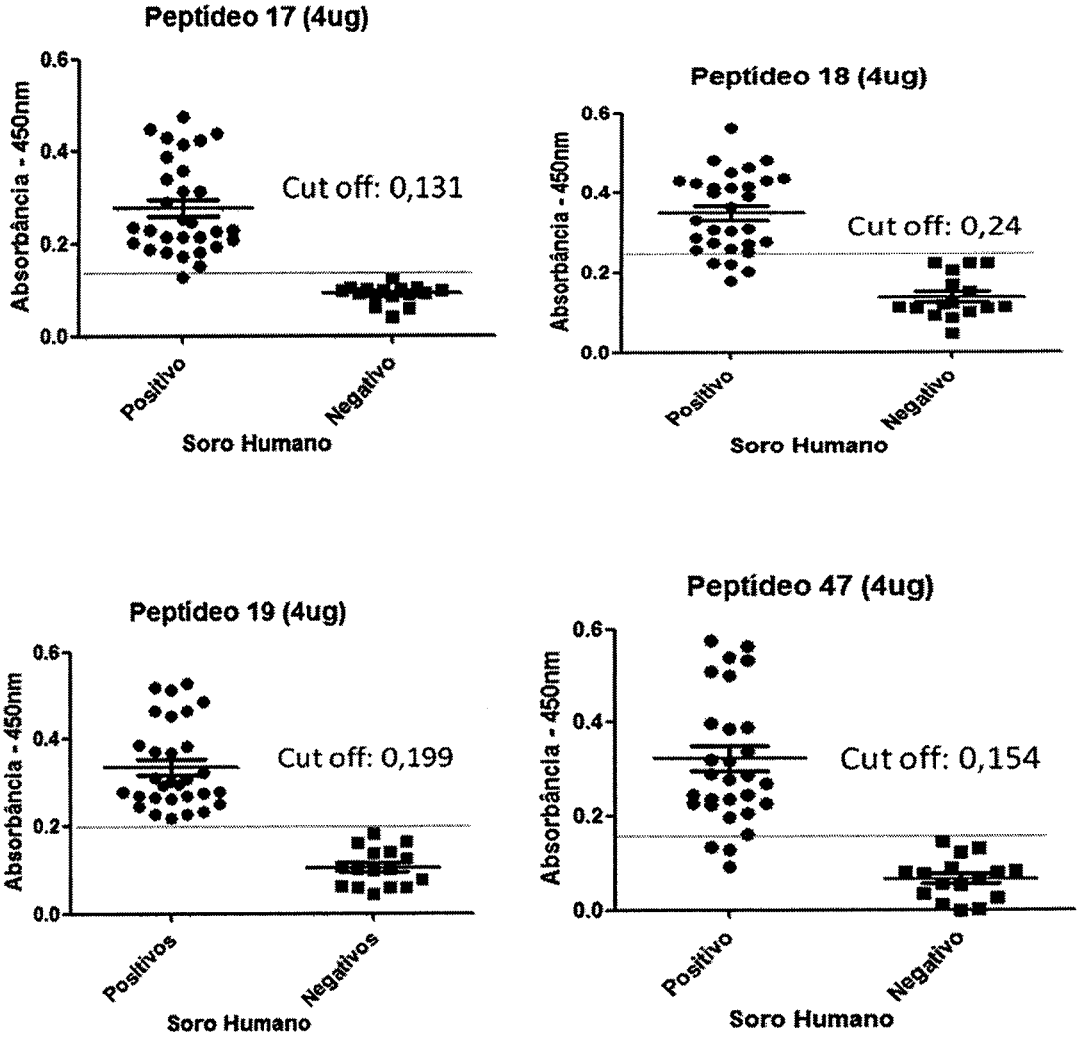

Peptídeo 17 (4ug)
Absorbância - 450nm
0.6
0.4
0.2
0.0
Cut off: 0,131
Positivo
Negativo
Soro Humano
Peptídeo 18 (4ug)
Absorbância - 450nm
0.6
0.4
0.2
0.0
Cut off: 0,24
Positivo
Negativo
Soro Humano
Peptídeo 19 (4ug)
Absorbância - 450nm
0.6
0.4
0.2
0.0
Cut off: 0,199
Positivos
Negativos
Soro Humano
Peptídeo 47 (4ug)
Absorbância - 450nm
0.6
0.4
0.2
0.0
Cut off: 0,154
Positivo
Negativo
Soro Humano

Figura 15

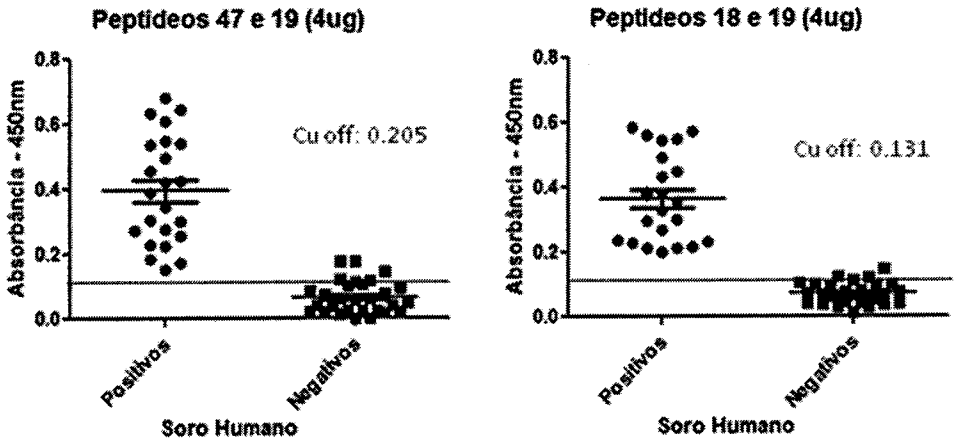

Peptideos 47 e 19 (4ug)
Absorbância - 450nm
0.8
0.6
0.4
0.2
0.0
Cu off: 0.205
Positivos
Negativos
Soro Humano
Peptideos 18 e 19 (4ug)
Absorbância - 450nm
0.8
0.6
0.4
0.2
0.0
Cu off: 0.131
Positivos
Negativos
Soro Humano

Figura 16

Resumo

Peptídeos Recombinantes, Método e Kit para Teste Imunodiagnóstico de Leishmaniose Visceral

A presente invenção descreve um método e kit para diagnosticar Leishmaniose visceral. Mais particularmente, a invenção trata da associação dos antígenos recombinantes definidos rA2, rNH e rLACK ou seus epítopos, peptídeos 47, 17, 18 e 19, permitindo o desenvolvimento de um método diagnóstico com maior especificidade, otimizando, assim, a reatividade dos testes sorológicos.

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PLoS NEGLECTED TROPICAL DISEASES

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

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Abstract

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

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

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

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Introduction

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

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

Author Summary

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

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

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

Methods

Ethics Statement

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

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

Mapping B-cell Epitopes

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

Synthetic peptides

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

Dog sera and infection status

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

Human sera

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

Enzyme-linked immunosorbent assays ELISA for canine sera

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

Enzyme-linked immunosorbent assays ELISA for human sera

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

Statistical analysis

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

Results

Bioinformatics analysis

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

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

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

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

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

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

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

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

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

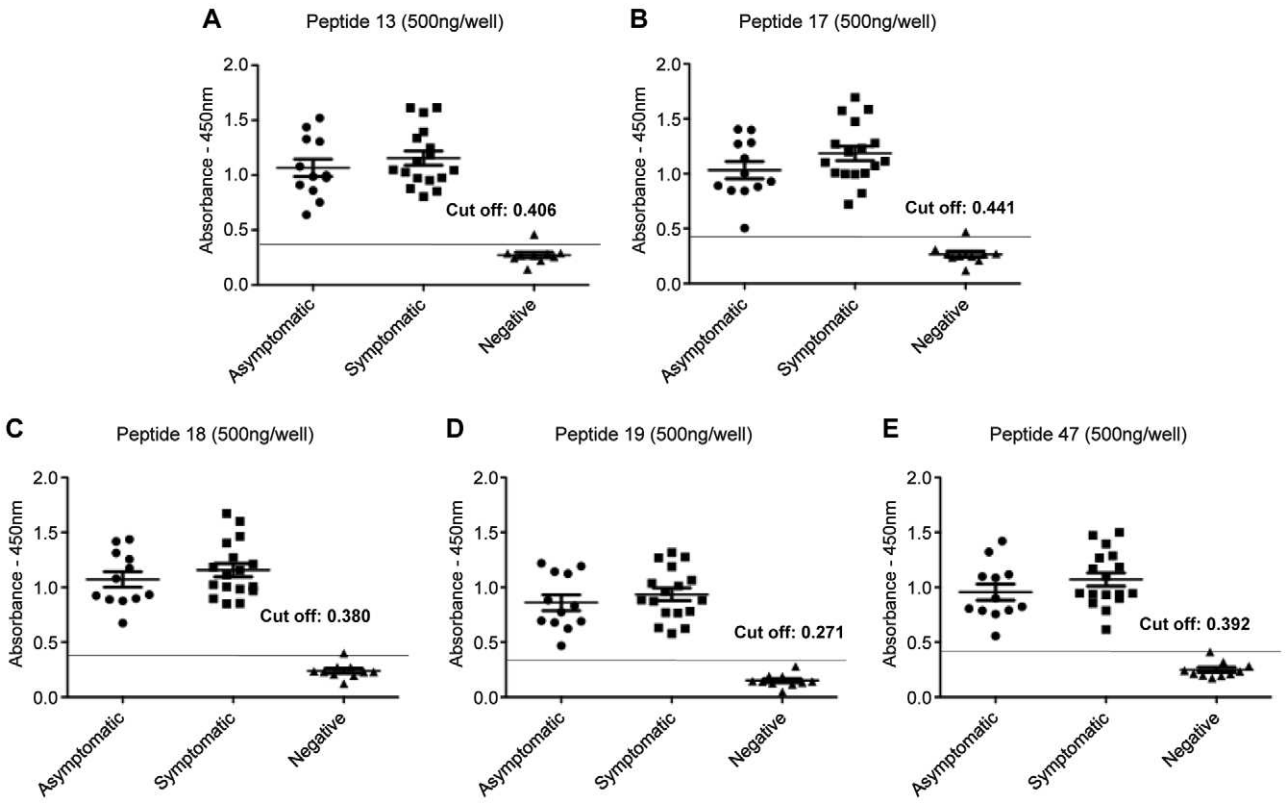

Figure 1. ELISA with individual synthetic peptides for detection of IgG-antibodies in dogs with *L. infantum* infection. Detection of anti-leishmanial total IgG antibodies with synthetic peptides by ELISA assay using sera from asymptomatic ($n = 13$) and symptomatic ($n = 17$) dogs and control group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

