UNIVERSIDADE FEDERAL DO PIAUÍ
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO
CENTRO DE CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

LEOPOLDO FABRÍCIO MARÇAL DO NASCIMENTO

IMUNOQUIMIOTERAPIA NA LEISHMANIOSE VISCERAL CANINA

TERESINA/PI
2019

LEOPOLDO FABRÍCIO MARÇAL DO NASCIMENTO

IMUNOQUIMIOTERAPIA NA LEISHMANIOSE VISCERAL CANINA

Tese apresentada ao Programa de Pós-Graduação em Ciência Animal do Centro de Ciências Agrárias da Universidade Federal do Piauí para obtenção do título de Doutor em Ciência Animal.
Área de Concentração: Sanidade e Reprodução Animal.

Orientadora: Profa. Dra. Maria do Socorro Pires e Cruz

TERESINA/PI
2019

FICHA CATALOGRÁFICA
Universidade Federal do Piauí
Biblioteca Setorial do Centro de Ciências Agrárias
Serviço de Processamento Técnico

N244i Nascimento, Leopoldo Fabrício Marçal do
Imunoquimioterepia na leishmaniose viceral canina / Leopoldo Fabrício Marçal do Nascimento - 2019.
84 f. : il.

Tese(Doutorado) – Universidade Federal do Piauí,Programa de Pós-Graduação em Ciência Animal, Teresina, 2019.
Orientação: Profª. Dr.ª Maria do Socorro Pires Cruz

1. Leishmaniose visceral canina 2. Vacina 3. Adjuvante 4.Droga 5. Sinais clínicos 6. Terapêutica I. Título.

CDD 636.08969364

IMUNOQUIMIOTERAPIA NA LEISHMANIOSE VISCERAL CANINA

LEOPOLDO FABRICIO MARCAL DO NASCIMENTO

Tese aprovada em: 26/03/2019

Banca Examinadora:

Profa. Dra. Maria do Socorro Pires e Cruz (Presidente) / DMV/CCA/UFPI

Prof. Dr. Airton Mendes Conde Junior (Interno) / DMORF/CCS/UFPI

Profa. Dra. Lucilene dos Santos Silva (Interna) / Bolsista PNPD-CAPES/UFPI

Profa. Dra. Alessandra Gutierrez de Oliveira (Externa) / UFMS

Profa. Dra. Luciana Pereira Machado (Externa) / UFFS

Pelo amor, conhecimento e apoio, dedico este trabalho,

Aos meus pais Luís e Francisca,

Por tudo isso e muito mais ...

AGRADECIMENTOS

Se hoje concluo mais uma etapa, agradeço a ti Senhor, que tão bem me guiaste e colocaste em meu caminho pessoas que só me fizeram o bem e me permitiram sonhar. Obrigado a ti por me mostrar a cada dia mais, quão certo é o caminho que escolhi.

À Universidade Federal do Piauí, ao Programa de Pós-Graduação em Ciência Animal, a CAPES, pelo apoio financeiro e condições para realização desta pesquisa e a FIOCRUZ-BAHIA, em nome da professora Aldina Barral e professor Manoel Barral por toda colaboração e ajuda necessária.

À minha querida orientadora, professora Maria do Socorro Pires e Cruz, pela confiança, competência, paciência e dignidade no ensino, na pesquisa e por acreditar no meu trabalho. Obrigado por todos os puxões de orelhas, palavras encorajadoras e conselhos compartilhados, sem dúvida alguma esses foram os anos mais felizes e proveitosos academicamente que poderia ter. Obrigado por me acolher como família, como filho e partilhar tantos momentos de alegria.

Aos companheiros de laboratório: Dayane Miranda, Flaviane Alves, Rebecca Tavares, Luana Moura, Nailson Melo, Samuel Souza, Aline Moreira, Marcielly Batista, que participaram ativamente dessa jornada e que fizeram esse sonho se tornar realidade, o trabalho foi mais leve por poder compartilhar tantos momentos juntos. Vocês foram essenciais para essa conquista.

Aos amigos que a vida me deu, Elis Rosélia, Emanuela Moura, Thyago David, Marcos Resende, Thiago Saraiva, Jucenira Duarte, Joilson Duarte e Nair Chaves, pelo incentivo, pela compreensão, pelos momentos de alegria e por terem segurado tantas barras difíceis.

Aos animais, que foram primordiais para esse estudo, a partida de vocês não será em vão. Aprendemos muito com vocês, assumimos o risco de parecer cruéis aos olhos daqueles que estão de fora, mas conscientes de que tudo sempre foi realizado com muito respeito. Obrigado por ensinarem um pouco mais da vida e por serem o maior e o melhor motivo que nos fez chegar onde chegamos.

Aos amigos que se fizeram presentes, mesmo na minha ausência, durante toda essa jornada, o meu muito obrigado.

A todos, minha sincera gratidão.

SUMÁRIO

INTRODUÇÃO 10
1. *Leishmania* e Leishmanioses 10
2. Agente Etiológico, Vetor e Ciclo Biológico 11
3. Leishmaniose Visceral no Cão 13
4. Aspectos Gerais da Imunidade contra a Leishmaniose Visceral 16
5. Diagnóstico da Leishmaniose Visceral Canina 18
5.1. Diagnóstico sorológico 18
5.2. Diagnóstico parasitológico 19
5.3. Métodos moleculares 19
6. Quimioterapia da Leishmaniose Visceral Canina 20
7. Imunoterapia da Leishmaniose Visceral Canina 23
8. Adjuvantes Imunológicos 27
9. Medidas de controle e profilaxia 31
10. Objetivos 33
10.1. Objetivo geral 33
10.2. Objetivos específicos 33
10.3. Desfecho primário 33
10.4. Desfecho secundário 33
Capítulo I: Combination chemo- and immunotherapy provides long term clinical improvement and sustained parasite clearance in L. infantum-infected dogs 36
Capítulo II: Scoring clinical sings can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil 62
Considerações Finais 72
Referências da Revisão de Literatura 73

RESUMO

A leishmaniose é uma doença crônica, causada por protozoários do gênero *Leishmania* e, de acordo com a Organização Mundial da Saúde (OMS), é uma das sete doenças tropicais mais importantes do mundo. O cão é considerado o principal reservatório urbano da enfermidade e os programas de controle da leishmaniose do Brasil ainda recomendam a eutanásia de animais soropositivos. Recentemente foi aprovado o uso do Miltefosine (Milteforan™, Virbac) para o tratamento de cães com LVC, sem, no entanto, avaliar a sua eficácia e a resistência do parasita. Devido à falta de tratamentos eficazes e de baixo custo, esforços consideráveis foram dedicados ao desenvolvimento de vacinas em que vários antígenos de leishmania foram explorados como candidatos promissores. Nesse contexto, considerando que a LV é uma doença fatal e que atualmente não existe uma cura para a doença no cão, o presente trabalho avaliou o uso de uma vacina imunoterápica de proteína recombinante de segunda geração associada com dois adjuvantes de receptores do tipo Toll, sendo uma um agonista TLR4 e outra com os agonistas TRL4 e TRL7, associadas ao alopurinol no tratamento de cães naturalmente infectados por *L. infantum.* A fim de avaliar sua eficácia quanto à cura parasitológica ou melhoria dos sinais clínicos da doença, vinte e oito cães naturalmente infectados por *L. infantum* foram incluídos no estudo e divididos em 4 grupos experimentais. Grupo I: cães que não receberam medicação (n=6); Grupo II: cães que receberam o alopurinol em monoterapia por via oral na dose 20 mg/Kg, 1x ao dia, durante 90 dias (n=8); Grupo III: cães que foram imunizados por injeções subcutâneas com a formulação Leish-F2 + SLA-SE (20 µg cada), 6 doses, com intervalo de 3 semanas mais o alopurinol na dose de 20 mg/Kg, 1x ao dia, durante 90 dias (n=8) e o Grupo IV: cães que foram imunizados por injeções subcutâneas da proteína recombinante Leish-F2 + SLA-IMQ-SE (20 µg cada), 6 doses, com intervalo de 3 semanas mais o alopurinol na dose de 20 mg/Kg, 1x ao dia, durante 90 dias (n=6). Esses cães foram acompanhados durante 1 ano e submetidos a avaliações clínicas e quantificação de carga parasitária de medula óssea em tempos determinados: antes do início do tratamento, 63, 180 e 360 dias após o tratamento. Os animais que receberam os tratamentos com o alopurinol em monoterapia ou em combinação com as formulações vacinas Leish-F2 + SLA-SE ou Leish-F2 + SLA-IMQ-SE, tiveram melhora clínica, mesmo após a suspensão do tratamento, mantendo um baixo escore durante todo o acompanhamento clínico. O alopurinol em monoterapia mostrou-se eficiente em reduzir a carga parasitária durante o tratamento, mas, após a suspensão do mesmo, a carga parasitária dos animais voltou a subir. Já os animais submetidos às formulações vacinas Leish-F2 + SLA-SE e Leish-F2 + SLA-IMQ-SE conseguiram reduzir a carga parasitária e promover melhora clínica, mantendo a carga em níveis indetectáveis mesmo após a suspensão do tratamento e durante todo o tempo de acompanhamento. Em conclusão, o presente estudo apresenta resultados promissores, demonstrando que a combinação do alopurinol com a proteína recombinante Leish-F2 + SLA-SE no tratamento de cães naturalmente infectados por *L. infantum* representa grande avanço para a terapêutica da doença, por promover melhora clínica e conseguir manter em níveis indetectáveis a carga parasitária nesses animais mesmo após a suspensão da terapia. Estudos posteriores devem ser realizados para elucidar os mecanismos da resposta imune responsável pelo sucesso desse estudo.

Palavras-chaves: Leishmaniose Visceral Canina; Vacina; Adjuvante; Droga; Sinais clínicos; Terapêutica.

ABSTRACT

Leishmaniasis is a chronic disease caused by protozoa of the genus *Leishmania* and, according to the World Health Organization (WHO) is one of the seven major tropical diseases in the world. The dog is considered the main urban reservoir of the disease and the programs for leishmaniasis control in Brazil, recommend euthanasia of seropositive animals. Recently the use of Miltefosine (MilteforanTM, Virbac) was approved for the treatment of dogs with LVC, however, its efficacy and resistance for the parasite has not been evaluated. Due to the lack of effective and inexpensive treatments, considerable efforts were devoted to the development of vaccines and several leishmania antigens were explored as promising candidates. In this context, considering that LV is a fatal disease and that there is currently no cure for the disease in the dog, the present study evaluated the use of two second generation recombinant protein vaccines associated with two Toll like receptors adjuvants which one agonist TLR4 and another agonist TRL4 and TRL7, associated with allopurinol in the treatment of dogs naturally infected by *L. infantum.* Efficacy in terms of a parasitological cure or improvement of the clinical signs of the disease was evaluated. Twenty-eight dogs naturally infected by *L. infantum* were included in the study and divided into 4 experimental groups. Group I: dogs that did not receive medication (n=6); Group II: dogs that received allopurinol monotherapy orally at a dose of 20 mg/kg, 1x a day, for 90 days (n=8); Group III: dogs that were immunized by subcutaneous injections with the Leish-F2 + SLA-SE formulation (20 μg each), 6 doses, with a 3-week interval plus alopurinol at a dose of 20 mg/kg, 1x at day for 90 days (n=8) and Group IV: dogs immunized by subcutaneous injections of the recombinant Leish-F2 + SLA-IMQ-SE protein (20 μg each), 6 doses, with a 3-week interval plus allopurinol dose of 20 mg/kg, 1x daily, for 90 days (n=6). These dogs were followed up for 1 year and underwent clinical evaluations and quantification of parasite load in bone marrow samples before the start of treatment, 63, 180 and 360 days after treatment. Animals receiving allopurinol alone or in combination with the Leish-F2 + SLA-SE or Leish-F2 + SLA-IMQ-SE vaccine formulations had a clinical improvement, even after discontinuation of treatment, maintaining a low throughout the clinical follow-up. Allopurinol monotherapy proved to be effective in reducing parasitic burden during treatment, but discontinuation resulted in parasitic load increase. On the other hand, animals submitted to the Leish-F2 + SLA-SE and Leish-F2 + SLA-IMQ-SE vaccine formulations were able to reduce the parasitic load and maintain this load at undetectable levels even after discontinuation of treatment. In conclusion, this study presents promising results, showing that combination of allopurinol with the recombinant Leish-F2 + SLA-SE protein in the treatment of dogs naturally infected by *L. infantum* represents a major advance in the treatment of the disease, with parasite burden on undetectable levels even after the end of therapy. Further studies should be performed to elucidate the mechanisms of the immune response responsible for the success of this treatment schedule.

Key-words: Canine Visceral Leishmaniasis; Vaccine; Adjuvant; Drug; Clinical signs; Therapy.

INTRODUÇÃO

1. *Leishmania* E LEISHMANIOSE

A leishmaniose visceral (LV) é uma doença de curso crônico, causada por protozoários do gênero *Leishmania* e que, de acordo com a Organização Mundial da Saúde (OMS), é uma das sete doenças tropicais mais importantes e representa um grave problema de saúde mundial, possuindo amplo espectro de manifestações clínicas com resultado potencialmente fatal (ALVAR et al., 2012; TORRES-GUERRERO et al., 2017).

A doença é generalizada nas áreas tropicais e subtropicais e encontrada em 97 países da Europa, África, Ásia e América (ALVAR et al., 2012). Em 2015, 7 países (Brasil, Etiópia, Índia, Kênia, Somália, Sudão do Sul e Sudão) relataram mais de 90% dos casos de LV em todo o mundo (Figura 1) (WHO, 2017).