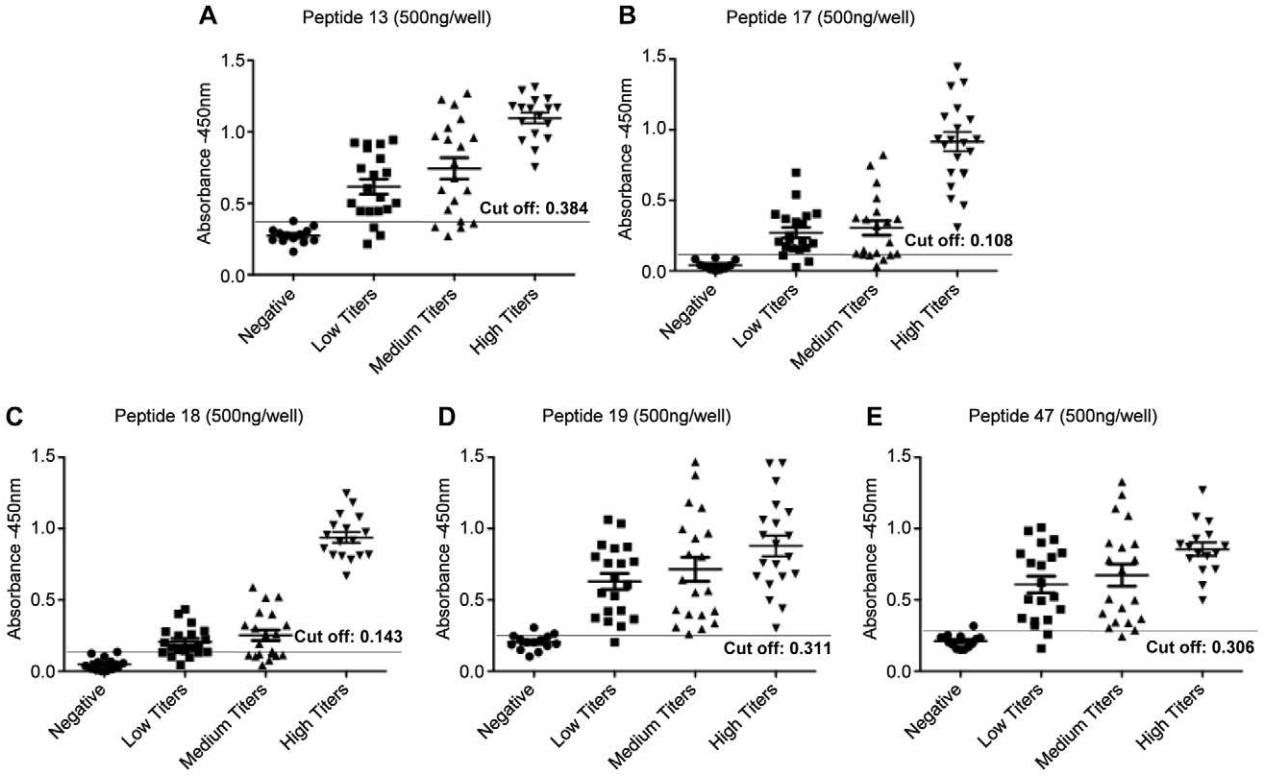

Figure 2. Reactivity of sera from infected dogs displaying different IFAT antibody titers with peptides. Detection of anti-leishmanial total IgG antibodies with synthetic peptides by ELISA assay using canine samples classified according to IFAT reactivity as low (<1:320 dilutions), intermediate (>1:320 <1:640) and high (>1:640) titers and 500 nanograms/per well of peptides 13 (panel **A**), 17 (panel **B**), 18 (panel **C**), 19 (panel **D**) and 47 (panel **E**).
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and 19, 18 and 19, 47 and 18, varying from 80% to 95%. The sensitivity of peptide 47 (85%) was not affected when combined with peptide 13 and decreased when associated to peptide 17 (Figure 3/Table 2).

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

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

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

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

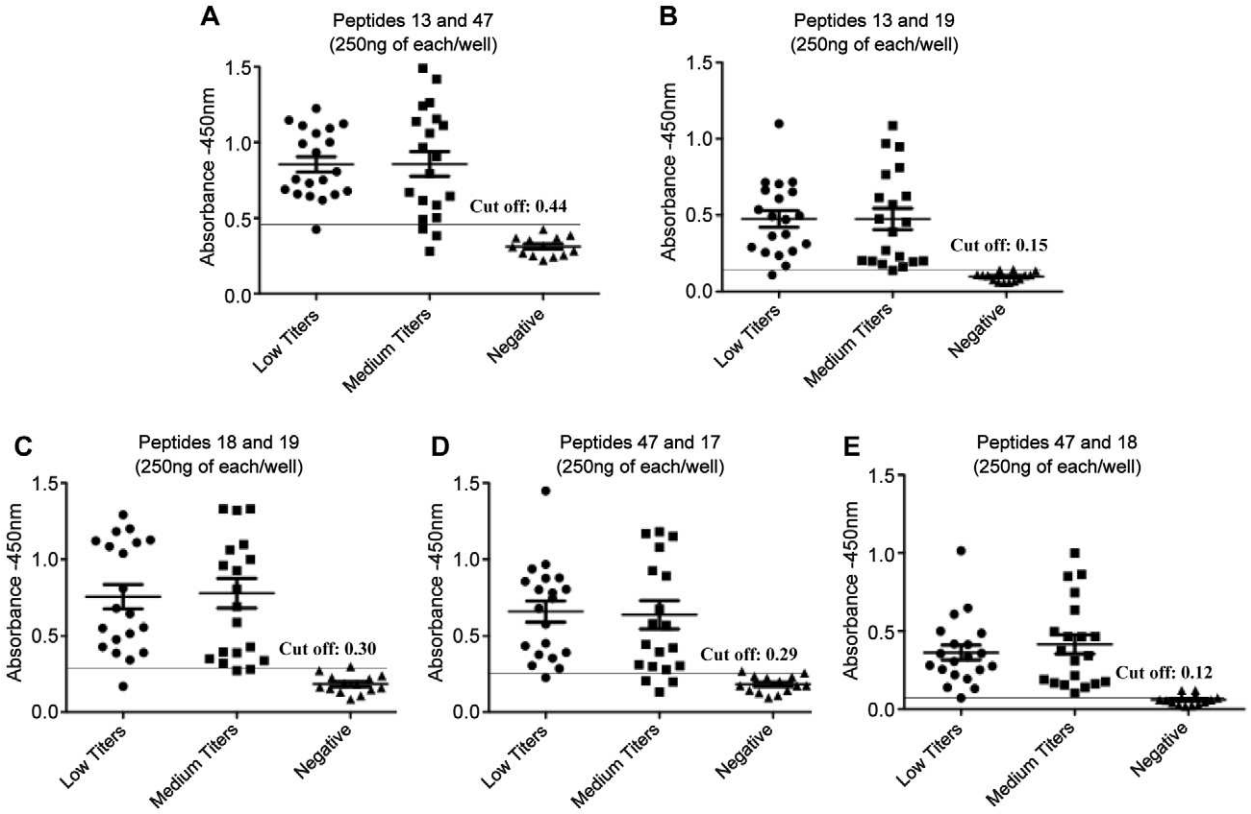

Figure 3. Reactivity of sera from infected dogs displaying different antibody titers with combined peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using canine samples classified according to IFAT reactivity as low (<1:320 dilutions) and intermediate (>1:320 <1:640) titers and 250 nanograms/per well from each peptide. Peptides were combined in pairs in the same reaction as following: peptides 13 and 47 (panel **A**), 13 and 19 (panel **B**), 18 and 19 (panel **C**), 47 and 17 (panel **D**) and 47 and 18 (panel **E**).
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and specificity between peptides 13 and 19, 18 and 19, 47 and 13, and 19 and 47 where we observe a sensitivity of 100% for the first three combinations and 95.65% for the last one, respectively. Only for the combination between peptides 17 and 47 a reduction in sensitivity to 70.83% was observed. Specificity values for all combination ranged from 93.10% to 100% (Figure 4 and 5/ Table 3).

Discussion

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

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

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

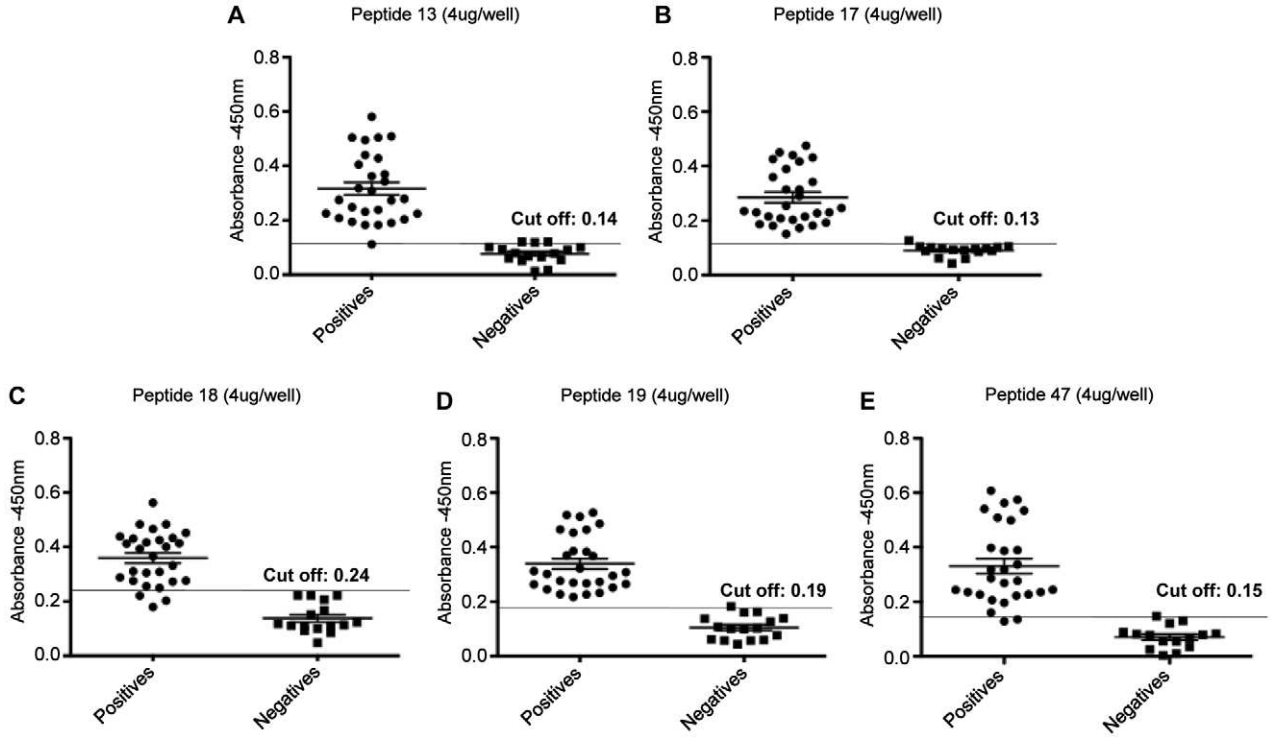

Figure 4. Detection of IgG-antibodies in sera of human patients with visceral leishmaniasis with individual peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using sera from patients with active visceral leishmaniasis ($n = 28$) and healthy individuals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