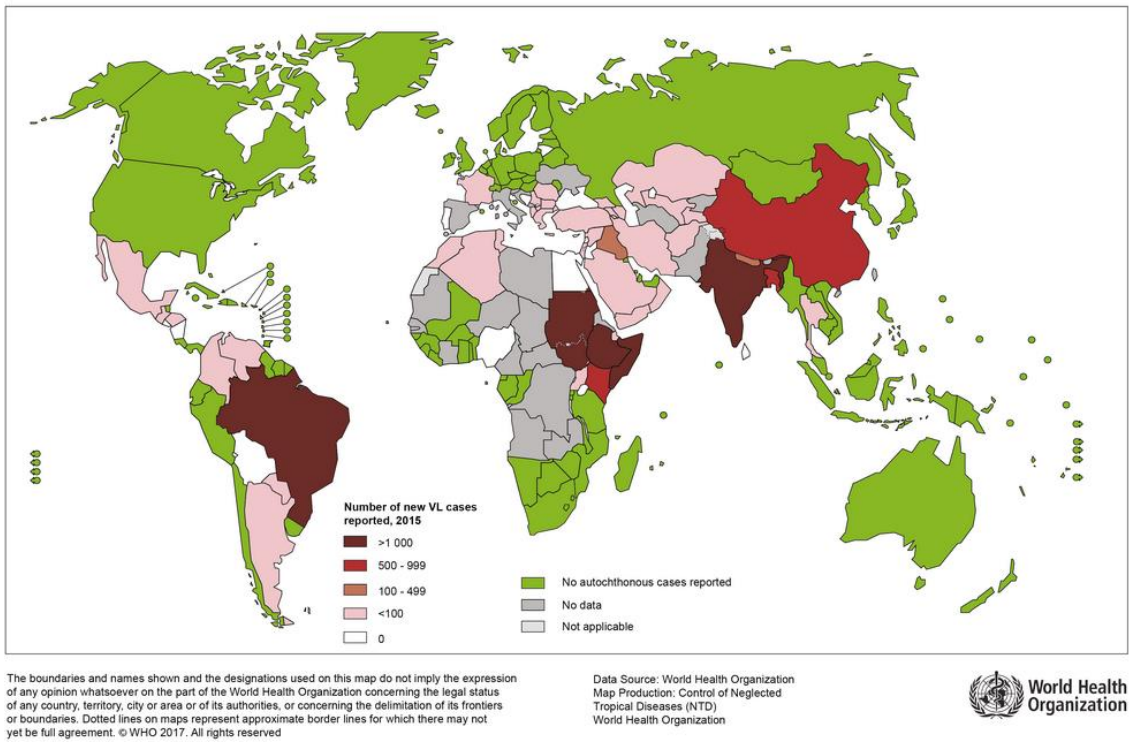


Figura 1: Status da endemicidade da leishmaniose visceral humana no mundo (WHO, 2015)

As primeiras grandes epidemias de LV nas áreas do Brasil ocorreram em Teresina, no Nordeste do Brasil, entre 1981 a 1986, quando foram relatados quase mil casos da doença, a maioria proveniente de bairros recentemente criados na época, resultados de assentamentos de migrantes (COSTA; PEREIRA; ARAÚJO, 1990; DRUMOND; COSTA, 2011). No Brasil, esta doença é um problema de saúde pública com altas taxas de transmissão e letalidade (MAIA-ELKHOURY et al., 2008; HARHAY et al., 2011).

A leishmaniose apresenta um espectro de manifestações clínicas causadas por diferentes espécies de *Leishmania*, que são parasitas intracelulares com ciclo de vida complexo, que requerem um hospedeiro susceptível e um vetor permissivo (AKHOUNDI et al., 2016; TORRES-GUERRERO et al., 2017). Assim as leishmanioses podem assumir diferentes formas clínicas que são englobadas em dois principais grupos: a Leishmaniose Visceral (LV) e a Leishmaniose Tegumentar (LT).

A LV geralmente afeta o baço, fígado ou outros tecidos linfoides e, se não for tratada, é fatal (MURRAY et al., 2005; ANTINORI; SCHIFANELLA; CORBELLINO, 2012). Já a LT pode ser classificada com base em seus aspectos clínicos, patológicos e imunológicos em leishmaniose cutânea localizada, quando o paciente apresenta lesões ulcerosas indolores únicas ou múltiplas; a cutaneomucosa, caracterizada por lesões mucosas agressivas nas regiões nasofaríngeas ou ainda como cutânea difusa, que se apresenta em lesões nodulares não ulceradas espalhadas pelo corpo (GHARBI et al., 2015).

2. AGENTE ETIOLÓGICO, VETOR E CICLO BIOLÓGICO

Os parasitas do gênero *Leishmania* (ROSS, 1903) são flagelados da Família Trypanosomatidae, Ordem Kinetoplastida, Filo Protozoa e incluem vários protozoários obrigatórios de seres humanos e mamíferos domésticos e selvagens. Com base na localização no intestino do vetor, (LAINSON; RYAN; SHAW, 1987) distinguiram dois subgêneros: *Leishmania* (que inclui o complexo *donovani*, *tropica*, *major*, *aethiopica*, *mexicana*), que se replica no intestino médio e o *Viannia* (que inclui o complexo *braziliensis, guyanensis, lainsoni, naifi*), que se replica no intestino posterior (READY, 2013; GHARBI et al., 2015).

No Velho Mundo as principais espécies são: *Leishmania (Leishmania) aethiopica, Leishmania (Leishmania) major, Leishmania (Leishmania) donovani* e nas Américas são *Leishmania (Leishmania) infantum chagasi, Leishmania* (*Viannia*) braziliensis, *Leishmania* (*Viannia*) *pananmensis, Leishmania* (*Viannia*) *guyanensis, Leishmania (Leishmania) amazonensis, Leishmania (Leishmania) mexicana* (SARIDOMICHELAKIS, 2009).

Considerando a presença ou ausência de reservatórios de animais para *Leishmania*, dois tipos básicos de ciclos epidemiológicos são observados: a forma zoonótica, geralmente causada pela *Leishmania infantum*, sinônimo de *Leishmania chagasi*, nas

Américas, Oriente Médio, Ásia Central, China e Mediterrâneo, tendo cães como os principais reservatórios e a forma antropozoonótica com transmissão de humanos para humanos sem o reservatório animal envolvido no ciclo de transmissão, causada principalmente pela *Leishmania donovani* que é prevalente na África Ocidental, Bangladesh, Índia e Nepal (GRAMICCIA, 2011; PALATINIK-DE-SOUSA; DAY, 2011; VAN GRIENSVEN; DIRO, 2012; ROATT et al., 2014) .

A transmissão dos protozoários desse gênero se dá entre hospedeiros humanos e animais pela picada de fêmeas de flebotomíneo infectadas no ato do repasto sanguíneo (Figura 2), sendo estas pertencentes ao gênero *Phlebotomus*, encontrado nos continentes europeus e *Lutzomyia*, nas Américas (MACHADO-COELHO et al., 1999; MAROLI et al., 2013; MCGWIRE; SATOSKAR, 2014). Das aproximadamente 900 espécies de flebotomíneos, em torno de 70 têm sido implicadas na transmissão da leishmaniose (READY, 2013). No Brasil, o principal vetor envolvido na transmissão do parasita é o flebotomíneo da espécie *Lutzomyia longipalpis*, mas no Estado do Mato Grosso do Sul (MS) já se tem registrado transmissão através da espécie *Lutzomyia cruzi* (BRITO et al., 2014; FALCÃO DE OLIVEIRA et al., 2017).

Os parasitas desse gênero são digenéticos (heteróxenos) e apresentam em seu ciclo de vida apenas duas formas evolutivas: a forma promastigota, que é flagelada e extracelular, e a forma amastigota, que é intracelular e sem movimento. As promastigotas apresentam corpo alongado, medindo entre 14 e 20µm e flagelo livre. As amastigotas têm corpo ovoide, medindo entre 2,1 e 3,2µm e flagelo interno (PESSOA; MARTINS, 1982).

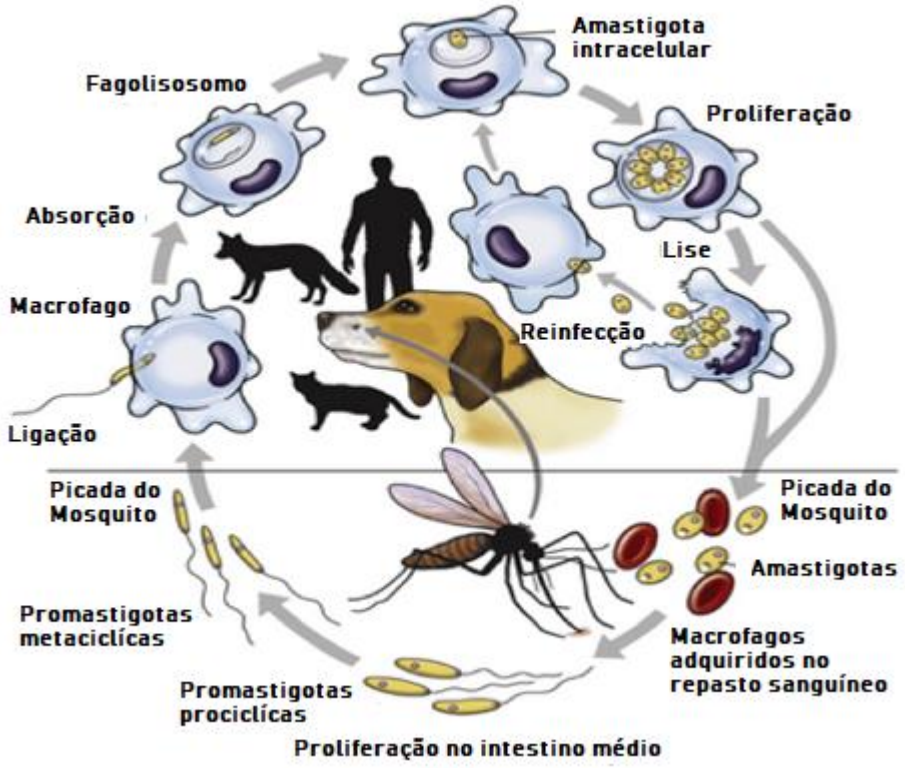


Figura 2: Ciclo de vida da *Leishmania infantum*, onde cães, gatos e humanos são infectados quando picados pelo flebotomíneo (SYKES; BANETH; PETERSEN, 2014).

3. LEISHMANIOSE VISCERAL NO CÃO

Como muitas outras doenças zoonóticas transmitidas por vetores, a transmissão da infecção para as populações humanas está ligada a múltiplos fatores sociais e econômicos que colocam o ser humano em contato frequente com os insetos transmissores da doença (COSTA; PEREIRA; ARAÚJO, 1990). No caso particular da leishmaniose visceral, o ciclo natural de transmissão da floresta entre mamíferos silvestres e vetores flebotomíneos tem gradualmente mudado o modo de transmissão enzoótica para periurbana e urbana.

A adaptação do vetor aos ambientes urbanos e a migração de seres humanos para a periferia das cidades são consideradas as principais razões para esta mudança epidemiológica, em que os cães se tornaram o principal reservatório conhecido da doença (BELO et al., 2013b; TRAVI, 2014), representando assim, a principal fonte de infecção para os vetores desta enfermidade potencialmente fatal.

Em virtude da alta prevalência de infecção e o intenso parasitismo cutâneo, independentemente de sua apresentação clínica (DE QUEIROZ et al., 2011; LAURENTI et al., 2013; COURA-VITAL et al., 2014), o Ministério da Saúde adota no Brasil a

eliminação de cães quando soropositivos para *L. infantum* como medida de controle em áreas endêmicas (BRASIL, 2014, 2016).

Embora preconizada no Brasil, a eliminação de cães soropositivos é cada vez mais discutida e avaliada (NUNES et al., 2010; COSTA et al., 2013; MACHADO; SILVA; VILANI, 2016), particularmente quanto ao seu efeito na redução da prevalência de doenças humanas e caninas e sua aceitação pelos proprietários de animais e instituições de proteção animal (TESH, 1995; ZUBEN; DONALÍSIO, 2016).

A patogênese da Leishmaniose Visceral Canina (LVC) envolve vários fatores, sendo um dos mais decisivos a progressão da doença estar associada à resposta imune que o animal desenvolve contra o parasita. Neste caso os anticorpos, em vez de ter a função de proteção, tornam-se altamente prejudiciais, ao participarem nos processos inflamatórios e serem responsáveis por muitos sinais clínicos associados à LVC (FREITAS et al., 2012; CANTOS-BARREDA et al., 2018), que podem acometer a pele, órgãos viscerais como: fígado, rins ou intestino, olhos, articulações e membranas mucosas.

Os depósitos de complexos imunes, compostos, principalmente, por anticorpos IgG e IgM em diferentes zonas anatômicas (especialmente nos rins e olhos) são muito característicos (BRITO et al., 2004; CIARAMELLA et al., 1997; GARCÍA-ALONSO et al., 1996; KASZAK; PLANELLAS; DWORECKA-KASZAK, 2015).

As manifestações clínicas da LVC são muito variáveis, pois as características clínicas de cada animal, bem como a evolução da doença dependem de complexas interações entre o parasita e as respostas imunológicas e genéticas de cada hospedeiro (BELO et al., 2013a; DE VASCONCELOS et al., 2017; HOSEIN; BLAKE; SOLANO-GALLEGO, 2017).

As lesões cutâneas são as principais queixas dos proprietários. Os cães infectados podem apresentar diferentes características clínicas que variam de aparentemente saudáveis (cães assintomáticos) a vários sinais característicos (cães sintomáticos), dentre os distúrbios da pele, as manifestações mais frequentes são: dermatite esfoliativa não pruriginosa com ou sem alopecia, dermatite erosiva-ulcerativa, dermatite nodular, papular ou pústula (SOLANO-GALLEGO et al., 2011; SILVA et al., 2017; RIBEIRO et al., 2018).

Dentre os problemas oculares a blefarite, uveíte e ceratoconjuntivite são observadas. Às vezes, observam-se também claudicação, epistaxis, problemas vasculares

ou neurológicos. No entanto, os sinais clínicos podem ser variáveis e inespecíficos (Figura 3). Na maioria dos casos, a linfadenomegalia, a apatia, a emaciação e a atrofia muscular também são observadas (CIARAMELLA et al., 1997; GHARBI et al., 2015; KASZAK; PLANELLAS; DWORECKA-KASZAK, 2015) e falhas renais são as causas de morte mais frequentes (GIUNCHETTI et al., 2008; SOLANO-GALLEGO et al., 2011). Ambos os cães, assintomáticos e sintomáticos, podem ser infecciosos para os flebotomíneos, transmissores da leishmaniose (GRAMICCIA, 2011; LAURENTI et al., 2013; RIBEIRO et al., 2018).



Figura 3: Principais sinais clínicos encontrados em cães com leishmaniose visceral. (**A)** Emagrecimento; **(B)** Onicogrifose; **(C)** Dermatite esfoliativa; **(D)** úlcera de lábio; **(E)** úlcera de focinho; **(F)** úlcera em ponta de orelhas; **(G)** despigmentação de lábios; **(H)** blefarite/uveite; **(I)** hiperqueratose de focinho (Arquivo Pessoal).

As alterações hematológicas e bioquímicas na leishmaniose canina não são específicas (PALTRINIERI et al., 2016) e embora limitados para diagnóstico de LVC, são muito úteis para avaliação do estado clínico do animal e extensão das lesões, podendo fornecer indicações sobre o prognóstico da doença (REIS et al., 2006a; IKEDA-GARCIA et al., 2007; BRAZ et al., 2015).

As alterações mais frequentemente relatadas em relação ao eritrograma de animais infectados são anemia normocítica e normocrômica e trombocitopenia (ABBEHUSEN et al., 2017; MELÉNDEZ-LAZO et al., 2018; MIRÓ; LÓPEZ-VÉLEZ, 2018; RIBEIRO et al., 2018). Em relação à contagem diferencial de leucócitos, deve-se considerar que o comportamento leucocitário varia com diversos fatores, como: status imunológico, gravidade dos sinais clínicos, presença de quadros infecciosos e ou parasitários associados (BUSH, 2004; BRAZ et al., 2015). Já a hiperglobulinemia associada à hipoalbuminemia, com diminuição da relação albumina globulina são as principais características bioquímicas da leishmaniose canina (SARIDOMICHELAKIS, 2009; TORRECILHA et al., 2017; MELÉNDEZ-LAZO et al., 2018; RIBEIRO et al., 2018).

4. ASPECTOS GERAIS DA IMUNIDADE CONTRA A LEISHMANIOSE VISCERAL

A eficácia da resposta imune é um aspecto fundamental na patogênese da doença e sua progressão, pois desempenha um papel crucial nas manifestações clínicas da LVC. Nem todos os cães expostos a *L. infantum* desenvolvem manifestações clínicas e as infecções subclínicas são mais frequentes que as doenças clínicas (SARIDOMICHELAKIS, 2009). Portanto, *L. infantum* em cães pode se manifestar como infecção subclínica crônica, doença autolimitada ou doença grave (SOLANO-GALLEGO et al., 2009; RIBEIRO et al., 2018). Essas diferenças são devidas à resposta imune individual, onde uma resposta imune celular apropriada é essencial para controlar a infecção (KASZAK; PLANELLAS; DWORECKA-KASZAK, 2015; HOSEIN; BLAKE; SOLANO-GALLEGO, 2017; RIBEIRO et al., 2018).

Em humanos (KAYE; AEBISCHER, 2011), camundongos (LIEW; O'DONNELL, 1993) e cães (KOUTINAS; KOUTINAS, 2014; HOSEIN; BLAKE; SOLANO-GALLEGO, 2017; PEREIRA-FONSECA et al., 2017), a imunidade protetora contra a leishmaniose depende do desenvolvimento de respostas imunes mediadas por linfócitos T CD4+, tipo T Helper 1 (Th1), caracterizadas pela produção inicial de Interluecina-12

(IL-12) pelas células apresentadoras de antígenos (APCs), que induzem a secreção de interferon gama (IFN-γ) e fator de necrose tumoral-α (TNF-α) (RODRIGUES et al., 2016; HOSEIN; BLAKE; SOLANO-GALLEGO, 2017; PEREIRA-FONSECA et al., 2017), estas por sua vez, induzirão a ativação dos mecanismos microbicidas dos macrófagos.

Induzem a produção de óxido nítrico (NO) e espécies reativas de oxigênio (ROS), que são altamente eficazes na morte de formas amastigotas intracelulares (KAYE; SCOTT, 2011; PEREIRA-FONSECA et al., 2017), enquanto a resposta imune mediada por células de perfil Th2 e predomínio de linfócitos T CD8+ (KAYE; SCOTT, 2011; RODRIGUES et al., 2016), com produção de citocinas anti-inflamatórias, como a IL-4, IL-10, IL-13 e fator transformador de crescimento (TGF)-β, com resposta humoral exuberante, está relacionada à doença progressiva (KOUTINAS; KOUTINAS, 2014; RODRIGUES et al., 2016; HOSEIN; BLAKE; SOLANO-GALLEGO, 2017; TORRES-GUERRERO et al., 2017; CARVALHO et al., 2018).

Dessa forma a LVC grave geralmente está associada a títulos significativos de anticorpos de todas as classes, enquanto níveis baixos de anticorpos são característicos de infecções subclínicas ou cães expostos, mas não infectados (SARIDOMICHELAKIS, 2009; LIMA et al., 2017). Embora o uso de diferentes isotipos de anticorpos tenha sido proposto para melhorar as avaliações sorológicas (INIESTA et al., 2005; DAY, 2007; FREITAS et al., 2012), os dados mais recentes de uma coorte de 134 cães sugerem que as respostas dos isotipos não têm maior valor preditivo (LARANJEIRA et al., 2014).

O sistema imune inato tem mecanismos específicos para reconhecer rapidamente o parasita. Um componente importante do reconhecimento de patógenos compreende a família de receptores Toll-like (TLRs), receptores de reconhecimento de padrões (PRRs) e responsáveis por detectar padrões moleculares associados a patógenos (PAMPs) de vários grupos de microrganismos (OSPELT; GAY, 2010), incluindo *Leishmania spp.* (TUON et al., 2008; CHAUHAN et al., 2017) e que reconhecem Padrões Moleculares Associados a Danos (DAMPs), que são proteínas derivadas do hospedeiro (MIYAKE, 2007; MIDWOOD; PICCININI, 2010) na inflamação crônica, desencadeando uma cascata de reações imunes (FUKATA; VAMADEVAN; ABREU, 2009).

Até o momento, 13 TLRs foram descritos em mamíferos. Dez TLRs são expressos em humanos, 12 em camundongos (CHAUHAN et al., 2017) e 10 em cães (CUSCÓ et al., 2014).

5. DIAGNÓSTICO DA LEISHMANIOSE VISCERAL CANINA

Os métodos diagnósticos realizados para a LVC podem ser classificados em métodos sorológicos [quantitativos tais como o reação de imunofluorescência indireta (RIFI) e o ensaio imunoabsorvente ligado à enzima (ELISA) e qualitativos como os testes rápidos]; métodos parasitológicos e métodos moleculares [reação em cadeia da polimerase (PCR) convencional, nested e em tempo real]; (SOLANO-GALLEGO et al., 2011).

5.1. Diagnóstico sorológico

Apesar da grande variedade de métodos diagnósticos disponíveis, no Brasil, os testes sorológicos autorizados e recomendados pelo Ministério da Saúde (MS) possuem sensibilidade e especificidade bastante variáveis. Por meio da nota técnica conjunta N° 1/2011 – CGDT-CGLAB/DEVIT/SVS/MS, o MS substituiu o protocolo de diagnóstico da LVC, sendo utilizado atualmente o Teste Imunocromatográfico TRDPP® Leishmaniose Visceral Canina (Dual Path Plataform, Biomanguinhos), que utiliza a proteína recombinante rK28 (fusão do rK9, rK39 e rK26) como teste de triagem e o ELISA® – (Kit EIE Leishmaniose Visceral Canina - Biomanguinhos) como teste confirmatório, no lugar da reação de imunofluorescência indireta - Biomanguinhos (RIFI®) (Brasil 2011). Falhas nesses resultados podem causar a eliminação de animais não infectados, e, por outro lado, não detectar casos positivos, favorecendo a disseminação da doença (PEIXOTO; DE OLIVEIRA; ROMERO, 2015).

O TRDPP® foi desenvolvido para detectar anticorpos contra antígenos rK9, rK39 e rK26 e apresenta alta especificidade (96%) e baixa sensibilidade (47%) para identificar cães sem sinais clínicos de LV. No entanto, na presença de sinais, a sensibilidade do teste aumenta (98%) (GRIMALDI et al., 2012).

Por outro lado, Laurenti et al. (2014), em um estudo comparativo do desempenho do TRDPP® com o ELISA produzido pela BioManguinhos (Brasil), utilizando amostras de soro de cães infectados clinicamente sintomáticos e assintomáticos de uma área endêmica de LVC, mostraram que o TRDPP® apresentou sensibilidade de 90,6% e especificidade de 95,1%, enquanto o ELISA mostrou a mesma sensibilidade (90,6%), mas baixa especificidade (77,8%). Além disso, eles mostraram que o TRDPP® forneceu uma maior precisão de 92,7%. Esses dados indicam que o TRDPP® teve bom desempenho como teste sorológico para LVC e detectou cães assintomáticos e

sintomáticos em proporções iguais em área endêmica (LAURENTI et al., 2014). O TRDPP® como um teste de triagem tem vantagens sobre o ELISA, devido à sua fácil manipulação e capacidade de mostrar resultados em 15 minutos; podendo ser usado tanto no campo quanto no laboratório (SANTIS et al., 2013).

5.2 Diagnóstico parasitológico

O diagnóstico parasitológico continua sendo o padrão ouro no diagnóstico da leishmaniose, devido à sua alta especificidade (HERWALDT, 1999). Os esfregaços de lesões sugestivas, aspirados do baço, medula óssea e linfonodos são examinados ao microscópio após a coloração com Giemsa, Leishman ou Panótico® (AKHOUNDI et al., 2017).

É uma abordagem rápida e barata, e fornece apenas informações quantitativas parciais sobre a carga parasitária, não permitindo discriminação entre espécies de *Leishmania*. Além disso, a sensibilidade é altamente dependente do número e da dispersão de parasitas na biópsia, bem como das habilidades técnicas de quem executa os testes. No entanto, em áreas endêmicas, ainda é uma abordagem diagnóstica útil devido à sua relação custo-benefício e simplicidade (HERWALDT, 1999; AKHOUNDI et al., 2017).

Embora o teste parasitológico seja considerado 100% específico, sua sensibilidade pode variar de menos de 30% a 100% em esfregaços obtidos por punção de linfonodos, e de 6,2% a 100% em esfregaços obtidos de medula óssea (REALE et al., 1999; SARIDOMICHELAKIS et al., 2005), a sensibilidade é menor em animais assintomáticos (SARIDOMICHELAKIS et al., 2005).

No isolamento em meio de cultura *in vitro* as formas amastigotas do parasita modificam-se em formas promastigotas a partir de aspirados teciduais, raspados ou biópsias. Infelizmente, a recuperação de parasitas em cultura raramente demonstra eficiência superior a 70%, mesmo para cepas que são facilmente mantidas *in vitro*. A sensibilidade mais alta (98%) é alcançada quando se utiliza aspirado do baço. A punção esplênicas é considerada um procedimento invasivo e exige ambientes apropriados para a coleta (BRASIL, 2014).

5.3. Métodos moleculares

A PCR é uma técnica de biologia molecular para a replicação enzimática de DNA sem o uso de organismos vivos, como *Escherichia coli* ou levedura. A técnica permite

que uma pequena quantidade da molécula de DNA seja amplificada muitas vezes, de forma exponencial. Com mais DNA disponível, a análise é muito mais fácil. A PCR é comumente usada em laboratórios de pesquisa médica e biológica para uma variedade de tarefas, como a detecção de doenças hereditárias, a identificação de impressões digitais genéticas, o diagnóstico de doenças infecciosas, a clonagem de genes, testes de paternidade e computação de DNA (RAHMAN et al., 2013).

A PCR permite a amplificação de DNA ou RNA através de ciclos repetitivos *in vitro*. A descrição de vários fragmentos de DNA específicos da espécie tornou este método viável para o diagnóstico da leishmaniose desde o início da década de 1990 (SMYTH et al., 1992). A PCR é superior a outros métodos parasitológicos, como microscopia ou cultivo, particularmente para amostras com baixa carga parasitária (PAIVA-CAVALCANTI; REGIS-DA-SILVA; GOMES, 2010).

Além do diagnóstico, a PCR tem aplicações na quantificação dos parasitas e, portanto, também monitora a progressão da doença, prevendo e controlando o resultado da terapia anti-Leishmania, definindo características específicas do parasita, como virulência ou resistência a drogas e discriminação de espécies (AKHOUNDI et al., 2017).

Três diferentes técnicas de PCR estão disponíveis: PCR convencional, nested-PCR e PCR em tempo real (GOMES et al., 2008; MAIA; CAMPINO, 2008). A PCR em tempo real é uma PCR quantitativa, o que permite a medição confiável de produtos de DNA gerados pelo monitoramento da amplificação da molécula de DNA alvo durante cada ciclo de PCR, podendo ser usada quantitativamente ou semi-quantitativamente (PAIVA-CAVALCANTI; REGIS-DA-SILVA; GOMES, 2010).

6. QUIMIOTERAPIA DA LEISHMANIOSE VISCERAL CANINA

A quimioterapia consiste em fármacos com efeitos leishmanicidas ou leishmanistáticos que são capazes de reduzir a carga parasitária em cães doentes, onde uma alta porcentagem de animais tratados passam a atingir um estado de remissão de doença clínica por longos períodos de tempo (GUARGA et al., 2002; MIRÓ et al., 2011). Atualmente não há tratamento disponível para alcançar uma cura 100% em cães.

Poucos medicamentos estão disponíveis para o tratamento da leishmaniose e a maioria deles está em uso há bastante tempo, dentre eles estão o antimonial pentavalente que foi a primeira classe de drogas aplicadas ao tratamento da leishmaniose e juntamente com a anfotericina B, pentamidina, miltefosine e paromomicina constituem os principais

recursos disponíveis para a quimioterapia das leishmanioses (ULIANA; TRINCONI; COELHO, 2017; RIBEIRO et al., 2018).

No Brasil o tratamento da Leishmaniose Visceral Canina (LVC) é regulamentado pela portaria Interministerial n° 1.426 de 11/07/2008, que proíbe o tratamento da LVC com drogas de uso humano (Anfotericina B, Anfotericina Lipossomal e Antimoniato) ou outras drogas não registradas no MAPA.

Recentemente o licenciamento do medicamento Miltefosine (Milteforan^{TM}, Virbac) foi emitido, através da Nota Técnica Conjunta n° 001/2016 – MAPA/MS, permitindo assim o tratamento de cães com LVC. No entanto, foram levantadas questões sobre a sua eficácia e a resistência do parasita, incluindo outras desvantagens como recaídas ou normalização mais lenta de variáveis clínico-patológicas (ANDRADE et al., 2011; PROVERBIO et al., 2014; MANNA et al., 2015; MONDELAERS et al., 2016).

Vários protocolos terapêuticos foram usados para a LVC, a maioria estabelecidos em países europeus (REGUERA et al., 2016; MIRÓ et al., 2017) utilizando drogas como antimonial pentavalente, anfotericina B convencional ou encapsuladas em lipossomas, sulfato de aminosidina, alopurinol, pentamidina, marbofloxacina e metronidazol, isoladamente ou em combinação, com recuperação clínica variável (OLIVA et al., 2010; ROATT et al., 2014; REGUERA et al., 2016; PINEDA et al., 2017; ULIANA; TRINCONI; COELHO, 2017; RIBEIRO et al., 2018).

Na medicina veterinária, o alopurinol (um análogo de purina) é considerado o medicamento de primeira linha para tratamento de longo prazo da LVC. O alopurinol é uma droga hipoxantínica administrada por via oral que é metabolizada por parasitas de *Leishmania* para produzir um análogo inativo da inosina, que é incorporado ao RNA da *Leishmania*, causando assim uma tradução deficiente da proteína (MANNA et al., 2009).

O alopurinol é uma droga amplamente utilizada para o tratamento da LVC, isoladamente (em monoterapia) ou em combinação com antimoniais pentavalentes ou miltefosine, devido à sua toxicidade relativamente limitada, sua eficiência em melhorar o estado clínico, baixo custo e a vantagem da administração oral (SOLANO-GALLEGO et al., 2011; MANNA et al., 2015).

A droga é comumente usada dentro de um intervalo de dose de 10 a 30 mg/kg por dia para terapia de manutenção de longo prazo e os efeitos colaterais são incomuns (SOLANO-GALLEGO et al., 2011; MANNA et al., 2015). Em cães, a eficácia do alopurinol em produzir remissão clínica é semelhante à dos antimoniais pentavalentes,

embora ambos os fármacos, usados isoladamente ou em associação, não alcancem a erradicação completa dos parasitas (KOUTINAS et al., 2001).