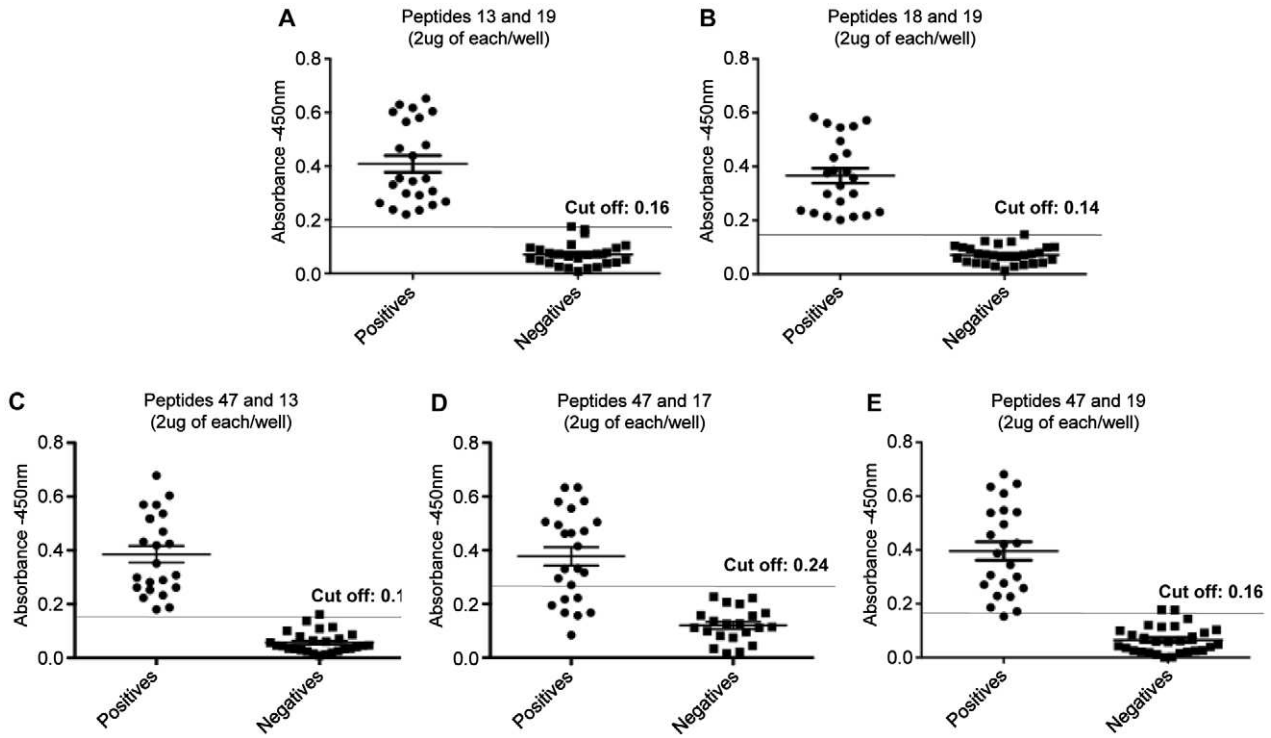

Figure 5. Detection of IgG-antibodies in sera of human patients with visceral leishmaniasis with combined peptides. Detection of anti-leishmania total IgG antibodies with synthetic peptides by ELISA assay using sera from patients with active visceral leishmaniasis ($n = 28$) and healthy individuals with previous negative results in ELISA to *Leishmania* ($n = 16$) and 2 μg/per well from each peptide. Peptides were combined in pairs in the same reaction as following: peptides 13 and 19 (panel **A**), 18 and 19 (panel **B**), 47 and 13 (panel **C**), 47 and 17 (panel **D**) and 47 and 19 (panel **E**).
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Table 3. Performance of ELISA employing synthetic peptides and sera of patients with visceral leishmaniasis.

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

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

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

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

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

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

Author Contributions

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

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Epitope Mapping of the HSP83.1 Protein of *Leishmania braziliensis* Discloses Novel Targets for Immunodiagnosis of Tegumentary and Visceral Clinical Forms of Leishmaniasis

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Gold standard serological diagnostic methods focus on antigens that elicit a strong humoral immune response that is specific to a certain pathogen. In this study, we used bioinformatics approaches to identify linear B-cell epitopes that are conserved among *Leishmania* species but are divergent from the host species *Homo sapiens* and *Canis familiaris* and from *Trypanosoma cruzi*, the parasite that causes Chagas disease, to select potential targets for the immunodiagnosis of leishmaniasis. Using these criteria, we selected heat shock protein 83.1 of *Leishmania braziliensis* for this study. We predicted three linear B-cell epitopes in its sequence. These peptides and the recombinant heat shock protein 83.1 (rHSP83.1) were tested in enzyme-linked immunosorbent assays (ELISAs) against serum samples from patients with tegumentary leishmaniasis (TL) and visceral leishmaniasis (VL) and from dogs infected with *Leishmania infantum* (canine VL [CVL]). Our data show that rHSP83.1 is a promising target in the diagnosis of TL. We also identified specific epitopes derived from HSP83.1 that can be used in the diagnosis of human TL (peptide 3), both human and canine VL (peptides 1 and 3), and all TL, VL, and CVL clinical manifestations (peptide 3). Receiver operating characteristic (ROC) curves confirmed the superior performance of rHSP83.1 and peptides 1 and 3 compared to that of the soluble *L. braziliensis* antigen and the reference test kit for the diagnosis of CVL in Brazil (EIE-LVC kit; Bio-Manguinhos, Fiocruz). Our study thus provides proof-of-principle evidence of the feasibility of using bioinformatics to identify novel targets for the immunodiagnosis of parasitic diseases using proteins that are highly conserved throughout evolution.

Leishmaniasis is a neglected vector-borne tropical disease that is caused by parasites of the *Leishmania* genus. Currently, it has a major impact on human health in tropical regions and affects approximately 12 million people worldwide (1). Two million new cases are reported annually, with an incidence of 1 to 1.5 million cases of tegumentary leishmaniasis (TL) and 500,000 cases of visceral leishmaniasis (VL) (1). The clinical forms of leishmaniasis range from self-healing cutaneous lesions to fatal visceral infections. In TL, a wide variety of skin manifestations, such as cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML), have been described (2). The CL form is characterized by one or more painless ulcers with raised borders and a bed of granulation tissue that appear near the area of the sand fly bite, while ML is characterized by the progressive destruction of the nasopharyngeal mucosa (3, 4). Although the determining factors involved in the development of each clinical form have not been elucidated, it is likely that host and parasite genetics are involved (2, 5).

In Brazil, TL is distributed throughout the country, and among the various *Leishmania* species that can cause the disease, *Leishmania braziliensis* is responsible for the majority of the cases. *L. braziliensis* infection results in higher clinical severity due to larger ulcerated areas and a higher proportion of patients with mucosal involvement in the upper airway (6, 7). VL is caused by the *Leishmania infantum* parasite and is a zoonotic disease that has shown significant changes in transmission with progressive urbanization and geographic expansion; it now affects regions in which it was previously quite rare (2). Dogs are the main urban reservoirs and represent the major source of infection for the vector due to the high prevalence of canine infections and intense cutaneous parasitism that may contribute to urban spread of the disease (8, 9).

The major prophylactic practice recommended by the World Health Organization to control the human disease and canine visceral leishmaniasis (CVL) (8) involves early accurate diagnosis, systematic treatment of human cases, vector control with insecticide, and the elimination of seropositive dogs (10). At present, there is no gold standard serological test for diagnosing leishmaniasis, and a combination of different techniques is frequently necessary to obtain precise results. Therefore, the development of a new serological technique with higher sensitivity and specificity

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than the available commercial tests and that is able to discriminate postvaccination reactivity from active infections would represent an important innovation in the serological diagnosis of leishmaniasis (11). Additionally, due to the high conservation of proteins among the various species of *Leishmania*, the selection of an antigen that is able to simultaneously diagnose the various clinical forms of the disease would represent an interesting strategy for the technological development and large-scale production of tests for diagnosis (12, 13).

In this context, we performed genome-wide screening to identify linear B-cell epitopes in the predicted proteome of *L. braziliensis* in an attempt to identify conserved targets within the *Leishmania* genus for the serodiagnosis of the tegumentary and visceral forms of leishmaniasis. The protein selected in this study was heat shock protein 83.1 (HSP83.1), a highly conserved molecule in prokaryotes and eukaryotes that plays important roles in protein folding, assembly of protein complexes, and the translocation of proteins across cellular compartments (14). We mapped three B-cell linear epitopes in this protein whose sequences are divergent from its orthologs in *Homo sapiens* and *Canis familiaris* and in *Trypanosoma cruzi*, the etiologic agent of Chagas disease (CD), which is frequently associated with cross-reactivity with leishmaniasis (15, 16). The reactivities of recombinant HSP83.1 (rHSP83.1) and three derived B-cell epitopes with serum samples from TL and VL patients and from *Leishmania*-infected dogs were evaluated in enzyme-linked immunosorbent assays (ELISAs). rHSP83.1 demonstrated excellent performance for diagnosing TL. Additionally, we identified specific epitopes derived from HSP83.1 that may be useful in the diagnosis of human TL (peptide 3), both human and canine VL (peptides 1 and 3), and all TL, VL, and CVL clinical manifestations (peptide 3). Receiver operating characteristic (ROC) curves confirmed the superior performance of rHSP83.1 and peptides 1 and 3 compared to that of the soluble *L. braziliensis* antigen and the reference test for diagnosing CVL in Brazil (EIE-LVC kit; Bio-Manguinhos, Fiocruz) (17).

MATERIALS AND METHODS

Ethics statement and human and dog serum samples. All samples that were used were anonymous and were obtained from the serum bank of the Laboratory of Immunology and Genomics of Parasites, Federal University of Minas Gerais. Approval to use the samples was obtained from the Human Research Ethics Committee (protocol CAAE – 00842112.2.0000.5149) and the Committee on Ethics of Animal Experimentation from the Federal University of Minas Gerais (protocol 44/2012).

The human serum panel consisted of 65 samples from tegumentary leishmaniasis patients infected with *L. braziliensis* that presented cutaneous (CL) ($n = 45$) or mucosal (ML) ($n = 20$) clinical manifestations obtained from the Centro de Referência em Leishmaniose (Januária, Minas Gerais, Brazil) and 55 samples from visceral leishmaniasis patients infected with *L. infantum* obtained from the University Hospital (Montes Claros, Minas Gerais, Brazil). The infection was confirmed by microscopic analyses of biopsy specimens from cutaneous lesions (for TL) or bone marrow aspirate samples (for VL), followed by specific PCR assays for the kinetoplastid DNA (kDNA) of *Leishmania* parasites (18). These individuals were known to be noninfected with *T. cruzi*. Information regarding the clinical evaluation and PCR results were obtained from the medical records of the patients. To evaluate the cross-reactivity with Chagas disease, serum samples from patients with chronic Chagas disease (CD) ($n = 20$) with infections confirmed by hemoculture or the Chagatest recombinant ELISA version 3.0 kit and the Chagatest hemagglutination inhibition (HAI) were used in this study. Serum samples from healthy Brazilian individuals (controls [CT]) ($n = 50$) from areas where TL and CD are not endemic (Belo Horizonte, Minas Gerais, Brazil) were used as negative controls in these assays. The resulting optical density (OD) values were compared with those obtained with the panel of TL and VL samples.

Male and female beagle dogs from an area where VL is not endemic that had *Leishmania*-negative tissue smears (bone marrow) were considered to be noninfected and were used as the control group (CT, $n = 30$). Serum samples from *Leishmania*-infected dogs (CVL) ($n = 30$) were obtained from the area where CVL is endemic in Minas Gerais in southeastern Brazil. The main inclusion criterion for the CVL serum samples used in this study was parasitological positivity for *L. infantum* confirmed by microscopic analysis of bone marrow aspirate samples. Samples from dogs that were experimentally infected with *T. cruzi* (CD, $n = 15$) but that were parasitologically negative for *Leishmania* were included in this study to evaluate possible cross-reactivity.

***In silico* prediction of linear B-cell epitopes.** Linear B-cell epitopes were predicted for the HSP83.1 protein of *L. braziliensis* (TriTrypDB identification [ID] [19] LbrM.33.0340) using the BepiPred 1.0 program with a cutoff of 1.3 (20). A BLASTp (21) search was performed against GeneDB (http://www.genedb.org/) to retrieve the HSP83.1 sequences of *T. cruzi*, *H. sapiens*, and *C. familiaris* using *L. braziliensis* HSP83.1 as a query. Multiple alignments of HSP83.1 were performed using the Clustal X 2.0 program (22), with the default parameters (23).

Soluble *L. braziliensis* antigen. Soluble *L. braziliensis* antigen (SLbA) was prepared from *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2904) stationary-phase promastigotes maintained in Schneider's insect medium (Sigma-Aldrich) supplemented with 10% inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The parasites were initially subjected to three cycles of freezing (with liquid nitrogen) and thawing (42°C), followed by ultrasonication (Ultrasonic processor model GEX-600) with 10 alternating cycles of 30 s on/off sonication in an ice water bath at 35 MHz. The lysate was then centrifuged at 6,000 × *g* at 4°C for 15 min. The supernatant containing SLbA was collected, and the protein content was quantified using the Pierce BCA protein assay (Thermo Scientific).

Cloning, protein expression, and purification. The primers used to amplify the HSP83.1 gene from the *Leishmania* genomic DNA were HSP83.1-Forward (5′-**GCTAGC**ATGACGGAGACGTTCGCGTT) and HSP83.1-Reverse (5′-**AAGCTT**TCAGTCCACCTGCTCCATG). The sites for restriction enzymes (NheI and HindIII) that were added to the forward and reverse primers, respectively, to facilitate cloning, are shown in bold type. The DNA fragments obtained were excised from the gel, purified, digested with the restriction enzymes, and cloned into the pET28a-TEV vector (24). Electrocompetent *Escherichia coli* BL21 Arctic Express (DE3) cells (Agilent Technologies, USA) were transformed with the recombinant plasmid pET28a-TEV-HSP83.1 by electroporation using a Micro-Pulser electroporation apparatus (Bio-Rad Laboratories, USA). Gene insertion was confirmed by colony PCR and sequencing using T7 primers (Macrogen, South Korea).

Protein expression and purification were performed as previously described (25). Briefly, expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM, and the bacterial culture was incubated for 24 h at 12°C with shaking at 200 rpm per min. The cells were then ruptured by sonication, the debris was removed by centrifugation, and the recombinant protein was purified using a HisTrap HP affinity column connected to an ÄKTAprime chromatography system (GE Healthcare, USA). The eluted fractions containing rHSP83.1 (705 amino acids, 80.6 kDa) were concentrated using the Amicon Ultra 15 centrifugal filters 10,000 nominal molecular weight limit (NMWL) (Millipore, Germany) and further purified on a Superdex 200 gel filtration column (GE Healthcare Life Sciences, USA).

Soluble peptide synthesis. The soluble peptides were manually synthesized by 9-fluorenylmethoxy (Fmoc) chemistry in solid phase on a 30-μmol scale (26). Briefly, Fmoc-amino acids were activated with a 1:2

solution of Oxyme to *N*,*N*′-diisopropylcarbodiimide (DIC). The active amino acids were then incorporated into Rink amide resin with a substitution degree of 0.61. Fmoc deprotection was then performed using 25% 4-methylpiperidine. These steps were repeated until the synthesis of each peptide was completed. The peptides were then deprotected and released from the resin by treatment with a solution of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane and precipitated with cold diisopropyl ether. The peptides were obtained with ≥90% purity, as confirmed by matrix-assisted laser desorption ionization–tandem time of flight mass spectrometry (MALDI-TOF-TOF MS) Autoflex III equipment (Bruker Daltonics, USA). Instrument calibration was achieved using peptide calibration standard II (Bruker Daltonics, USA) as a reference, and α-cyano-4-hydroxycinnamic acid was used as the matrix.

Serological assay and depletion ELISA. rHSP83.1 and SLbA were coated onto 96-well microplates (Nalge Nunc International, USA) overnight at 2 to 8°C at a concentration of 250 ng/well for rHSP83.1 and 50 ng/well for SLbA. The peptides were coated onto flat-bottom plates (CoStar, USA) overnight at 37°C at a concentration of 10 μg/well. The plates were blocked with 150 μl of phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 1 h at 37°C and then treated with 1:100 dilutions of human or canine serum samples for 1 h at 37°C. Peroxidase-conjugated antibodies specific for human or dog IgG (Sigma-Aldrich, USA) were diluted at 1:5,000 and added to the plates for 1 h at 37°C. The wells were washed, and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, USA) in citrate buffer containing hydrogen peroxide was added to the plate. The plates were incubated for 30 min in the dark at 37°C. The reaction was halted by adding 4 N H_2SO_4, and the absorbance at 450 nm was read with an automatic microplate reader (VersaMax; Molecular Devices, USA). Each serum sample was assayed in duplicate. The results of the ELISA using rHSP83.1 as an antigen were compared with those using SLbA. The results of the ELISA using rHSP83.1 as an antigen for the canine serum samples were compared with those with the EIE-LVC kit immunoassay (Bio-Manguinhos, Fiocruz, Brazil), which is the test currently recommended by the Brazilian Ministry of Health for the screening of seroreactive animals (17). The assays were carried out according to the manufacturer's instructions.

Depletion ELISAs were performed as previously described (27, 28). Briefly, flat-bottom plates (CoStar, USA) were coated overnight at 37°C with 10 μg/well of peptide 1 (EEDESKKKSCGDEGEPKVE), peptide 2 (VTEGGEDKKK), and peptide 3 (EVAEAPPAEAAPA) and then washed and blocked as described above. All serum samples from individuals in the TL, VL, and CVL groups that were parasitologically positive for *Leishmania* and reactive for the tested peptide were used in the assay. Pools of sera were added to the plates at a 1:100 dilution and incubated overnight at 2 to 8°C. The depleted and undepleted sera were transferred to plates coated overnight with rHSP83.1 (50 ng/well) after appropriate washing and blocking, and the ELISAs were performed as described above.

Statistical analysis. The lower limits of positivity (cutoff) for rHSP83.1, SLbA, and peptides 1, 2, and 3 were established for optimal sensitivity and specificity using the receiver operating characteristic (ROC) curve. The cutoff was chosen based on the point that provides the maximum of the sum of the sensitivity and specificity (29). The EIE-LVC cutoff was obtained according to the manufacturer (twice the average of the negative control provided by the kit). The performance of each test was evaluated according to the sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), area under the curve (AUC), and accuracy (AC). The degree of agreement between the ELISAs using rHSP83.1, SLbA, or the EIE-LVC kit with the parasitological test (biopsy specimen, aspirate sample, or PCR) was determined by the Kappa index (k) values with 95% confidence intervals and interpreted according to the following Fleiss scale: 0.00 to 0.20, poor; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; 0.81 to 0.99, very good; and 1.00, perfect (30). The one-sample Kolmogorov-Smirnov test was used to determine whether a variable was normally distributed. For the depletion assays, significant differences were detected using a two-way analysis of variance (ANOVA). The differences were considered statistically significant at a P value of <0.05. All of the statistical analyses were performed using GraphPad Prism (version 5.0) and the GraphPad QuickCalcs software.

RESULTS

Sequence divergence and prediction of B-cell linear epitopes in *L. braziliensis* HSP83.1 protein and its orthologs. Gold standard serological diagnostic methods focus on markers that are able to elicit a strong antibody response specific to the pathogen. Although HSP83.1 is conserved throughout evolution, B-cell epitopes derived from *Leishmania* HSP83.1, with sequences that are divergent from those of the parasite hosts and other closely related parasites, may bind to specific antibodies and thus allow for the specific identification of samples from patients with leishmaniasis. To identify potential epitopes that can increase the accuracy of the currently available serological tests, we scanned the HSP83.1 sequence of *L. braziliensis* for B-cell linear epitopes that are divergent in the host orthologs and that cooccur with intrinsically unstructured regions (Fig. 1). The presence of these unstructured regions suggests that a given protein region is in an unfolded structure and therefore possibly accessible for antibody binding. As expected, the HSP83.1 ortholog of *L. braziliensis*, LbrM.33.0340, displayed a high level of identity to the orthologous proteins from *L. infantum*, *T. cruzi*, *C. familiaris*, and *H. sapiens*, with identities of >63.5%, as shown in Fig. 1 and Table 1. Despite this overall high level of similarity, we identified three potential linear B-cell epitopes (peptide 1, EEDESKKKSCGDEG EPKVE; peptide 2, VTEGGEDKKK; and peptide 3, EVAEAPPAE AAPA) in the LbrM.33.0340 protein that are highly divergent in *T. cruzi* as well as in dog and human orthologs, with identities ranging from 21.4 to 60.0% (Fig. 1 and Table 1). This result leads us to speculate that despite the high overall sequence similarity between HSP83 in *Leishmania* species and its orthologs in *T. cruzi* and in humans and dogs, this protein and the three predicted B-cell linear epitopes might be potential targets in the immunodiagnosis of leishmaniasis.