Poucos efeitos colaterais do alopurinol são conhecidos em cães, mas o uso prolongado dessa droga pode resultar na formação de urólitos xantínicos (TORRES et al., 2016). Recentemente foi publicado o primeiro relato de resistência ao alopurinol em parasitas de *L. infantum* isolados de cães, e isso foi associado à recidiva clínica (YASUR-LANDAU et al., 2016).

O antimoniato de meglumina (Glucantime®) é amplamente utilizado no tratamento da LVC na Europa e em outras partes do mundo (REGUERA et al., 2016; MIRÓ et al., 2017). O mecanismo de ação dos antimoniais pentavalentes continua pouco compreendido, mas parece ser um evento que envolve diversos aspectos do metabolismo do parasita. Tem sido sugerido que algumas particularidades químicas na composição destes fármacos podem contribuir para seus efeitos farmacológicos. Por exemplo, os carboidratos, como o ácido glicônico (presente no Pentostan® ou no Glucantime®), são capazes de formar complexos solúveis em água com o átomo de antimônio, o que pode distribuir os agentes antimoniais para os macrófagos infectados (ROBERTS; BERMAN; RAINEY, 1995; SERENO et al., 2001).

Vários estudos avaliaram a eficácia do antimoniato de meglumina com protocolos que variavam na administração da via, dose e duração. O antimoniato de meglumina tem sido usado por via subcutânea (SC) (a cada 12, 24 ou 48 h), intramuscular (IM) (a cada 24 ou 48 h) ou por via intravenosa (IV) por um período inicial variando entre uma semana e meia e 7 semanas, até 25 semanas. A dose diária total varia entre 50 mg e 100 mg/kg/dia (NOLI; AUXILIA, 2005; PARADIES et al., 2012; MANNA et al., 2015; SOLANO-GALLEGO et al., 2016).

No cão, a farmacocinética do antimoniato de meglumina foi estabelecida (TASSI et al., 1994; VALLADARES et al., 1996), tendo uma meia vida curta: 21, 42 e 122 minutos, quando administrada por via IV, IM e SC respectivamente e sendo 80% a 95% do antimônio excretada pela urina entre 6 e 9 horas após a administração (TASSI et al., 1994; VALLADARES et al., 1996; SLAPPENDEL; TESKE, 1997). Injeções intramusculares de antimoniais pentavalentes podem causar efeitos colaterais graves, incluindo fibrose muscular e formação de abscesso. Outros efeitos colaterais dos antimoniais incluem distúrbios gastrointestinais, dor muscular tardia e rigidez articular (SLAPPENDEL; TESKE, 1997).

O Miltefosine foi inicialmente desenvolvido como uma droga anticancerígena, sendo a primeira droga oral eficaz para Leishmaniose Visceral Canina (LVC), e representa um grande avanço no tratamento da doença (CROFT; COOMBS, 2003).

O nome químico da miltefosine é hexadecil 2-(trimetilazanil) etil fosfato, um éter de fosfocolina com cadeia alquílica de 16 carbonos, também conhecido como hexadecilfosfocolina de fórmula empírica ($C_{21}H_{45}NO_4P$), resultando em um peso molecular de 407,57 g/mol (DORLO et al., 2012; NO, 2016).

A principal atividade antileishmania da droga deve-se à modulação dos receptores da superfície celular, do metabolismo do inositol, da ativação da fosfolipase e da proteína cinase-C em vias mitogênicas, resultando em apoptose (VERMA; DEY, 2004; DORLO et al., 2012).

Os principais efeitos colaterais da droga incluem distúrbios gastrointestinais, mas os sintomas são transitórios ou reversíveis; entretanto, a teratogenicidade é um problema grave (SUNDAR; OLLIARO, 2007). Em cães não foram relatados efeitos nefrotóxicos e o vômito foi o efeito colateral mais comum (WOERLY et al., 2009). No entanto, questões sobre sua eficiência e resistência do parasita foram levantadas (ANDRADE et al., 2011; PROVERBIO et al., 2014).

Além das drogas citadas, existem outros produtos que são propostos para modular a resposta imune, imunoestimulando o organismo animal, como a domperidona, citocinas e vacinas (imunoterapia) (GÓMEZ-OCHOA et al., 2009; SABATÉ et al., 2014; REGUERA et al., 2016).

7. IMUNOTERAPIA DA LEISHMANIOSE VISCERAL CANINA

A vacinação é considerada uma das formas mais econômicas/efetivas de controlar a infecção por *Leishmania*. No entanto, nenhuma vacina contra a leishmaniose humana está atualmente disponível. Vários candidatos foram propostos nas últimas décadas (KUMAR; ENGWERDA, 2014). Alguns se mostraram imunogênicos e conferiram proteção contra *Leishmania* em modelos de roedores. No entanto, a maioria deles foi descartada depois de provar ser ineficaz em grandes animais (GRADONI et al., 2005; KUMAR; ENGWERDA, 2014).

Devido à falta de tratamentos eficazes e de baixo custo, esforços consideráveis foram dedicados ao desenvolvimento de vacinas e, embora estudos iniciais tenham sofrido com efeitos colaterais desagradáveis, resultando em complicações clínicas, vários

antígenos de *Leishmania* foram explorados como candidatos promissores para uma vacina. Esses antígenos incluíam parasitas de *Leishmania* mortos ou vivos atenuados (primeira geração), proteínas recombinantes de *Leishmania* (segunda geração), DNA codificador de proteínas *Leishmania* (terceira geração) e imunomoduladores (ABDIAN et al., 2011; ARAÚJO et al., 2011; NAGILL; KAUR, 2011; SINGH; SUNDAR, 2012; ALVAR et al., 2013; KUMAR; ENGWERDA, 2014; GRADONI, 2015).

Nas últimas décadas, as vacinas de segunda geração passaram a ser amplamente utilizadas e são baseadas na definição de subunidades sintéticas ou recombinantes geneticamente modificada de *Leishmania sp*., através de bactérias recombinantes ou de vírus que carreiam genes do antígeno de *Leishmania* (CONNELL et al., 1993; MCMAHON-PRATT et al., 1993) e nas frações nativas purificadas dos parasitas (PALATNIK et al., 1989; JARDIM et al., 1991; RACHAMIM; JAFFE, 1993).

Várias proteínas de *Leishmania* foram identificadas, com base na abundância e localização de superfície (HANDMAN et al., 1986), clones de células T, triagem de pools de antígenos (MELBY et al., 2000) e seleção de bibliotecas de expressão com soros de animais e humanos infectados (REED; BADARO; LLOYD, 1987; SKEIKY et al., 1995a, 1998, WEBB et al., 1996, 1997, 1998; RAFATI et al., 2001).

Estes incluem a glicoproteína 63 (gp63), glicoproteína 46 da membrana (gp46, também conhecida como M-2), ligante fucose manose (FML), homólogo de receptores *Leishmania* para C quinase ativada (p36/LACK), NH36, quimera Proteína Q , proteinase cisteína B (CP) e antígenos CPA, GRP78, LD1, proteína de superfície acilada hidrofílica B1 (HASPB1), LCR1, proteína salivar 15 (SP15), antígeno de superfície promastigota 2 (PSA-2), A-2, histona H1, MML, fator de alongamento e iniciação de *L. brasiliensis* (LeIF), homólogo de *L. major* da proteína indutível por estresse eucariota-1 (LmSTI1), homólogo de *L. major* do antioxidante específico de tiol eucariótico (TSA) e Leish-111f (NAGILL; KAUR, 2011; JAIN; JAIN, 2015).

Entre os animais domesticados afetados pela infecção por *Leishmania*, os cães são as espécies mais comuns e as opções de tratamento para a LVC são limitadas, por isso os cães infectados são comumente eutanasiados (MORENO; ALVAR, 2002).

A quimioterapia na LVC não é amplamente recomendada por ser apenas parcialmente eficaz e, além disso, se o tratamento canino for realizado com os mesmos medicamentos usados para tratar a infecção humana, pode-se aumentar o risco do surgimento de parasitas resistentes a drogas (NOLI; AUXILIA, 2005).

Uma vacina canina para prevenção ou tratamento pode ser extremamente benéfica, uma vez que os cães que abrigam *L. infantum* servem como um reservatório intimamente ligado à infecção humana (América do Sul e Sul da Europa) (JAIN; JAIN, 2015). O uso de uma vacina canina para reduzir o papel deste reservatório de infecção e interromper a transmissão da *Leishmania* para humanos poderia eliminar a necessidade de eutanásia, dessa forma, vários estudos demonstraram graus de eficácia variáveis em abordagens de vacinas profiláticas e terapêuticas na LVC (SILVA et al., 2000; GRADONI et al., 2005; PALATNIK-DE-SOUSA et al., 2009; BORJA-CABRERA et al., 2010; TRIGO et al., 2010).

Assim, se a imunização é capaz de prevenir a transmissão do parasita ao vetor, a vacinação dos cães pode ser o principal método para o controle da doença em áreas endêmicas (DE LUCA et al., 2001; GRADONI, 2015; GRIMALDI et al., 2017). Como resultado da pesquisa sobre o desenvolvimento de vacinas contra a LVC, o Brasil tornou-se o primeiro país do mundo a oferecer vacinas disponíveis comercialmente para imunizar cães (FERNANDES et al., 2014).

Em 2014, duas vacinas disponíveis comercialmente foram licenciadas pelo Ministério da Agricultura para uso em cães: Leish-Tec® (Hertape SA, Juatuba, Brasil), que contém uma proteína amastigota recombinante específica para o estágio de diferentes espécies de *Leishmania* (rA2) mais saponina como adjuvante (COELHO et al., 2003; ZANIN et al., 2007; FERNANDES et al., 2008) e Leishmune® (Zoetis, Campinas, Brasil), composto de glicoproteínas semi-purificadas do ligante fucose manose (FML) de *Leishmania donovani* e saponina (SILVA et al., 2000; BORJA-CABRERA et al., 2002; PARRA et al., 2007).

No entanto, em novembro de 2014, o Ministério da Agricultura do Brasil suspendeu a licença provisória concedida à vacina Leishmune® por não atender totalmente aos requisitos de um ensaio clínico de vacina de fase III (DE MENDONÇA et al., 2016). Em um estudo prospectivo (FERNANDES et al., 2014), 92,9% dos cães imunizados com Leish-tec® permaneceram saudáveis durante o período de 11 meses de acompanhamento. Já no estudo de fase III randomizado, realizado em cães de uma área endêmica no estado de Minas Gerais, foi observado que a vacina possui uma eficácia de 80,8% e uma redução de 46,6% na transmissão para flebotomíneos alimentados de cães vacinados durante o xenodiagnóstico (REGINA-SILVA et al., 2016).

Estudos avaliando Leishmune® como uma vacina profilática em cães expostos à infecção natural mostraram taxas similares de resultados clínicos, variando de 83,3% (DE AMORIM et al., 2010) a 95% (BORJA-CABRERA et al., 2002). Dessa forma, o sucesso das vacinas contra a LVC encorajaram os cientistas para o desenvolvimento da vacina contra a leishmaniose humana.

É improvável que uma vacina ideal contra a leishmaniose consista em um único antígeno. Então, vários autores têm buscado selecionar antígenos com capacidade para proteger camundongos e primatas, e testar combinações de antígenos em modelos profiláticos e terapêuticos (COLER; REED, 2005).

Das muitas proteínas testadas, três das que apresentaram melhor resposta (TSA, LmSTI1 e LeIF) para a leishmaniose, foram selecionadas para inclusão em uma vacina baseada em subunidades (SKEIKY et al., 2002).

Uma mistura de TSA e LmSTI1 também foi demonstrada como indutora de boa proteção tanto nos modelos de primatas e murinos da leishmaniose cutânea como uma vacina profilática (CAMPOS-NETO et al., 2001). O terceiro componente, LeIF, mostrou conferir proteção parcial no modelo BALB/c como vacina terapêutica, uma propriedade atribuída à sua atividade adjuvante promotora de resposta do tipo Th1 (BORGES et al., 2001; PROBST et al., 1997; SKEIKY et al., 1995a, 1998). No entanto, do ponto de vista prático, uma vacina que consiste em múltiplas proteínas recombinantes seria difícil e cara de fabricar.

Uma proteína de fusão recombinante de 111kDa, a Leish-111f (conhecida também como Leish-F1 ou MML), composta de uma única poliproteína recombinante que compreende as sequências de três proteínas geneticamente ligadas em série (na ordem TSA – LmSTI1 – LeIF) (Figura 4) que estão presentes nas formas amastigota e promastigota do parasita e são altamente conservadas entre espécies do gênero *Leishmania*, um requisito para assegurar a proteção entre espécies (SKEIKY et al., 1995b, 1998; WEBB et al., 1996, 1998) surge como candidato promissor a uma vacina de segunda geração.

Esta vacina em formulação com MPL-SE demonstrou proteger ratos, hamsters e macacos rhesus contra LV e LC (COLER et al., 2007; SKEIKY et al., 2002), embora não protegesse consistentemente os cães contra a LV (GRADONI et al., 2005; MIRET et al., 2008; MORENO et al., 2007). Dessa forma a Leish-111f foi a primeira vacina de subunidade para leishmaniose que alcançou estudos clínicos de fase I e II em seres

humanos (VÉLEZ et al., 2009; LLANOS-CUENTAS et al., 2010; NASCIMENTO et al., 2010).

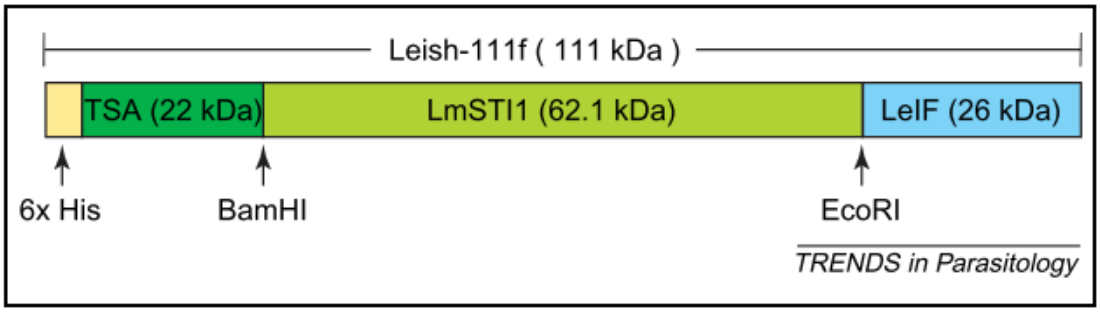


Figura 4. Representação esquemática da construção de Leish-111f contendo genes ligados de forma tandem de TSA, LmSTI1 e LeIF (COLER; REED, 2005).