Expression and purification of rHSP83.1 protein. To verify whether the HSP83.1 protein is a candidate for the development of new diagnostic assays, we first expressed this protein as a His-tagged recombinant protein. The full-length coding region of the LbrM.33.0340 gene was amplified by PCR and cloned into the pET28a-TEV expression vector. rHSP83.1 was overexpressed in *E. coli* BL21 Arctic Express (DE3) cells as a soluble protein and purified by Ni^{2+} affinity chromatography, followed by gel filtration on a Superdex 200 column. The recombinant protein, with a predicted molecular mass of 80.6 kDa, was successfully expressed and obtained at a high level of purity (Fig. 2).

ELISA performance using rHSP83.1, peptides 1, 2, 3, and SLbA in TL diagnosis. To evaluate the specific antibody responses against rHSP83.1 and the derived peptides 1, 2, and 3 in TL, ELISAs with these antigens were compared to SLbA ELISAs (Fig. 3 to 5 and Tables 2 and 3). When tested with serum samples from CL patients, the rHSP83.1 and peptide 1, 2, and 3 antigens showed sensitivities of 95.55%, 71.11%, 64.44%, and 95.55%, respectively. The serum samples from ML patients presented lower sensitivity values to all antigens tested (90.00%, 55.00%, 50.00%, and 75.00%, respectively). The antigens presented total sensitivity (CL + ML) for rHSP83.1 and sensitivities of 93.85% (95% confidence interval [CI], 84.99 to 98.30%), 63.08% (95% CI, 50.20 to 74.72), 63.08% (95% CI, 50.20 to 74.72), and 89.23% (95% CI, 79.06 to 95.56) for peptides 1, 2, and 3,

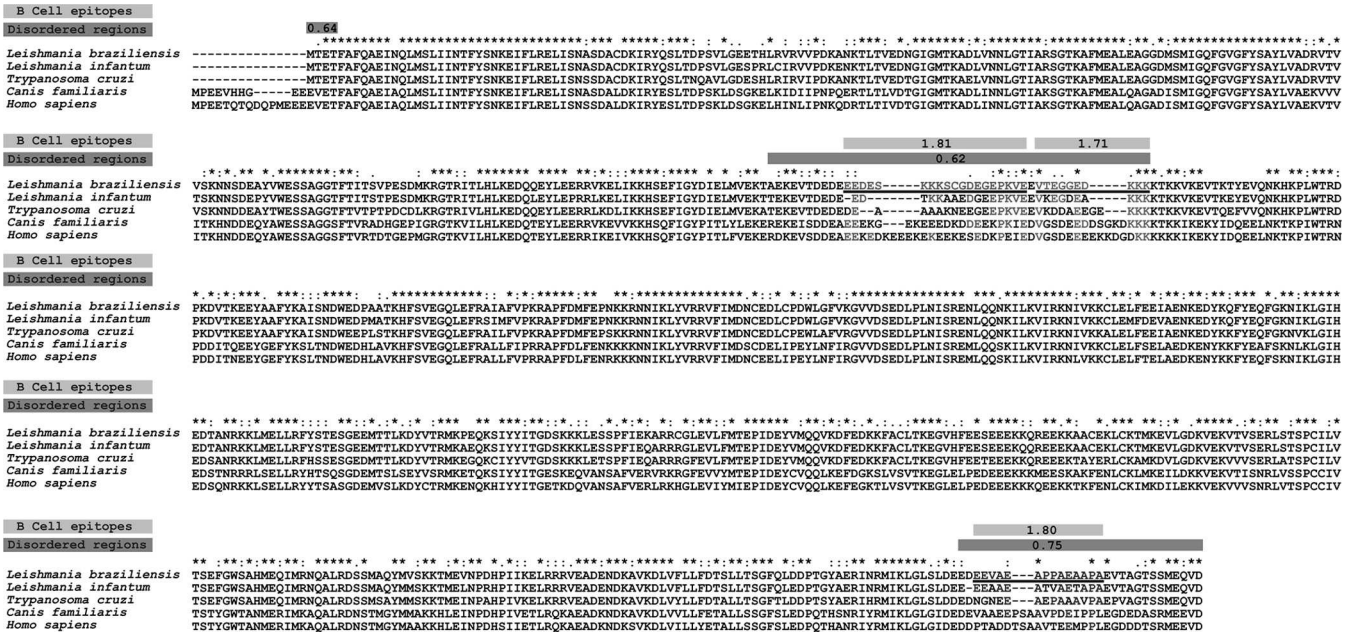

FIG 1 Sequence divergence and prediction of B-cell linear epitopes and intrinsically unstructured/disordered regions in *L. braziliensis* HSP83.1 and its orthologs. Alignment between *L. braziliensis* HSP83.1 (TriTrypDB ID LbrM.33.0340) and orthologous proteins present in *L. infantum* (TriTrypDB ID Linj.33.0360), *T. cruzi* (TriTrypDB ID TcCLB.509105.140), *H. sapiens* (RefSeq ID NP_005339.3), and *C. familiaris* (RefSeq ID XP_532154.4). The yellow boxes mark predicted B-cell epitopes, and the gray boxes mark predicted disordered regions. The continuous black underlined amino acid sequences represent three potential B-cell epitopes predicted by BepiPred in the LbrM.33.0340 protein, and red, green, and blue highlight amino acid conservations in the *T. cruzi*, *C. familiaris*, and *H. sapiens* sequences in relation to the *L. braziliensis* sequence.

respectively (Table 2). ELISAs using SLbA extract presented sensitivities of 75.55% and 60.00% for detecting the CL and ML clinical forms, respectively. The total sensitivity of SLbA in TL was 70.77% (95% CI, 58.17 to 81.40%), which was lower than that for rHSP83.1 and peptide 3 (Table 2). To evaluate specificity (Sp), serum samples from chagasic patients and negative-control individuals were also tested. rHSP83.1 and peptides 1, 2, and 3 showed total specificity values of 95.71% (95% CI, 87.98 to 99.11%), 94.29% (95% CI, 86.01 to 98.42), 90.00% (95% CI, 80.48 to 95.88%), and 91.43% (95% CI, 82.27 to 96.79%), respectively. The ELISA using SLbA presented a much lower specificity value of 68.57% (95% CI, 56.37 to 79.15%) (Table 2).

The maximum PPV was achieved with rHSP83.1 (95.31%), followed by peptide 1 (91.11%), peptide 3 (90.63%), peptide 2 (85.42%), and SLbA (67.65%). High NPVs were also observed for rHSP83.1 (94.37%), followed by peptide 3 (90.14%), peptide 1 (73.33%), peptide 2 (72.41%), and SLbA (71.64%) (Table 2).

Based on the ELISA results, ROC curves were calculated to evaluate the capacity of the tested antigens to discriminate TL patients from healthy volunteers (Fig. 5 and Table 3). The area under the curve (AUC) and accuracy (AC) were used to compare the efficiencies of the different diagnostic antigens or tests (29) (Table 3). rHSP83.1 presented the highest AUC value (0.989; 95%

TABLE 1 Sequence identity of the B-cell linear epitopes predicted in LbrM.33.0340 HSP83 protein of *Leishmania braziliensis* and its orthologs

Species	% identity with LbrM.33.0340	% identity with peptide[a]:		
		1	2	3
Leishmania infantum	93.9	52.3	70.0	71.4
Trypanosoma cruzi	85.2	31.6	50.0	50.0
Canis familiaris	64.1	36.8	60.0	35.7
Homo sapiens	63.5	36.8	40.0	21.4

[a] Peptide 1, EEDESKKKSCGDEGEPKVE; peptide 2, VTEGGEDKKK; peptide 3, EEVAEAPPAEAAPA.

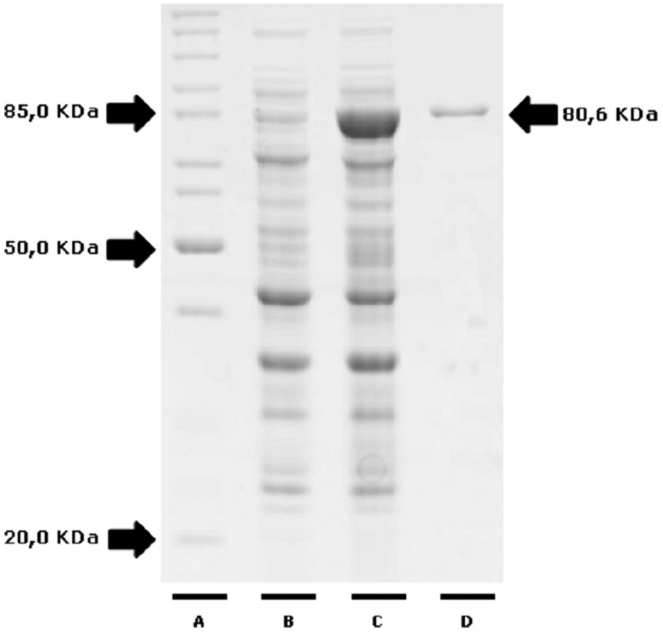

FIG 2 Expression and purification of recombinant HSP83.1 protein. Protein samples were separated by 12.5% SDS-PAGE. Lane A, molecular weight standard, lysate of culture before (lane B) and after (lane C) induction with IPTG and recombinant HSP83.1 protein (mass, 80.6 kDa) purified by gel filtration (lane D).