Posteriormente, outras modificações à proteína de fusão Leish-111f foram feitas para simplificar a fabricação; as melhorias do processo incluíram a remoção do marcador seis-His N-terminal e a mutação de um ponto quente proteolítico para reduzir a degradação proteica. A nova construção de 110kDa foi nomeado Leish-110f (conhecida também com F2) (VEDVICK et al., 2008).

Outro grande avanço no desenvolvimento de vacinas candidatas contra a leishmaniose, assim como outras doenças que requerem respostas de células T potentes e dirigidas, ocorreu com a identificação de adjuvantes capazes de induzir respostas Th1. A descoberta de que agonistas apropriadamente formulados do receptor do tipo Toll (TLR) podem estimular respostas imunes Th1 tem impactado profundamente o desenvolvimento de vacinas contra patógenos intracelulares como *Leishmania* (BETHONY et al., 2011).

8. ADJUVANTES IMUNOLÓGICOS

Os adjuvantes de vacina são críticos para o desenvolvimento efetivo de respostas protetoras com muitos antígenos. Os adjuvantes do agonista do receptor do tipo Toll-like (TLR) são particularmente promissores, pois envolvem o sistema imune inato para estimular uma resposta imune adaptativa mais forte e durável (REED; ORR; FOX, 2013).

Os TLRs participam do controle de infecções pela regulação da resposta imune inata mediada por padrões moleculares associados ao patógeno (PAMPs), como glicolipídios, peptidoglicanos e lipopeptídeos, comuns em um grande grupo de microrganismos e são responsáveis por mediar as respostas imunes pela produção de fator de necrose tumoral-α (TNF-α), IL-12 e óxido nítrico (NETEA et al., 2004; JAIN; JAIN, 2015).

Os TLRs são divididos em dois subgrupos, dependendo da sua localização celular e dos respectivos ligantes de PAMPs. Um grupo é composto por TLR1, TLR2, TLR4, TLR5, TLR6 e TLR11, que são expressos na superfície celular e reconhecem principalmente componentes da membrana microbiana, como lipídios, lipoproteínas e proteínas; outro grupo é composto de TLR3, TLR7, TLR8 e TLR9, que são expressos exclusivamente em vesículas intracelulares, como o retículo endoplasmático (ER), endossomos, lisossomos e endolisossomos, e reconhecem os ácidos nucléicos microbianos (MOGENSEN, 2009; KAWAI; AKIRA, 2010). O ligante para o TLR10 é desconhecido (Figura 5) (KAWAI; AKIRA, 2010).

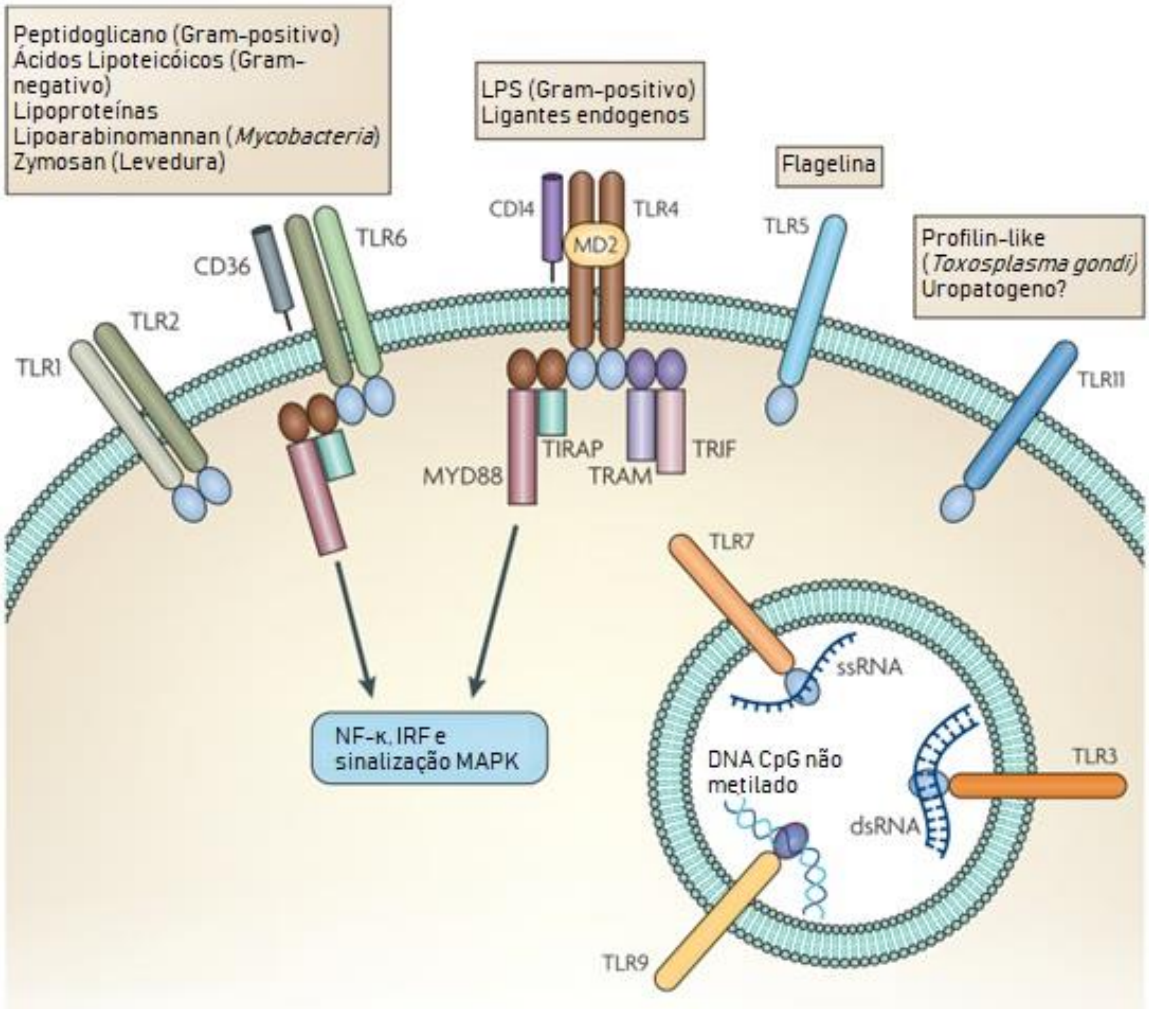


Figura 5: Funções fisiológicas dos receptores Toll-like (TLRs). Localização celular e respectivos ligantes de PAMPs. Esta figura foi adaptada da Ref (RAKOFF-NAHOUM; MEDZHITOV, 2009).

Desde então, ligantes para TLR 7/8 (Imiquimod, Resiquimod) (VASILAKOS; TOMAI, 2013), TLR 9 (CpG) (SCHEIERMANN; KLINMAN, 2014; SHIROTA;

KLINMAN, 2014), TLR 5 (Flagellin) (MIZEL; BATES, 2010) e TLR 4 (ALVING et al., 2012; IRETON; REED, 2013; REED; ORR; FOX, 2013) foram avaliados pré-clinicamente como componentes de adjuvantes de vacinas.

Dos 11 membros da família TLR, o TLR4 e o TLR9 contribuem principalmente na resposta imune contra a infecção por *Leishmania*. Também foi sugerido que esses dois TLRs desempenham um papel possível no mecanismo antileishmanial da miltefosine. Assim, esses receptores também poderiam ser fator importante na concepção da vacina candidata adequada contra a infecção leishmanial, bem como no sistema de liberação direcionada de drogas para o tratamento da leishmaniose (SAHA; MUKHOPADHYAY; CHATTERJEE, 2011; MUKHERJEE et al., 2012).

O agonista de TLR4 mais conhecido é o lipopolissacarídeo (LPS), que se apresenta na membrana externa das bactérias Gram-negativas, mostrando muitos efeitos adversos e, para supera-los, foram introduzidas variações modificadas do LPS, cada uma com características únicas e potencial de ativação de TLR4, incluindo derivados biológicos da molécula LPS original, como o monofosforil lipídeo A (MPL) (COLER et al., 2011), bem como substitutos totalmente sintéticos. O MPL é um derivado do LPS obtido a partir da *Salmonella minnesota* R595 (BALDRIDGE; CRANE, 1999) e exibe apenas ~ 0,1% da toxicidade inflamatória do LPS (QURESHI et al., 1997; EVANS et al., 2003). Quando usado como adjuvante, o MPL aumenta as respostas imunológicas específicas do imunógeno, promovendo o desenvolvimento de células T CD4+ Th1 (REED et al., 2009).

Moléculas de segunda geração que são sintéticas e alvo para TLR4 também estão sendo desenvolvidas. Algumas delas, como os agonistas sintéticos glicopiranosil lipídico (GLA, também chamado de PHAD ou MPLA sintético) (FOX et al., 2017) e o adjuvante lipídico de segunda geração (SLA) estão sendo desenvolvidos pelo Infectious Disease Research Institute (IDRI) e se mostraram promissores em estudos pré-clínicos e clínicos em seres humanos (ANDERSON et al., 2010; COLER et al., 2011; LOUSADA-DIETRICH et al., 2011).

O GLA é um análogo sintético do Lipídeo A e é baseado simplesmente na estrutura da molécula natural (COLER et al., 2011; LOUSADA-DIETRICH et al., 2011). Depois que a estrutura do complexo humano MD2/TLR4 foi publicada (PARK et al., 2009), o uso dessa estrutura foi utilizado para projetar um novo agonista. O SLA foi desenvolvido *in silico* (CARTER et al., 2016) através de modificação racional das cadeias acilares terminais do GLA (Figura 6) (COLER et al., 2011), uma molécula precursora agonística

de TLR4 atualmente em desenvolvimento clínico de fase II (PAES et al., 2016). O SLA foi desenvolvido para se encaixar melhor no receptor MD2/TLR4 humano e parece ter algumas propriedades benéficas para induzir citocinas de perfil Th1, enquanto mantém baixos níveis de citocinas anti-inflamatórias (FOX et al., 2017).

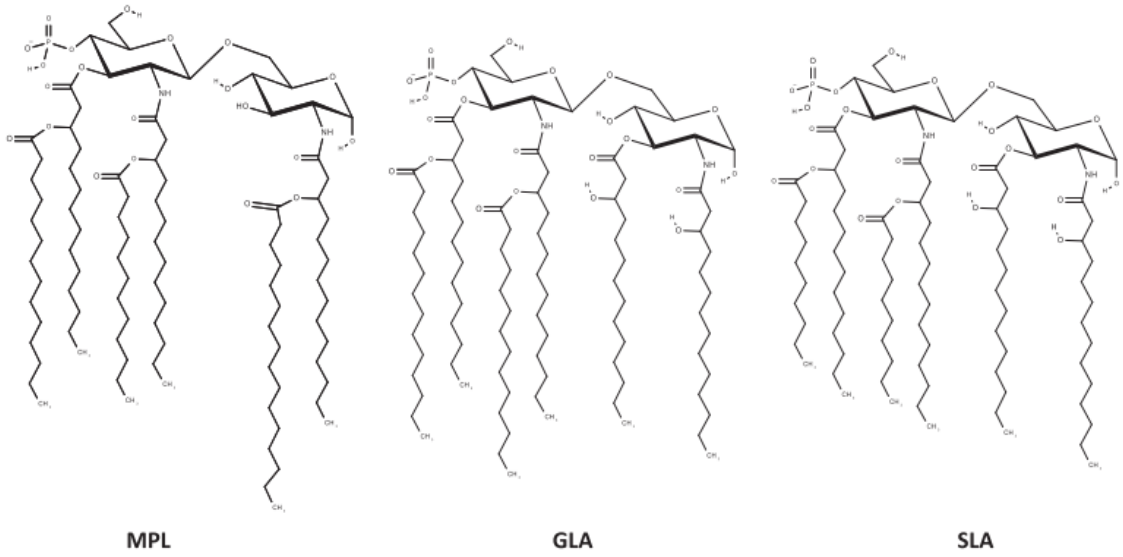


Figura 6. Estruturas comparativas de ligantes de TLR4 (FOX et al., 2017).

A molécula resultante, SLA, tem várias propriedades desejáveis e foi desenvolvida através de ensaios clínicos em humanos (BALDWIN et al., 2016; PAES et al., 2016). Nesses estudos, a molécula de SLA foi capaz de induzir respostas adaptativas mais robustas em humanos do que agonistas de TLR4 similares, e tornou-se um dos principais candidatos para algumas indicações de vacinas em humanos (CARTER et al., 2016; FOX et al., 2017).

Os TLRs 7 e 8 são filogenética e estruturalmente relacionados (HEIL et al., 2004). Ambos reconhecem sequências de RNA de cadeia simples (ssRNA) contendo sequências ricas em poli-U ou GU e são ativadas por imidazoquinolinas sintéticas incluindo imiquimod (R-837) e resiquimod (R-848) bem como por análogos de guanosina como o loxoribina (LEE et al., 2003; DIEBOLD et al., 2004; HEIL et al., 2004; JURK et al., 2006).

As imidazoquinolinas são atrativas como novos adjuvantes para vacinas proteicas fracamente imunogénicas (JOHNSTON; BYSTRYN, 2006). As imidazoquinolinas são uma família de compostos orgânicos sintéticos, dos quais o imiquimod é o mais conhecido (HUSSEIN et al., 2014). O imiquimod é uma pequena molécula que ativa os receptores Toll-like 7 e 8 (TLR 7/8) em células apresentadoras de antígenos e medeia a

produção de uma variedade de citocinas, incluindo IFN-α, IFN-γ, TNF-α, IL-1 e IL-12 levando à indução de respostas imunes Th1 aumentadas (HEMMI et al., 2002; TOMAI et al., 2007; HUSSEIN et al., 2014).

Como os TLRs são os primeiros receptores inatos que reconhecem a *Leishmania*, uma terapia direcionada por TLR possui um forte potencial de sucesso. Da mesma forma, como os ligantes TLR aumentam a resposta imune, eles podem ser usados como adjuvantes nas estratégias profiláticas, sendo essa interação entre *Leishmania* e TLR de grande interesse e foco de pesquisa (CHAUHAN et al., 2017).

9. MEDIDAS DE CONTROLE E PROFILAXIA

As medidas de controle atualmente empregadas pelo Ministério da Saúde para o controle da LV consistem em: diagnóstico e tratamento precoce de casos humanos; redução das populações de flebotomíneos por meio da pulverização de inseticidas residuais em domicílios; levantamentos sorológicos de cães e eutanásia de animais soropositivos e educação em saúde (BRASIL, 2014).

Estudos mostraram que essas medidas de controle apresentam limitações significativas, o que compromete sua eficácia e não impede a disseminação da doença. Entre essas limitações, podemos citar o alto custo, a oposição de donos de cachorros, médicos veterinários e instituições de proteção animal, uma alta rotatividade de cães e intervalos prolongados entre o diagnóstico e a remoção dos cães (GONTIJO; MELO, 2004; OLIVEIRA; MORAIS; MACHADO-COELHO, 2008; ZUBEN; DONALÍSIO, 2016).