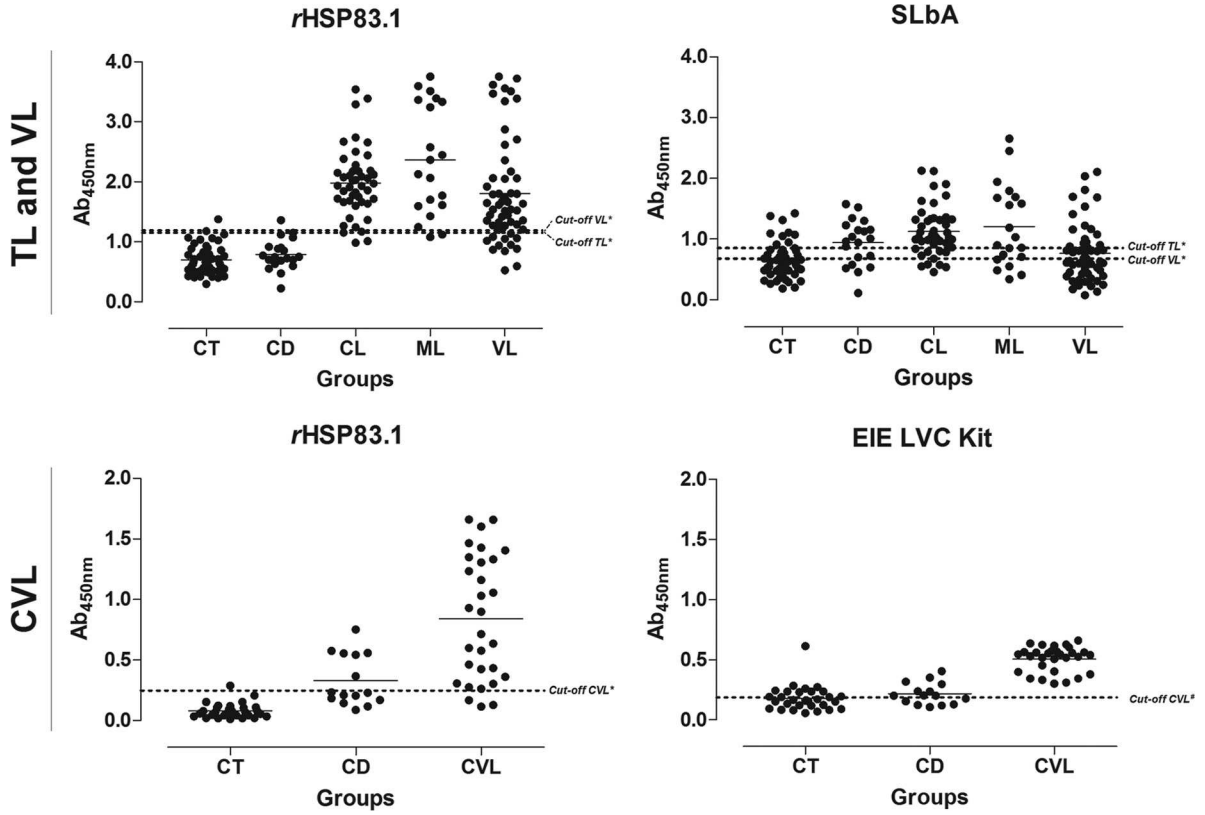

FIG 3 Comparison of the reactivity of ELISAs against rHSP83.1 and SLbA and the EIE-LVC kit against serum samples from TL and VL patients and from *L. infantum*-infected dogs. (Top) ELISAs were performed on samples from different groups of individuals (CT [control] group, $n = 50$; CD [Chagas disease] patients, $n = 20$; CL [cutaneous leishmaniasis], $n = 45$; ML [mucosal leishmaniasis], $n = 20$; VL [visceral leishmaniasis], $n = 55$). (Bottom) ELISAs were performed on samples from different groups of dogs (CT [control group], $n = 30$; CD [*T. cruzi*-infected dogs], $n = 15$; CVL [canine visceral leishmaniasis], $n = 30$). An asterisk indicates a cutoff value obtained by ROC curve; a pound sign indicates a cutoff value obtained according to the manufacturer. Dots represent individual absorbance values. The dotted line represents the cutoff value. The solid line corresponds to the mean values. Ab_{450nm}, absorbance at 450 nm.

CI, 0.979 to 1.000), followed by peptide 3 (0.961; 95% CI, 0.933 to 0.989), peptide 1 (0.881; 95% CI, 0.826 to 0.936), peptide 2 (0.819; 95% CI, 0.748 to 0.889), and SLbA (0.753; 95% CI, 0.673 to 0.834). The accuracy value for rHSP83.1 was also the highest (94.81%), followed by peptide 3 (90.37%), peptide 1 (79.26%), peptide 2 (77.04%), and SLbA (69.63%) (Table 2).

ELISA performance using rHSP83.1, peptides 1, 2, and 3, and SLbA in VL diagnosis. We also tested the performance of ELISAs using rHSP83.1 and the derived peptides 1, 2, and 3 in VL diagnosis and compared the results to those of the SLbA ELISA (Fig. 3 to 5 and Tables 2 and 3). rHSP83.1 and peptides 1, 2, and 3 presented total sensitivities of 78.18% (95% CI, 64.99 to 88.19%), 83.64% (95% CI, 71.20 to 92.23%), 85.45% (95% CI, 73.34 to 93.50%), and 87.27% (95% CI, 75.52 to 94.73%), respectively, for detecting VL (Table 2). The total sensitivity of SLbA for detecting VL was 52.73% (95% CI, 38.80 to 66.35%), which is much lower than those for the other tested antigens. rHSP83.1 and peptides 1, 2, and 3 presented specificity values of 97.14% (95% CI, 90.06 to 99.65%), 95.71% (95% CI, 87.98 to 99.11), 92.86% (95% CI, 84.11 to 97.64%), and 94.29% (95% CI, 86.01 to 98.42%), respectively; again, these values were much higher than that obtained for SLbA (50.00%; 95% CI, 37.80 to 62.20%) (Table 2).

The maximum PPV was achieved with rHSP83.1 (95.56%), followed by peptide 1 (93.88%), peptide 3 (92.31%), peptide 2 (90.38%), and SLbA (45.31%). The highest NPV was observed for peptide 3 (90.41%), followed by peptide 2 (89.04%), peptide 1 (88.16%), rHSP83.1 (85.00%), and SLbA (57.38%). Peptide 3 also presented the highest AUC value (0.943; 95% CI, 0.892 to 0.995), followed by rHSP83.1 (0.937; 95% CI, 0.894 to 0.981), peptide 1 (0.934; 95% CI, 0.880 to 0.988), peptide 2 (0.924; 95% CI, 0.867 to 0.981), and SLbA (0.510; 95% CI, 0.405 to 0.614) (Fig. 4 and Table 3). The accuracy value for peptide 3 was the highest (91.20%), followed by peptide 1 (90.40%), peptide 2 (89.60%), rHSP83.1 (88.80%), and SLbA (51.20%) (Table 2).

ELISA performance using rHSP83.1, peptides 1, 2, and 3, and the EIE-LVC kit in CVL diagnosis. Next, we evaluated the performance of ELISAs using rHSP83.1 and the derived peptides 1, 2, and 3 in CVL diagnosis and compared the results to those obtained with the EIE-LVC kit (Fig. 3 to 5 and Tables 2 and 3). Serological assays using rHSP83.1 and peptides 1, 2, and 3 presented total sensitivities of 90.00% (95% CI, 73.47 to 97.89%), 96.67% (95% CI, 82.78 to 99.92%), 63.33% (95% CI, 43.86 to 80.07%), and 93.33% (95% CI, 77.93 to 99.18%), respectively, for detecting CVL (Table 2). All of the tested antigens showed sensitivity values for detecting CVL that were inferior to those obtained with the EIE-LVC kit (100.00% sensitivity; 95% CI, 88.43 to 100.00%). In contrast, ELISAs using the antigens tested in this study showed a better specificity value (84.44%; 95% CI, 70.54 to 93.51%) than that of the EIE-LVC kit (53.33%; 95% CI, 37.87 to 68.34%) (Table 2).

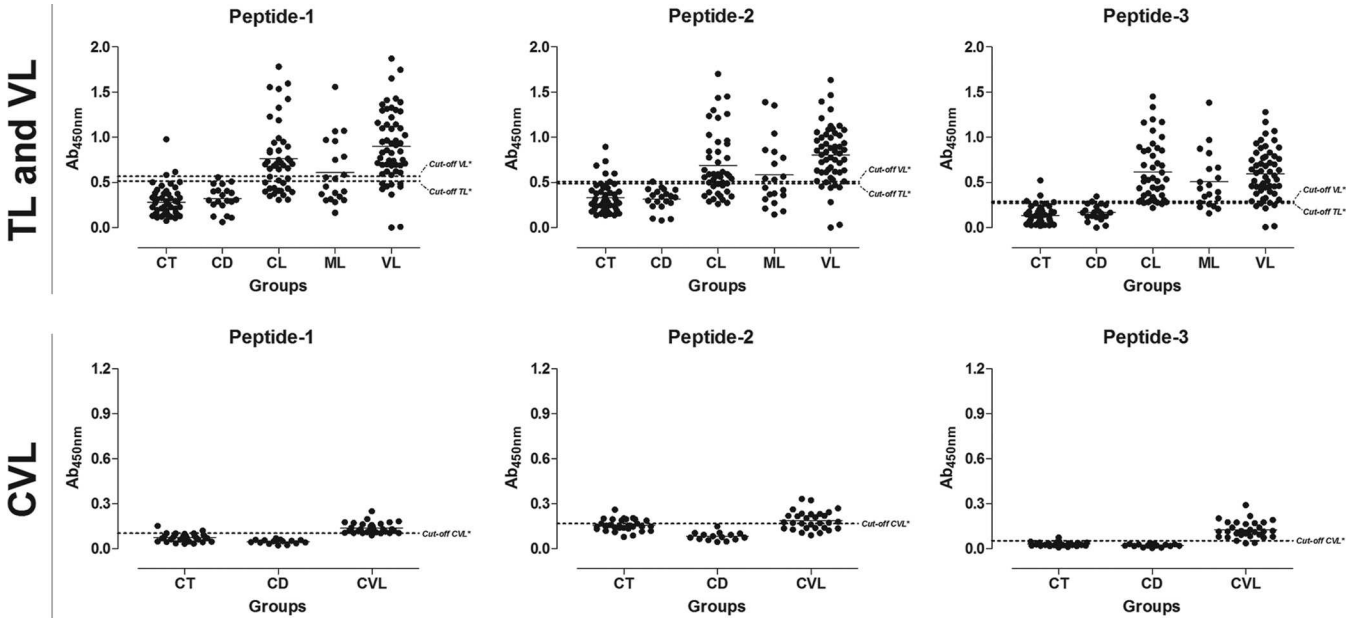

FIG 4 Comparison of the reactivities of the ELISAs against peptides 1, 2, and 3 against serum samples from TL and VL patients and from *L. infantum*-infected dogs. (Top) ELISAs were performed on samples from the different groups of individuals (CT [control group], $n = 50$; CD [Chagas disease] patients, $n = 20$; CL [cutaneous leishmaniasis], $n = 45$; ML [mucosal leishmaniasis], $n = 20$; VL [visceral leishmaniasis], $n = 55$). (Bottom) ELISAs were performed on samples from the different groups of dogs (CT [control] group, $n = 30$; CD [*T. cruzi*-infected dogs], $n = 15$; CVL [canine visceral leishmaniasis], $n = 30$). An asterisk indicates a cutoff value obtained by ROC curve.