Considerando que a picada do flebotomíneo é a mais importante via de transmissão da LV, as medidas de controle da infecção devem ser primariamente focadas na prevenção do contato com o inseto vetor, seja através de barreiras físicas (redes de malha fina nas janelas e canis), barreiras químicas (repelentes) ou manuseio (evitando a exposição ao crepúsculo, eliminando o material peridomiciliar orgânico).

Prevendo uma grande possibilidade de falha dessas medidas, o cão ainda precisa ser capaz de responder ao desafio de infecção causado pelas picadas de flebotomíneos infectados, preferencialmente por uma resposta imune adaptativa previamente desenvolvida através da vacinação, ou como última alternativa, por quimioterápicos, que podem impulsionar o sistema imunológico para ajudar a combater a infecção (RIBEIRO et al., 2018).

As coleiras impregnadas com deltametrina a 4% são atualmente consideradas uma medida importante para a prevenção do animal, uma vez que elas atuam como repelente eficaz contra o vetor, promove o aumento da mortilidade de flebotomíneos, além de diminuir a prevalência e a incidência da infecção canina (DAVID et al., 2001; WERNECK, 2014; SEVÁ et al., 2016), o que seria uma alternativa a eutanásia para o controle da LVC no Brasil (COSTA et al., 2013).

O mecanismo de repelência é pouco estudado, mas supõe-se que o inseticida atue nos órgãos sensitivos do vetor juntamente com a combinação de gás carbônico e odores exalados pelo hospedeiro afetando sua atração à picada. (KILLICK-KENDRICK, 1999). O longo tempo de efeito que a coleira apresenta é explicado pela liberação da deltametrina induzida pelo atrito do colar com o pescoço do cão, espalhando-se por todo corpo do animal e protegendo-o contra picadas (KILLICK-KENDRICK, 1999; DAVID et al., 2001).

Alguns experimentos demonstram que o efeito das coleiras (repelente e letal) sobre os flebotomíneos ocorre uma a duas semanas após a colocação (KILLICK-KENDRICK et al., 1997; DAVID et al., 2001), que corresponde ao tempo necessário para que a deltametrina se espelhe pelo corpo do cão. No entanto, o uso de colares impregnados com deltametrina como parte dos programas nacionais ou regionais de controle em países endêmicos apresenta desafios: os colares são caros, precisam ser usados por 80% da população canina para causar impacto em casos humanos, e eles precisam ser substituídos a cada 6 meses ou antes, se forem perdidos (MAROLI et al., 2001; FOGLIA MANZILLO et al., 2006).

Nesse contexto, considerando que a LV é uma doença fatal e que atualmente não existe cura para a doença no cão, o presente trabalho avaliou o uso de duas vacinas imunoterápicas de proteína recombinante de segunda geração associadas com dois adjuvantes de receptores do tipo Toll, sendo uma um agonista TLR4 e outra com os agonistas TRL4 e 7, associadas ao alopurinol no tratamento de cães naturalmente infectados por *L. infantum*, a fim de tentar obter a cura parasitológica ou melhora dos sinais clínicos da doença.

10. OBJETIVOS

10,1. Objetivo Geral:

Avaliar o potencial terapêutico da proteína recombinante de fusão Leish-F2 com diferentes adjuvantes para tratamento de cães com Leishmaniose Visceral canina, naturalmente infectados em área endêmica.

10.2. Objetivos Específicos:

- Avaliação da evolução dos sinais clínicos dos animais nos diferentes grupos
- Avaliação da carga parasitária antes, durante e 245 dias após o final do esquema terapêutico em diferentes órgãos.
- Comparar o melhor sítio de detecção de *L.infantum* para fins de diagnóstico.

10.3. Desfecho Primário:

- Cura parasitológica.

10.4. Desfecho Secundário:

- Redução do escore clínico de gravidade da LVC;
- Redução de carga parasitária nos diferentes órgãos como: fígado, baço, rins, pele e linfonodos.

Os dados serão apresentados na forma de dois artigos intitulados:

1. **Combination chemo- and immunotherapy provides long term clinical improvement and sustained parasite clearance in L. infantum-infected dogs**, seguindo as normas para publicação da Revista njp Vaccines.
2. **Scoring clinical sings can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil**, publicada na Revista Memórias do Instituto Oswaldo Cruz.

CAPÍTULO I*

*Manuscrito redigido conforme as normas do periódico njp Vaccines

Combination chemo- and immunotherapy provides long term clinical improvement and sustained parasite clearance in *L. infantum*-infected dogs

Leopoldo Fabrício Marçal do Nascimento[1],
Dayane Francisca Higino Miranda[1],
Luana Dias de Moura[1],
Flaviane Alves de Pinho[2],
Guilherme Loureiro Werneck[3],
Ricardo Khouri[4 5]
Steven G. Reed[6],
Malcolm S. Duthie[6 7],
Aldina Maria do Prado Barral[4 5 8],
Manoel Barral-Netto[4 5 8],
Maria do Socorro Pires e Cruz[1 *]

[1]Universidade Federal do Piauí, Departamento de Morfofisiologia Veterinária, Teresina, PI, Brasil

[2]Universidade Federal da Bahia, Faculdade de Medicina, Salvador, BA, Brasil

[3]Universidade Federal do Rio de Janeiro, Instituto de Estudos em Saúde Coletiva, Rio de Janeiro, RJ, Brasil

[4]Fundação Oswaldo Cruz- Fiocruz, Instituto Gonçalo Moniz, Salvador, BA, Brasil

[5]Universidade Federal da Bahia, Faculdade de Medicina, Salvador, BA, Brasil

[6]Infectious Diseases Research Institute, Seattle, WA 98102, USA

[7]HDT Biotech Corporation, Seattle, WA 98102, USA

[8]Instituto Nacional de Ciência e Tecnologia, Instituto de Investigação em Imunologia, São Paulo, SP, Brasil

*Address correspondence and reprint requests:
Universidade Federal do Piauí, Departamento de Morfofisiologia Veterinária, Teresina, PI, Brasil
Email address: mspcruz@ufpi.edu.br

Running title: Chemo-immunotherapy for CVL

Keywords: canine visceral leishmaniasis; vaccine; adjuvant; drug; clinical signs

Author contributions

Experimental design: Leopoldo Fabrício Marçal do Nascimento, Dayane Francisca Higino Miranda, Luana Dias de Moura, Flaviane Alves de Pinho, Guilherme Loureiro Werneck, Ricardo Khouri, Steven G. Reed, Malcolm S. Duthie, Aldina Maria do Prado Barral, Manoel Barral-Netto, Maria do Socorro Pires e Cruz

Veterinary procedures: Leopoldo Fabrício Marçal do Nascimento, Dayane Francisca Higino Miranda, Luana Dias de Moura, Flaviane Alves de Pinho, Maria do Socorro Pires e Cruz

Laboratory procedures: Leopoldo Fabrício Marçal do Nascimento, Dayane Francisca Higino Miranda, Luana Dias de Moura, Flaviane Alves de Pinho, Ricardo Khouri, Maria do Socorro Pires e Cruz

Data analyses: Leopoldo Fabrício Marçal do Nascimento, Luana Dias de Moura, Guilherme Loureiro Werneck, Ricardo Khouri, Aldina Maria do Prado Barral, Manoel Barral-Netto, Maria do Socorro Pires e Cruz

Manuscript preparation: Leopoldo Fabrício Marçal do Nascimento, Luana Dias de Moura, Guilherme Loureiro Werneck, Ricardo Khouri, Steven G. Reed, Malcolm S. Duthie, Aldina Maria do Prado Barral, Manoel Barral-Netto, Maria do Socorro Pires e Cruz

Abbreviations used in this paper:

CVL, canine visceral leishmaniasis

GLA, glycopyranosyl lipid

IMQ, imiquimod

IFN, interferon

IL, interleukin

MPL, monophosphoryl lipid A

SLA, Second generation Lipid Adjuvant

SE, stable emulsion

Th1, T helper 1-like cells

TLR, Toll-like receptor

VL, visceral leishmaniasis

Abstract

Dogs are an important reservoir for *L. infantum* and support transmission to humans to cause VL. There is little evidence that current control strategies for canine visceral leishmaniasis (CVL), the veterinary disease caused by *L. infantum* infection, are having a positive impact and new measures are needed. Drugs intended primarily for human use are prohibited for the treatment of CVL because of concerns over the propagation of resistant parasites. Allopurinol is effective in decreasing parasite burden in CVL but the treatment need to be maintained for life. We hypothesized that during the allopurinol-induced parasite reduction dogs may be prone to develop a more robust immune response if properly activated which may lead to more effective control of parasite load. Herein we report on the therapeutic capacity of allopurinol combined with immunotherapeutic approaches involving defined subunit vaccines, for the treatment of dogs naturally infected with *L. infantum*. As vaccines we used the Leish-F2 antigen and as adjuvant Second generation Lipid Adjuvant (SLA) formulated with stable emulsion (SE; SLA-SE, an agonist of Toll-like receptor (TLR) 4 or SLA combined with imiquimod (IMQ; an agonist of TLR7). Our study consisted of 28 dogs distributed as follows: Untreated; oral allopurinol alone (20 mg/kg, once each day for 90 days); allopurinol combined to Leish-F2 + SLA-SE; and allopurinol plus Leish-F2 + SLA/IMQ-SE. While the animals that did not receive treatment had a progressive decline in their clinical condition with an increase on their parasite load, treatment with allopurinol alone alleviated the clinical symptoms of CVL and provided long-term improvement in clinical condition but did not maintain a low parasite load for long term. Concomitant vaccination with Leish-F2 and SLA-SE (with or without IMQ) was, however, able to lead to improved clinical condition and to provide long-term clearance of *L. infantum* from the lymphoid tissues and systemic organs. These results have important implications for both the management of CVL in individual animals and for limiting potential transmission of *L. infantum* from infected dogs to the general population.

Introduction

Visceral leishmaniasis (VL), the clinical consequence of infection with *Leishmnaia infantum*, is a serious public health problem in several countries. The infection can also cause canine VL (CVL) in dogs, a multisystemic disease that manifests with a range of signs that can include dermatitis, lymphadenomegaly, general muscular atrophy and kidney failure. Dogs are incriminated as the main domestic reservoirs of *L. infantum* and are responsible for sustained transmission to humans (TRAVI, 2014). Several preventative and control measures have been recommended to combat CVL as a means to limit parasite transmission and disease in humans. In Brazil, the treatment of CVL is regulated by Interministerial Order No. 1,426 of July 11, 2008. Due to the perceived risk of the potential emergence of drug resistant parasites, this order prohibits the treatment of CVL with drugs intended primarily for human use (NOLI; AUXILIA, 2005). Given that currently available chemotherapies have only been partially effective to date, several alternative treatment options have been investigated (NOLI; AUXILIA, 2005).

Vaccines can potentially be used more broadly than drugs, and could possibly protect both animals and humans, however, vaccines used in either prophylactic or therapeutic approaches for VL, have demonstrated varying efficacies (GHORBANI; FARHOUDI, 2018).

Among the first defined vaccine candidate antigens against leishmaniasis was the recombinant fusion protein Leish-111f (L111f; LEISH-F1; MML) comprising the *L. major* homolog of the eukaryotic thiol-specific antioxidant (TSA), Leishmania major homologue of the yeast stress-inducible protein STI1 (LmSTI1), and *L. braziliensis* elongation and initiation factor (LeIF) (SKEIKY et al., 2002). When appropriately adjuvanted this chimeric fusion protein promotes protective immune responses in mice, hamsters and rhesus monkeys, and LEISH-F1 + monophosphoryl lipid A (MPL)-SE was the first subunit vaccine against *Leishmania* to enter human trial (CAMPOS-NETO et al., 2001; CHAKRAVARTY et al., 2011; SKEIKY et al., 2002). Leish-F1 did not provide consistent prophylactic protection against CVL (GRADONI et al., 2005; MORENO et al., 2007). Subsequent modifications of the Leish-F1 polyprotein were made to simplify manufacturing including removal of the N-terminal six-His label and mutation of a proteolytic hot spot to reduce protein degradation. The modified construct was named Leish-110f (also known as Leish-F2) (VEDVICK et al., 2008).

Concurrent with advances in molecular biology techniques that have made protein production more efficient, the development of defined adjuvants has provided a major additional

129 breakthrough in vaccine development. Adjuvants can now be designed to enhance and bias T cell
130 responses, with the ability of properly formulated Toll-like receptor (TLR) agonists to promote
131 antigen-specific T helper (Th) 1 immune responses that can potentially impart protective
132 responses against intracellular pathogens such as *Leishmania* species (REED et al., 2009). In
133 particular, monophosphoryl lipid A (MPL), a multiple species TLR4 agonist obtained from the
134 cell wall of Salmonella, has a long history has been safely incorporated into approved vaccines
135 (GARÇON; VAN MECHELEN, 2011). Second generation, single species synthetic TLR4
136 agonists such as glycopyranosyl lipid (GLA, also called PHAD or synthetic MPLA) and second
137 generation lipid adjuvant (SLA) have now been developed and advanced into clinical trials (FOX
138 et al., 2017) .
139 This study was conducted to determine if immunization could be used in conjunction
140 with drug therapy to improve the clinical and parasitological outcome of CVL. As vaccines we
141 used the second generation Leish-F2 polyprotein and contrasted its ability when formulated with
142 the second-generation lipid adjuvant in stable emulsion (SLA-SE) or SLA/ imiquimod (IMQ)-SE
143 adjuvants that have the ability to stimulate either TLR4 alone or TRL4 and TRL7, respectively.
144 As drug, we used allopurinol as it has previously been used in the treatment of dogs infected with
145 *L. infantum.*
146

Materials and Methods

Ethics statement. This project was approved by the Ethics Committee on Animal Experimentation of the Universidade Federal do Piaui (UFPI), under opinion No. 093/15. All procedures were conducted in accordance with the recommendations of the Brazilian College of Animal Experimentation (COBEA).