The maximum PPV was achieved with peptide 3 (96.65%), followed by peptide 1 (90.63%), rHSP83.1 (79.41%), peptide 2 (67.86%), and the EIE-LVC kit (58.82%). The highest NPV was observed for the EIE-LVC kit (100.00%), followed by peptide 1 (97.67%), peptide 3 (95.65%), rHSP83.1 (92.68%), and peptide 2 (76.60%) (Table 2). Peptide 3 presented the highest AUC value (0.987; 95% CI, 0.967 to 1.007), followed by peptide 1 (0.950; 95% CI, 0.894 to 1.005), rHSP83.1 (0.924; 95% CI, 0.867 to 0.981), and pep-

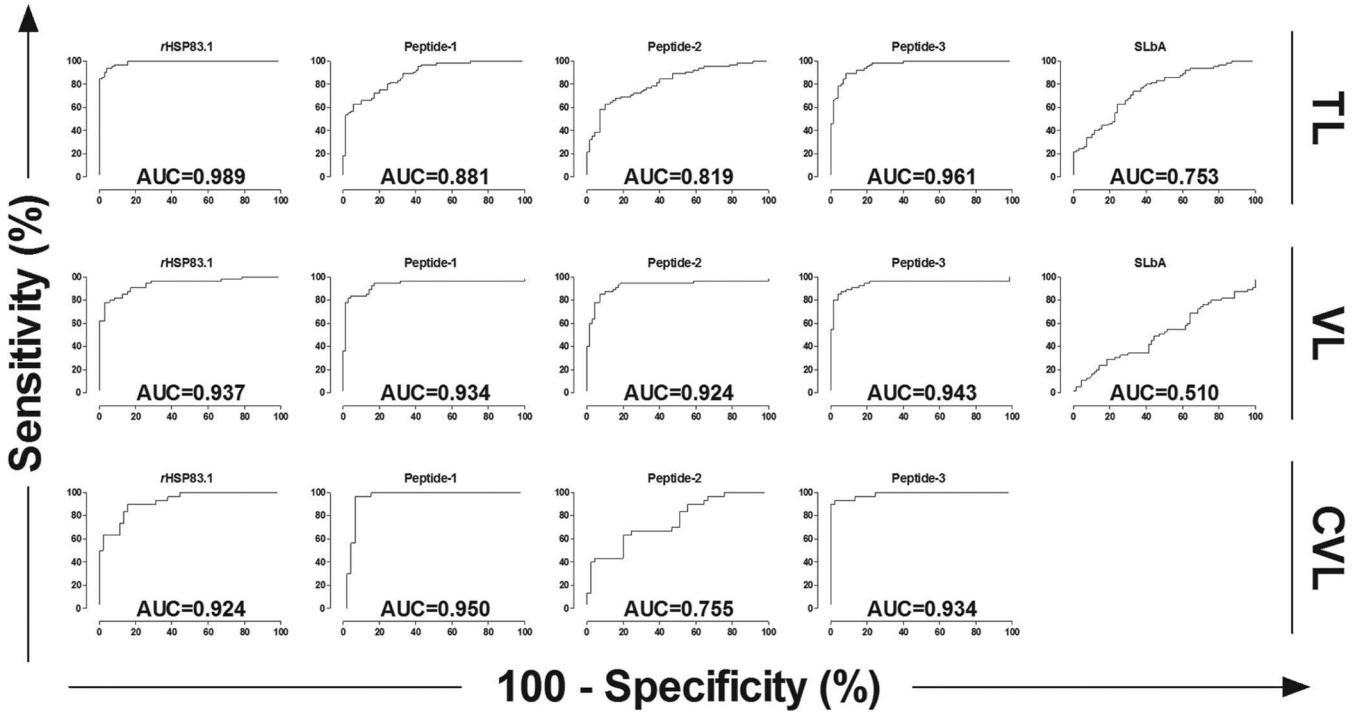

FIG 5 Comparison of ROC curves obtained from rHSP83.1, peptides 1, 2, and 3, and SLbA. The ROC curves were used to determine the ELISA cutoff, sensitivity, specificity, and AUC. Ab_{450nm}, absorbance at 450 nm.

TABLE 2 Measure of diagnostic performance for rHSP83.1, peptides 1, 2, and 3, SLbA, and the EIE-LVC kit

Test[a]	Disease	Cutoff value	Test performance parameter (% [95% CI])[b]				
			TSe	TSp	PPV	NPV	AC
rHSP83.1	TL	1.1510	93.85 (84.99–98.30)	95.71 (87.98–99.11)	95.31	94.37	94.81
Peptide 1	TL	0.5165	63.08 (50.20–74.72)	94.29 (86.01–98.42)	91.11	73.33	79.26
Peptide 2	TL	0.4884	63.08 (50.20–74.72)	90.00 (80.48–95.88)	85.42	72.41	77.04
Peptide 3	TL	0.2713	89.23 (79.06–95.56)	91.43 (82.27–96.79)	90.63	90.14	90.37
SLbA	TL	0.8530	70.77 (58.17–81.40)	68.57 (56.37–79.15)	67.65	71.64	69.63
rHSP83.1	VL	1.1900	78.18 (64.99–88.19)	97.14 (90.06–99.65)	95.56	85.00	88.80
Peptide 1	VL	0.5693	83.64 (71.20–92.23)	95.71 (87.98–99.11)	93.88	88.16	90.40
Peptide 2	VL	0.5096	85.45 (73.34–93.50)	92.86 (84.11–97.64)	90.38	89.04	89.60
Peptide 3	VL	0.2901	87.27 (75.52–94.73)	94.29 (86.01–98.42)	92.31	90.41	91.20
SLbA	VL	0.6780	52.73 (38.80–66.35)	50.00 (37.80–62.20)	45.31	57.38	51.20
rHSP83.1	CVL	0.2473	90.00 (73.47–97.89)	84.44 (70.54–93.51)	79.41	92.68	86.67
Peptide 1	CVL	0.1031	96.67 (82.78–99.92)	93.33 (81.73–98.60)	90.63	97.67	94.67
Peptide 2	CVL	0.1669	63.33 (43.86–80.07)	80.00 (65.40–90.42)	67.86	76.60	73.33
Peptide 3	CVL	0.0520	93.33 (77.93–99.18)	97.78 (88.23–99.24)	96.65	95.65	96.00
EIE-LVC kit	CVL	0.1894	100.00 (88.43–100.0)	53.33 (37.87–68.34)	58.82	100.00	72.00

[a] Cutoff values for all tests obtained by ROC curve, except that for the EIE-LVC kit, which was obtained according to the manufacturer.
[b] Parameters were calculated using all samples presented in this work for TL (CT + CD + CL + ML) ($n = 135$), VL (CT + CD + VL) ($n = 125$), and CVL (CT + CD + LM + CVL) ($n = 75$). Tse, total sensitivity; CI, confidence interval; TSp, total specificity; PPV, positive predictive value; NPV, negative predictive value; AC, accuracy.

tide 2 (0.755; 95% CI, 0.644 to 0.866). The AUC value was not calculated for the EIE-LVC kit because the cutoff value not was established using an ROC curve but was instead calculated as twice the average of the negative control provided by the kit, as recommended by the manufacturer. The accuracy value for peptide 3 was the highest (96.00%), followed by peptide 1 (94.67%), rHSP83.1 (86.67%), peptide 2 (73.33%), and the EIE-LVC kit (72.00%) (Table 2).

Agreement between rHSP83.1, peptides 1, 2, and 3, SLbA, and the EIE-LVC kit with parasitological assays. The agreement (Kappa index) values between the serological tests using rHSP83.1, peptides 1, 2, and 3, SLbA, and the EIE-LVC kit with parasitological assays are shown in Table 3. Regarding TL diagnosis, the best agreement score was observed for rHSP83.1 (0.896, very good), followed by peptide 3 (0.807, very good), peptide 1 (0.580, moderate), peptide 2 (0.536, moderate), and SLbA (0.393, fair). For the diagnosis of human and canine VL, the best agreement scores were obtained for peptide 3 (0.820, very good for VL; 0.916, very good for CVL), followed by peptide 1 (0.803, very good for VL, and 0.890, very good for CVL).

Linear B-cell epitopes identified in this study contribute significantly to the overall antigenicity of rHSP83.1. To determine the contribution of the linear B-cell epitopes identified in this

TABLE 3 Diagnostic performance for rHSP83.1, peptides 1, 2, and 3, SLbA, and the EIE-LVC kit using ROC curves, data validation, and agreement using kappa index

Test[a]	Disease	AUC[b] (95% CI[c])	No. with result[d]:				κ[e] (95% CI)	Agreement[f]
			TP	TN	FP	FN		
rHSP83.1	TL	0.989 (0.979–1.000)	61	67	3	4	0.896 (0.821–0.971)	Very good
Peptide 1	TL	0.881 (0.826–0.936)	41	66	4	24	0.580 (0.448–0.712)	Moderate
Peptide 2	TL	0.819 (0.748–0.889)	41	63	7	24	0.536 (0.397–0.674)	Moderate
Peptide 3	TL	0.961 (0.933–0.989)	58	64	6	7	0.807 (0.707–0.907)	Very good
SLbA	TL	0.753 (0.673–0.834)	48	46	22	19	0.393 (0.238–0.548)	Fair
rHSP83.1	VL	0.937 (0.894–0.981)	43	68	2	12	0.768 (0.655–0.881)	Good
Peptide 1	VL	0.934 (0.880–0.988)	46	67	3	9	0.803 (0.697–0.908)	Very good
Peptide 2	VL	0.924 (0.867–0.981)	47	65	5	8	0.788 (0.679–0.897)	Good
Peptide 3	VL	0.943 (0.892–0.995)	48	66	4	7	0.820 (0.719–0.922)	Very good
SLbA	VL	0.510 (0.405–0.614)	29	35	35	26	0.027 (−0.147–0.200)	Poor
rHSP83.1	CVL	0.924 (0.867–0.981)	27	38	7	3	0.728 (0.572–0.884)	Good
Peptide 1	CVL	0.950 (0.894–1.005)	29	42	3	1	0.890 (0.786–0.995)	Very good
Peptide 2	CVL	0.755 (0.644–0.866)	19	36	9	11	0.438 (0.230–0.646)	Moderate
Peptide 3	CVL	0.987 (0.967–1.007)	28	44	1	2	0.916 (0.823–1.000)	Very good
EIE-LVC kit	CVL	NA	30	24	21	0	0.478 (0.316–0.639)	Moderate

[a] Cutoffs for all tests obtained by ROC curve, except that for the EIE-LVC kit, which was obtained according to the manufacturer.
[b] AUC, area under the curve; NA, not applicable.
[c] CI, confidence interval.
[d] TP, true positive; TN, true negative; FP, false positive; FN, false negative.
[e] Kappa index values were calculated using all samples presented in this work for TL (CT + CD + CL + ML) ($n = 135$), VL (CT + CD + VL) ($n = 125$), and CVL (CT + CD + CVL) ($n = 75$).
[f] Agreement was calculated using parasitological assays as a gold standard test.