Study population. The study was conducted in the Experimentation Kennel and in the Laboratory of Infectious Diseases, located in the Center of Agricultural Sciences, University Veterinary Hospital of the Universidad Federal do Piaui (UFPI). Twenty eight dogs, all naturally infected with *L. infantum*, were obtained from the Management of Zoonoses of the Municipal Health Foundation of Teresina, Piaui or from University Veterinary Hospital, UFPI. Dogs were obtained by donation from their original owner after objectives of the study were described to them and after they had signed the donation term. Collectively the dogs were male and female adults of unknown age, and of different defined or undefined breeds. Serum-reactive animals diagnosed by rapid serological tests for *Ehrlichia canis* (Alere Erliquiose Ac test KIT®) and for canine distemper (Alere cinomose Ag test kit®) were excluded. Animals were included based on the positive parasitological examination for *L. infantum* in aspirates of lymph node, skin and / or bone marrow, and if they did not present hostility that made the oral treatment and collection of samples unfeasible. Each animal was provided an identification tag by subcutaneous implantation of a microchip, as well as vermifugation (Dauverm Plus, Vansil®) and immunization against common viruses (Vanguard HTLP 5 / CV-L; Pfizer Animal Health, New York, USA) before housing in screened kennels measuring 1.5 m width / 3.0 m length. Water and food were provided *ad libitum.*

Experimental groups. Dogs were allocated to different treatment groups by a stratified randomization procedure that was conducted to ensure greater comparability in relation to the degree of evolution of the infection. Four strata were created based on the median of the two variables: parasite load in bone marrow aspirate and clinical score. Each group contained animals with: (1) high parasite load and high clinical score; (2) high parasite load and low clinical score; (3) low parasite load and high clinical score; or (4) low parasite load and low clinical score. Within each stratum the animals were subsequently randomly allocated to the different treatment

178 groups. Group I comprised 6 dogs that did not receive medication, while Groups II, III and IV all
179 received allopurinol therapy orally at a dose of 20 mg/kg, one time each day for 90 days. Group
180 II comprised 8 dogs that received allopurinol therapy only; Group III comprised 8 dogs that were
181 additionally subcutaneously injected with the Leish-F2 + second generation lipid adjuvant and
182 the stable oil-in-water emulsion (SLA-SE) (20 μg each) for a total of 6 doses, with a 3 week
183 interval between each dose; Group IV comprised 6 dogs were additionally subcutaneously
184 injected with Leish-F2 formulated with SLA-SE supplemented with imiquimod (SLA/IMQ-SE)
185 (20 μg each), for a total of 6 doses, with a 3 week interval between each dose.
186
187 **Clinical evaluation of animals.** Each dog was clinically evaluated by the following parameters:
188 nutritional status, lymph nodes, mucosal coloration, onychogryphosis, skin changes (hair, alopecia,
189 dermatitis, lesions and depigmentation of the skin), ophthalmic alterations (ocular secretion and
190 blepharitis) and bleeding. These evaluations were performed at days 0, 63, 180 and 360 relative
191 to initiation of treatment. After the evaluations, scores were assigned to clinical signs according
192 to Silva et al., (2017) and data compiled into an evaluation form.
193
194 **Sample collection.** Samples were collected from bone marrow on days 0, 63, 180 and 360. At
195 each time animals were physically restrained and the bone marrow sample was obtained by
196 inserting a needle into the sternum and slowly extracting a volume of 1.5 mls. At the end of the
197 monitoring period all dogs underwent sedation by intravenous injection of an anesthetic
198 composed of xyzaline hydrochloride (1mg/kg) and ketamine hydrochloride (10mg/kg), and after
199 10 minutes, the general anesthetic 2.5% sodium thiopental was administered intravenously at the
200 dose of 0.5 mL/kg. After reaching the appropriate anesthetic plane, T-61 (Hoechst Roussel Vet)
201 was administered and death was confirmed by the absence of heart beat and respiration. Samples
202 of spleen, liver, kidney, skin and lymph node were collected.
203
204 **DNA extractions.** DNA was extracted from samples using the PureLink® Genomic DNA
205 Minikit (Invitrogen™ Cat No. 1820-02), following the manufacturer's recommendations for each
206 particular sample type. DNA quantity was determined using Nanodrop™ 2000 / 2000c
207 spectrophotometer (Thermo Scientific), with the purity evaluated based on the ratio 260nm /
208 280nm. The efficiency of extraction was evaluated by conventional PCR amplifying the

housekeeping gene beta-actin. The primers were obtained from Invitrogen® (São Paulo, Brazil) and had the follwoing sequences: Primer β-Actin Forward GACCCTGAAGTACCCCATTGAG and Primer β-Actin Reverse TTGTAGAAGGTGTGGTGCCAGAT. For each PCR reaction, 20 ng DNA was amplified using the following reagents: 2 mM $MgCl_2$, 200 μM dNTPs, 0.6 μM of each primer, 1 IU Platinum Taq DNA Polymerase and specific buffer (all Invitrogen). The themrocycling program was 95°C for 5 minutes followed by 35 cycles of 30 seconds by 95°C; 58.8°C for 45 seconds, 72°C for 45 seconds; and final extension at 72°C for 7 minutes. The amplification product was then observed on 1.5% agarose gel stained with ethidium bromide.

Determination of parasite load. The parasite load of each sample was determined by real-time PCR according to a previously described protocol (MANNA et al., 2008). Each sample was evaluated in duplicate. *L. infantum* DNA was amplified using the primers: sense-5'-GGTTAGCCGATGGTGGTCTT-3 'and anti-sense - 5'-GCTATATCATATGTCCAAGCACTTACCT-3' and TaqMan probe (5'-ACCACCTAAGGTCAACCC- . In brief, 5 μL of DNA sample (100ng) was added to 16 μL of a mixture consisting of 12.5 μL of 2x TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 1.25 μL of each primer, 1.0 μL TaqMan MGB probe (FAMTM dye-labeled, Applied Biosystems) and 4 μl of milli-Q water, providing a final volume of 25 μL for each reaction. The runs were performed on an ABI 7500 real time PCR system using SDS 2.0 software (Applied Biosystems) and the following thermocycling program: denaturing at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Quantification was achieved by extraploation into a standard curve that was generated from cultured *L. infantum* (MCAN/BR/89/Ba-262) to provide a range of 1 to 1 x 10^6 parasites (DE PAIVA CAVALCANTI et al., 2009) and that was amplified in parallel to the test samples.

Statistical analysis. GraphPad Prism 7 software (GraphPad Software Inc.) was used for statistical analysis. Differences between the parasite load of each group, as well as the clinical scores, were evaluated by the Kruskal-Wallis test, with a Dunn's comparative post-test. Data were considered significantly different when p-values < 0.05 were obtained.

239 **Data availability.** The underlying data reported in this paper are available from the
240 corresponding author upon reasonable request.

Results

Treatment with either drug or drug plus vaccine prevents clinical progression of CVL. To evaluate if chemo- and or chemoimmunotherapeutic intervention could interrupt *L. infantum* infection and CVL development, we enrolled dogs that were confirmed to be infected with *L. infantum* upon examination of lymph node, skin and / or bone marrow aspirates. As expected, animals that did not receive treatment exhibited a steady worsening in their clinical score over time, indicating the natural progression of the disease (Figure 1A and 1B). In contrast, animals receiving allopurinol alone or in combination with either the Leish-F2 + SLA-SE or Leish-F2 + SLA-IMQ-SE vaccines demonstrated an improvement in clinical response that extended even after discontinuation of treatment, with all treated dogs maintaining a low clinical score throughout the monitoring period. Together, these data indicate successful intervention in the clinical advancement of CVL.

Treatments reduce parasite burdens within bone marrow but immunization is required for sustained elimination. Having demonstrated that treatment improved the clinical condition of *L. infantum*-infected dogs, we then evaluated if this was associated with reductions in the parasite burden. Given that *L. infantum* demonstrates a preference for multiplying in the lymphoid organs, we assessed parasite loads within bone marrow aspirates. At the beginning of the treatment, each group had low, but similar, parasite numbers in their bone marrow aspirates (Figures 2A and 2B). Consistent with their symptomatic progression, dogs that received no treatment exhibited a large expansion of parasites within the first 2 months of observation then retained large numbers throughout the 1-year monitoring period (Figure 2). Although allopurinol treatment led to a rapid reduction to undetectable levels by 63 of treatment, mean parasite numbers in the bone marrow rebounded after completion of the 90-day regimen (Figure 2). The addition of either the Leish-F2 + SLA-SE or Leish-F2 + SLA-IMQ-SE vaccine to the allopurinol treatment, however, provided a long-term suppression of parasite burdens.

At each time assessed after treatment initiation there was a significantly lower number of *L. infantum* in the bone marrow of dogs that received allopurinol and the Leish-F2 + SLA-SE vaccine relative to those quantified in untreated dogs (p-values = 0.0145, 0.0267 and 0.0017 at days 63, 90 and 360, respectively) (Figure 3). By day 360 statistically significant differences were observed between dogs provided either Leish-F2 + SLA-SE and allopurinol or Leish-F2 +

SLA-IMQ-SE and allopurinol versus dogs that received allopurinol alone (*p*-values = 0.0058 and 0.0264, respectively) (Figure 2E). Interestingly, close evaluation revealed that oral allopurinol monotherapy had cleared parasites to below detectable levels in only 5 of the 8 dogs by day 63 after treatment initiation (Figure 2C). All eight dogs treated with allopurinol alone then had detectable parasites one year after treatment (Figure 2E). In contrast, addition of either vaccine to the allopurinol regimen provided a long term 1 year clearance of parasites to levels below the limit of detection in all but one of the 16 immunized animals (in Leish-F2 + SLA-IMQ-SE group) (Figure 2E). Taken together, these data indicate that while treatment with allopurinol alone provided a transient reduction of parasite burdens, addition of either Leish-F2 + SLA-SE or Leish-F2 + SLA-IMQ-SE vaccine was required for the sustained elimination of *L. infantum* from the bone marrow.

Immunization provides sustained protection from parasite dissemination. To expand upon the observations made when evaluating bone marrow and provide a more complete determination of the impact of treatment on infection, various organs were collected at the termination of monitoring period. Parasites were found in the lymph node and spleen samples of all of the untreated animals, while a proportion of the untreated dogs also displayed infection in their liver, kidney, and skin samples (Figure 3). Consistent with the observation of detectable parasites in the bone marrow of dogs treated with allopurinol alone, parasites could also be detected in each of the other organs collected at termination. Unlike in the bone marrow, a majority (5 of 6) dogs provided Leish-F2 + SLA-IMQ-SE vaccine in conjunction with allopurinol had measurable *L. infantum* burdens in their lymph node samples. The presence of parasites in the other organs from 1 or 2 dogs treated with allopurinol and Leish-F2 + SLA-IMQ-SE resulted in the lack of statistically significant reductions in parasite burdens relative to untreated dogs (all *p*-values > 0.05). In contrast, parasites were significantly reduced relative to untreated dogs and were undetectable in organs from all but one of dogs treated with Leish-F2 + SLA-SE and allopurinol (a low number of parasites were detected in the spleen and lymph node samples from a single animal) (Figures 3 and 4). Taken together, these data indicate that treatment with Leish-F2 + SLA-SE and allopurinol provides a robust, sustained systemic reduction (potentially elimination) of *L. infantum* from infected dogs.

Discussion

Monotherapy with allopurinol is the preferred treatment for CVL. In this study, we evaluated the use of allopurinol alone or in conjunction with defined subunit of vaccines (Leish-F2 protein formulated with either SLA-SE or SLA/IMQ-SE) for the treatment of CVL. Each treatment regimen provided long-term clinical improvement of the infected dogs. When we evaluated the infectious status, however, our data revealed that although treated with allopurinol alone initially reduced parasite burden in the bone marrow, numbers had rebounded to pre-treatment levels one year later and systemic infection could be observed. In contrast, addition of vaccine, in particular Leish-F2 + SLA-SE, elicited a prolonged clearance of parasites from lymphoid and non-lymphoid organs. These results demonstrate an improved treatment regimen for CVL and strongly suggest the potential for inhibiting transmission of *L. infantum* from the canine reservoir in endemic regions. As such, these results have major implications for VL control programs.

In agreement with earlier reports that long-term allopurinol monotherapy generates a good clinical response, dogs treated with allopurinol alone in our study responded well and demonstrated clinical improvement. We did not observe clinical relapse as has been reported at a rate of up to 88.9% within 2-4 weeks of allopurinol withdrawal (CAVALIERO et al., 1999). Persistence of parasites long after clinical 'cure' of CVL by chemotherapy leaves many treated dogs still infective to sand flies for months after treatment completion (ALVAR et al., 1994). This persistence is apparent in our study because, despite clinical responsiveness and alleviation of symptoms and a transient reduction, we observed a resurgence of parasites in all animals after the allopurinol only treatment phase that ultimately returned infectious burdens in the bone marrow to pre-treatment levels one year after treatment initiation. CVL is a viscerotropic disease, making it necessary to evaluate parasite loads in different lymphoid compartments and systemic organs to fully assess the efficacy of treatment. At termination of the observation period the highest parasite loads were detected in lymphoid organs (bone marrow, lymph node, spleen), probably due to the abundance of phagocytic cells (macrophages and dendritic cells) for which the parasite presents a tropism (REIS et al., 2006). Parasites were also detected in kidneys, liver and skin, however, indicating widespread systemic dissemination and likely availability for uptake during the blood meal of sand flies. In contrast to chemotherapy alone, the return of parasites was observed in only a small proportion of dogs that had been immunized in addition to

their allopurinol treatment. Taken together these data suggest that while allopurinol generates an immediate, early restraint on the advancement of symptoms and parasite replication, the addition of immunization provides a response that likely promotes their clearance and a decreased transmission potential.

Vaccination has been previously studied as an immunotherapy for CVL, with conflicting results. It has been reported that either TLR agonist supplementation promoted success (MIRET et al., 2008) or contrarily that inclusion of a TLR4 agonist was not successful in halting disease progression (GRADONI et al., 2005; MORENO et al., 2007). However, none of these studies quantified the parasite load before treatment initiation and this may have an important impact on the success, or failure, of the treatment. The success of the vaccines used in our study could potentially be attributed to the use of the refined TLR4 agonist SLA, which was designed *in silico* and generated through modifications of acyl chains of GLA, which in itself is a second-generation synthetic single species evolution of *S. minnesota*-derived monophosphoryl lipid A (MPL) (CARTER et al., 2016). SLA is capable of inducing Th1 profile cytokines while maintaining low levels of anti-inflammatory cytokines (FOX et al., 2017). It is well documented that TLR4 agonists can exert synergistic effects when used simultaneously with TLR7 agonists such as IMQ, leading to elevated levels of IFNγ and IL-12 that would enhance anti-parasitic responses (FOX et al., 2014). Although not significantly different, in our study the SLA/IMQ-SE adjuvant did not appear to provide as much benefit as the use of SLA-SE alone. The group of animals that received the combination allopurinol and Leish-F2 + SLA-SE chemoimmunotherapy demonstrated statistically significant reductions in parasite burdens in bone marrow at all times following treatment initiation, with the sustained reduction at day 360 being significantly lower than the burden of animals treated with allopurinol only chemotherapy at the same time. Irrespective of this minor discrepancy, it is evident that our immunizations facilitated the long-term clearance of parasites from most dogs. Variables such as dose, administration frequency and interval, along with the adjuvant formulation used, could all influence the outcome. Further analyses of these appears important to determine the limitations of various strategies and guide broader application.