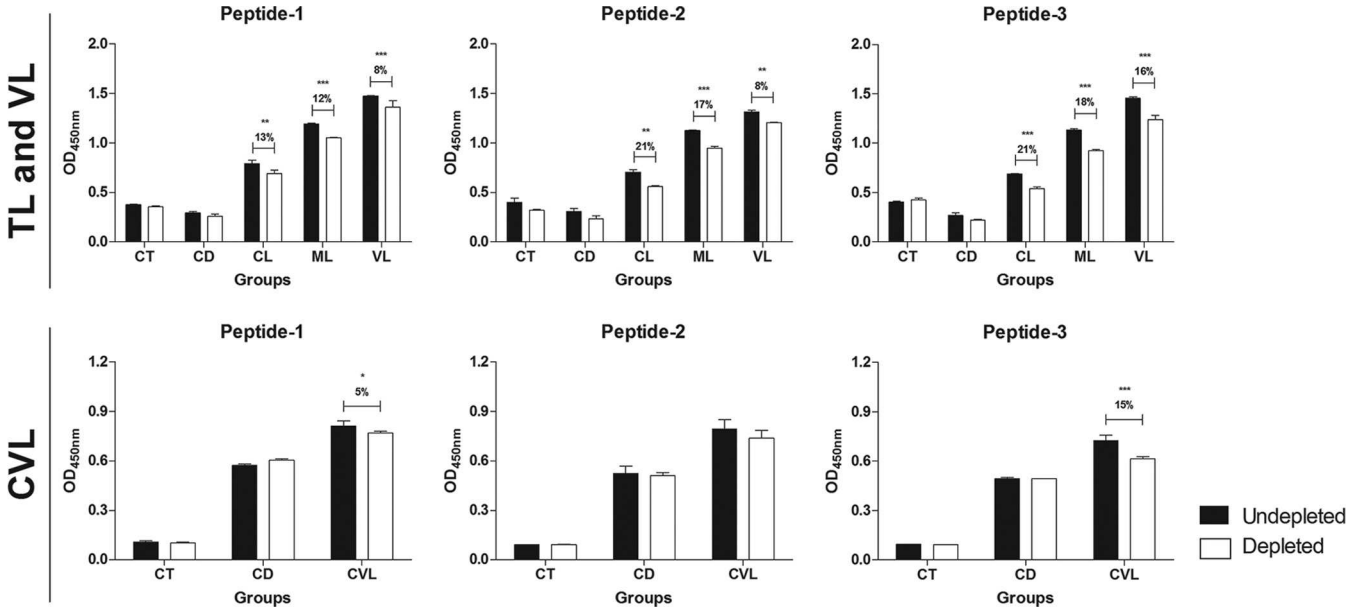

FIG 6 Immunodepletion assay showing specific IgG antibody recognition of the synthetic peptides with known reactivity to HSP83.1. Pools of serum ($n = 10$) from the different groups were depleted with peptide 1, 2, or 3 (CT [control] group; CD [Chagas disease]; CL [cutaneous leishmaniasis]; ML [mucosal leishmaniasis]; VL [visceral leishmaniasis]; CVL [canine visceral leishmaniasis]). The mean antibody OD values are shown on the *y* axis, and the error bars indicate the standard deviation. Significant differences are indicated on the graphs with *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

study to the antigenicity of rHSP83.1, ELISA depletion assays were performed using the synthetic peptides EEDESKKKSCGDEGEP KVE, VTEGGEDKKK, and EVAEAPPAEAAPA, which correspond to the predicted linear B-cell epitopes present in the protein (Fig. 6). In this assay, the IgG reactivity against rHSP83.1 after the depletion of antibodies against peptide 1 was reduced by 13% for the CL ($P < 0.01$), 12% for the ML ($P < 0.001$), 8% for the VL ($P < 0.001$), and 5% for the CVL ($P < 0.05$) group. For peptide 2, we observed a reduction of 21% for the CL ($P < 0.01$), 17% for the ML ($P < 0.001$), 8% for the VL ($P < 0.001$), and no reduction for the CVL group. The depletion of antibodies against peptide 3 resulted in a reduction of 21% for the CL ($P < 0.001$), 18% for the ML ($P < 0.001$), 16% for the VL ($P < 0.001$), and 15% for the CVL ($P < 0.001$) group in the reactivity against rHSP83.1. No significant differences in the reduction of reactivity after depletion were observed in the human and canine CT and CD groups for any of the peptides evaluated. Our results suggest that specific antibodies against peptides 1 and 3 contribute significantly to the overall antigenicity of rHSP83.1 in all *Leishmania*-infected groups; however, peptide 2 contributes to the antigenicity of this protein in the TL and VL groups only.

DISCUSSION

Genome-wide epitope predictions for pathogenic microorganisms have broadened the range of target epitopes and have provided clues to enhance peptide immunogenicity. Among the methods of selection, epitope mapping is a very useful procedure that has vast applications in the areas of antibody production, immunodiagnostics, epitope-based vaccine design, selective deimmunization of therapeutic proteins, and autoimmunity. The identification of these targets was facilitated by the publication of several parasite genomes that together with the availability of algorithms for epitope prediction allow for the use of large-scale approaches for the identification of new antigens (31–33). As an attempt to identify new targets for diagnosing multiple forms of leishmaniasis, we screened the *L. braziliensis* proteome to predict linear B-cell epitopes that are conserved in *Leishmania* spp. and divergent in *H. sapiens*, *C. familiaris*, and *T. cruzi*, the etiologic agent of Chagas disease, which often presents cross-reactivity with leishmaniasis due to sharing multiple common epitopes within the trypanosomatid species (34, 35).

Heat shock proteins (HSPs) are highly conserved molecules in prokaryotes and eukaryotes that play important roles in protein folding, assembly of protein complexes, and the translocation of proteins across cellular compartments (14). Despite the fact that their sequences are highly conserved throughout evolution, it has been proposed that the recognition of epitopes shared by HSPs from different pathogens may provide the immune system with a universal signal of infection (36). In this sense, HSP70 and HSP83 of *L. infantum* have been described as strong mitogens for B lymphocytes in a murine model (37). This phenomenon occurs even in the absence of T lymphocytes and adherent cells, suggesting a direct effect of *Leishmania* HSPs on B cells, and that *Leishmania* may serve to divert the immune response into the nonspecific activation of immune cells (37).

In our study, we identified three linear B-cell epitopes in HSP83.1 of *L. braziliensis* that are conserved within *Leishmania* species but are divergent from the orthologous proteins from human and canine hosts and from *T. cruzi*, the etiological agent of Chagas disease that has been responsible for several reports of cross-reactivity in the serological diagnosis of leishmaniasis. Previous studies have described that heat shock proteins, such as HSP83 and other chaperones, are among the most abundant proteins detected in *Leishmania* antigenic extracts and have obtained promising results that can be applied to the development of diagnostic kits (38–44). Here, the antigenicity of recombinant

HSP83.1 (rHSP83.1) and three epitopes predicted in this protein were tested with sera from the TL, VL, and CVL groups.

rHSP83.1 showed excellent performance in the diagnosis of TL. Not all of the epitopes mapped in this study were recognized by sera from all clinical forms of leishmaniasis. Nevertheless, our results allowed for the identification of epitopes that presented high performance depending upon the species of the parasite. Interestingly, it was possible to identify specific epitopes derived from HSP83.1 that presented high performance for the diagnosis of human TL (peptide 3), both human and canine VL (peptides 1 and 3), and all TL, VL, and CVL clinical manifestations (peptide 3).

In recent years, synthetic peptides have shown high sensitivity and specificity when used as antigens in diagnostic tests (23, 45). These findings have also been associated with several advantages over chemically purified or recombinant antigens, because their production does not involve the manipulation of living organisms, and the peptides can be obtained with a high level of purity. Thus, it is appealing to use these peptides to develop devices to diagnose one or multiple types of leishmaniasis (46).

Comparing conventional and new techniques proposed to be innovative is essential to justify the implementation of the novel methods. In this sense, several authors have described ELISAs using soluble antigens from promastigotes for serological diagnosis that showed high sensitivity for detecting *Leishmania*-infected individuals (34, 35, 47, 48). Despite these findings, the use of these antigens still represents a relevant obstacle because it is common to observe a large number of false-positive reactions in individuals infected with other trypanosomatids due to the sharing of multiple common epitopes used in immunofluorescence assays or ELISAs sensitized with crude antigens (34, 35, 49, 50). As expected, using crude antigens, such as the soluble *L. braziliensis* antigen or the EIE-LVC kit, which uses antigen prepared from *Leishmania major*-like promastigotes, leads to a pronounced number of false positives compared to with rHSP83.1. This is the main limitation to their use in epidemiological surveys in areas where leishmaniasis is endemic (47). Furthermore, different species and strains of *Leishmania* can be used for the production of crude antigen, thus limiting the standardization and reproducibility of test results (51, 52). Therefore, there is a need to standardize an antigen for use in different regions of the world (47).

The serologic enzyme-linked immunosorbent assay is an important tool in the diagnosis of leishmaniasis compared to methods currently used to directly reveal parasites in tissue smears that are invasive, are risky, require considerable expertise, and are inappropriate for use in epidemiological surveillance (53, 54). Given the above, the rational development of a new serological technique featuring high sensitivity and specificity that is also able to discriminate cross-reactivity with other human and canine infections, at the individual level, represents an important innovation in the serological diagnosis of CVL (11).

The present study demonstrates that rHSP83.1 and the epitopes mapped on its sequence might be among the target molecules that could be used in an immunodiagnostic test. This was confirmed by the high sensitivity for diagnosing *Leishmania* infections, the high specificity for discriminating between other human and canine infections, and the high degree of agreement with parasitological techniques for the diagnosis of leishmaniasis. Further prospective studies using large cohorts of negative and positive individuals from areas where leishmaniasis is endemic are necessary to better characterize this approach as a possible marker in the diagnosis of TL, VL, and CVL and for monitoring posttherapeutic cures of TL and VL.

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