It is also quite possible that the infection and clinical status of each particular dog influences the response to treatment, and it is well established that transition from asymptomatic

363 infection to clinically advancing disease is accompanied by the onset of immune exhaustion.
364 This is consistent with the observation that combinations of TLR agonists and vaccine antigens
365 can overcame *L. infantum*-induced cellular exhaustion *ex vivo*, allowing robust Th1 responses
366 from symptomatic dogs that previously had dampened responses to antigen alone (SCHAUT et
367 al., 2016). It is possible that a benefit of combined TLR agonist adjuvant formulation may
368 emerge in studies of dogs with higher parasite infection levels and/ or more advanced CVL. It is
369 worth noting, however, that the animal with the highest parasite burden in its bone marrow at
370 intake was the only 1 of the 6 dogs treated with allopurinol and Leish-F2 + SLA/IMQ-SE that
371 had detectable parasites at day 360. That same animal also had large numbers of parasites in its
372 liver, lymph node and spleen samples at the termination of the study. This fact reinforces the idea
373 that a marked reduction on parasite load using a chemotherapeutic as allopurinol influence on the
374 capacity of the immune response to maintain the infection controlled. Howerver the
375 chemotherapy alone cannot control the infection for long term after suspending the treatment.
376 Further studies are required to both determine the limits of a combined chemoimmunotherapeutic
377 approach and to elucidate the mechanisms responsible for the success of this treatment.

378 In conclusion, the present study presents promising results indicating that use of a
379 combined chemoimmunotherapeutic approach, involving allopurinol treatment in conjunction
380 with the Leish-F2 + SLA-SE vaccine formulation, for the treatment of CVL. This
381 chemoimmunotherapy of naturally infected dogs alleviated symptoms and reduced *L. infantum*
382 burdens in both the short and long term. As such, these results represent a major advance in the
383 treatment and management of CVL, as well as the management of *L. infantum* infection and
384 potential transmission to cause human disease.

Acknowledgements

This study was financial supported by grants from National Council for Scientific and Technological Development – CNPq under Award Number 4674463/2014-0 and Coordination of Superior Level Staff Improvement - CAPES. IDRI has been funded by for leishmaniasis vaccine development through grant support from the Bill and Melinda Gates Foundation (#631) and National Institute of Allergy And Infectious Diseases of the National Institutes of Health under Award Number R01AI025038. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding bodies.

Conflict of Interest Statement

Malcolm Duthie and Steven Reed are co-inventors on a patent for leishmaniasis vaccine development. The other authors have stated no conflict of interest.

399 **Figure Legends**

400 **Figure 1. Allopurinol, either alone or in combination with vaccination, alleviates clinical**
401 **signs of canine visceral leishmaniasis (CVL).** Dogs were confirmed to be infected with *L.*
402 *infantum* and provided a full clinical examination to generate a clinical score for each animal
403 before placement into treatment groups. A group of 6 dogs did not receive medication
404 (untreated), while all other animals received allopurinol therapy orally at a dose of 20 mg/kg,
405 once each day, for 90 days. A group of 8 dogs received allopurinol therapy alone (allopurinol); 8
406 dogs were additionally subcutaneously injected with the Leish-F2 + SLA-SE (Leish-F2 + SLA-
407 SE + allopurinol) and a final group of 6 dogs were additionally subcutaneously injected with
408 Leish-F2 + SLA/IMQ-SE (Leish-F2 + SLA/IMQ-SE + allopurinol). Vaccines were provided for
409 a total of 6 doses, with a 3 week interval between each dose. In A, dogs were monitored
410 throughout and full evaluations conducted to yield clinical scores. The allopurinol treatment
411 phase is indicated by the shaded box. In B, the cumulative clinical score for each dogs was
412 calculated. Each point represents data from one individual dog, and results are shown as mean
413 and SEM per group.
414
415 **Figure 2. Combined treatment with allopurinol and vaccine is required for sustained**
416 **parasite clearance from the bone marrow of *L. infantum*-infected dogs.** Bone marrow
417 aspirates were collected from dogs before, and at regular intervals after, intervention and *L.*
418 *infantum* burden determined by real time PCR. Animals were either not treated (n = 6) or were
419 treated with allopurinol alone (n = 8), Leish-F2 + SLA-SE + allopurinol (n = 8) or Leish-F2 +
420 SLA/IMQ-SE + allopurinol (n = 6). In (A), results are shown as median and interquartile range
421 over time for each group. In (B), data are displayed for each analyses time. Each point shows the
422 parasite number detected for an individual animal, with the box and whiskers representing the
423 minimal and maximal values. * and ** represent *p*-value 0.0332 and 0.0021, respectively, versus
424 untreated animals and treated with allopurinol alone at the same time.
425
426 **Figure 3. Combined treatment with allopurinol and vaccine is required for sustained**
427 **parasite clearance from lymphoid and non-lymphoid organs of *L. infantum*-infected dogs.**
428 Dogs were either not treated (n = 6) or were treated with allopurinol alone (n = 8), Leish-F2 +
429 SLA-SE + allopurinol (n = 8) or Leish-F2 + SLA/IMQ-SE + allopurinol (n = 6). Samples were

collected from at termination of the observation period and *L. infantum* burden determined by real time PCR. Parasite burdens were determined in samples from (A) kidney, (B) skin, (C) liver, (D) lymph nodes and (E) spleen. Each point shows the parasite number detected for an individual animal, with the box and whiskers representing the minimal and maximal values. * and ** represent *p*-value 0.0332 and 0.0021, respectively, versus untreated animals.

Figure 4. Schematic representation of *L. infantum* distribution in infected dogs at the end of the monitoring period. Dogs were either not treated (n = 6) or were treated with allopurinol alone (n = 8), Leish-F2 + SLA-SE + allopurinol (n = 8) or Leish-F2 + SLA/IMQ-SE + allopurinol (n = 6). Samples were collected from the indicated organs at termination of the observation period and *L. infantum* burden determined by real time PCR. Parasite burdens were catergorised by number and each category is displayed as a different color.

References

ALVAR, J. et al. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. **Annals of tropical medicine and parasitology**, v. 88, n. 4, p. 371–8, ago. 1994.

CAMPOS-NETO, A. et al. Protection against Cutaneous Leishmaniasis Induced by Recombinant Antigens in Murine and Nonhuman Primate Models of the Human Disease. **Infection and Immunity**, v. 69, n. 6, p. 4103–4108, 1 jun. 2001.

CARTER, D. et al. A structure-function approach to optimizing TLR4 ligands for human vaccines. **Clinical & Translational Immunology**, v. 5, n. 11, p. e108, 2 nov. 2016.

CAVALIERO, T. et al. Clinical, serologic, and parasitologic follow-up after long-term allopurinol therapy of dogs naturally infected with Leishmania infantum. **Journal of veterinary internal medicine / American College of Veterinary Internal Medicine**, v. 13, n. 4, p. 330–334, 1999.

CHAKRAVARTY, J. et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. **Vaccine**, v. 29, n. 19, p. 3531–3537, 2011.

DE PAIVA CAVALCANTI, M. et al. The development of a real-time PCR assay for the quantification of Leishmania infantum DNA in canine blood. **The Veterinary Journal**, v. 182, n. 2, p. 356–358, nov. 2009.

FOX, C. B. et al. A nanoliposome delivery system to synergistically trigger TLR4 AND TLR7. **Journal of Nanobiotechnology**, v. 12, n. 1, p. 17, 26 abr. 2014.

FOX, C. B. et al. Current Status of Toll-Like Receptor 4 Ligand Vaccine Adjuvants. In: **Immunopotentiators in Modern Vaccines**. [s.l.] Elsevier, 2017. p. 105–127.

GARÇON, N.; VAN MECHELEN, M. Recent clinical experience with vaccines using MPL- and QS-21-containing Adjuvant Systems. **Expert Review of Vaccines**, v. 10, n. 4, p. 471–486, 9 abr. 2011.

GHORBANI, M.; FARHOUDI, R. Leishmaniasis in humans: drug or vaccine therapy? **Drug Design, Development and Therapy**, v. 12, p. 25, 2018.

GRADONI, L. et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from Leishmania infantum infection and to prevent disease progression in infected animals. **Vaccine**, v. 23, n. 45, p. 5245–5251, 1 nov. 2005.

MANNA, L. et al. Real-time PCR assay in Leishmania-infected dogs treated with meglumine antimoniate and allopurinol. **Veterinary Journal**, v. 177, n. 2, p. 279–282, 2008.

MIRET, J. et al. Evaluation of an immunochemotherapeutic protocol constituted of N-methyl meglumine antimoniate (Glucantime®) and the recombinant Leish-110f®+MPL-SE® vaccine to treat canine visceral leishmaniasis. **Vaccine**, v. 26, n. 12, p. 1585–1594, 17 mar. 2008.

MORENO, J. et al. Immunization with H1, HASPB1 and MML Leishmania proteins in a vaccine trial against experimental canine leishmaniasis. **Vaccine**, v. 25, n. 29, p. 5290–5300, 20 jul. 2007.

NOLI, C.; AUXILIA, S. T. Treatment of canine Old World visceral leishmaniasis: a systematic review. **Veterinary Dermatology**, v. 16, n. 4, p. 213–232, ago. 2005.

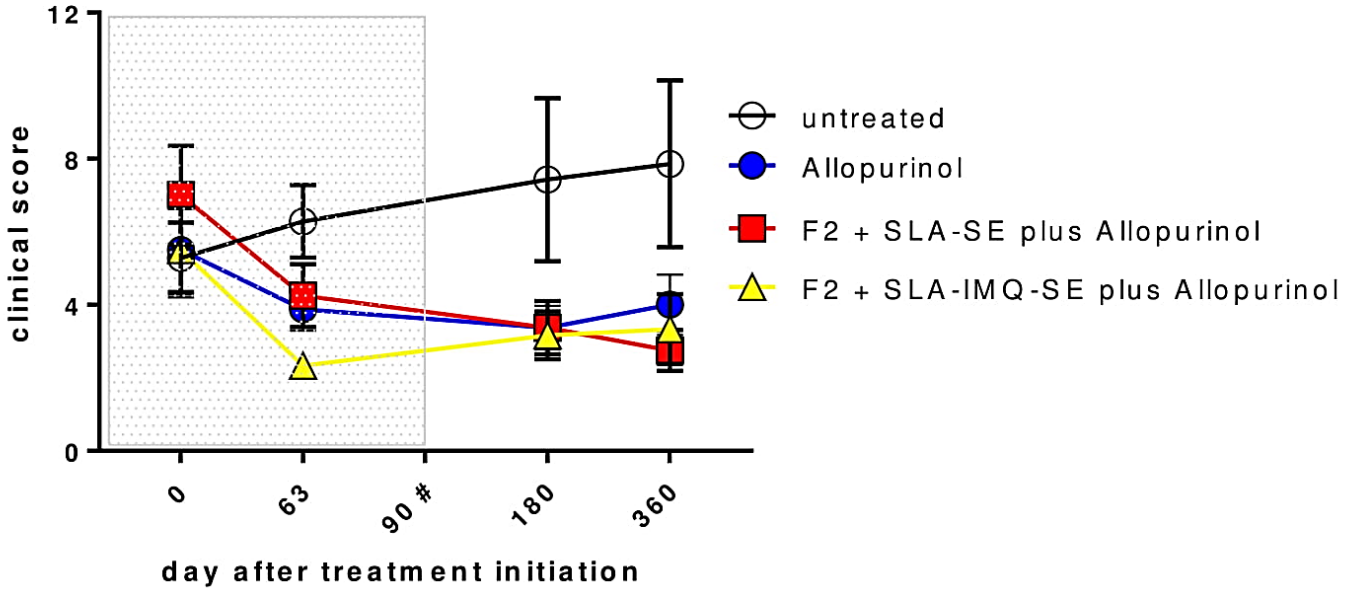
REED, S. G. et al. New horizons in adjuvants for vaccine development. **Trends in Immunology**, v. 30, n. 1, p. 23–32, jan. 2009.

REIS, A. B. et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. **Research in Veterinary Science**, v.

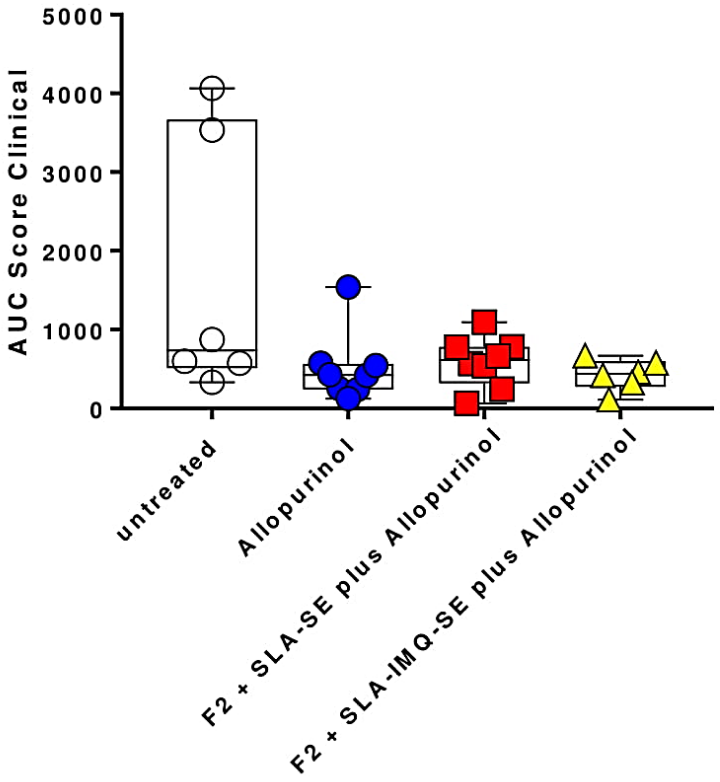
81, n. 1, p. 68–75, ago. 2006.
ROMERO, G. A. S.; BOELAERT, M. Control of Visceral Leishmaniasis in Latin America—A Systematic Review. **PLoS Neglected Tropical Diseases**, v. 4, n. 1, p. e584, 19 jan. 2010.
SCHAUT, R. G. et al. Recovery of antigen-specific T cell responses from dogs infected with Leishmania (L.) infantum by use of vaccine associated TLR-agonist adjuvant. **Vaccine**, v. 34, n. 44, p. 5225–5234, 17 out. 2016.
SILVA, K. R. DA et al. Scoring clinical signs can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil. **Memórias do Instituto Oswaldo Cruz**, v. 112, n. 1, p. 53–63, jan. 2017.
SKEIKY, Y. A. W. et al. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. **Vaccine**, v. 20, n. 27–28, p. 3292–303, 10 set. 2002.
TRAVI, B. L. Ethical and epidemiological dilemmas in the treatment of dogs for visceral leishmaniasis in Latin America. **Biomédica : revista del Instituto Nacional de Salud**, v. 34, n. 1, p. 7–12, 2014.
VEDVICK, T. et al. **An Improved Manufacturing Process for a Recombinant Polyprotein Vaccine**. [s.l: s.n.]. v. 21
WERNECK, G. L. The control of visceral leishmaniasis in Brazil: end of a cycle? **Cadernos de saude publica**, v. 32, n. 6, 20 jun. 2016.

Nascimento et al, Figure 1

A

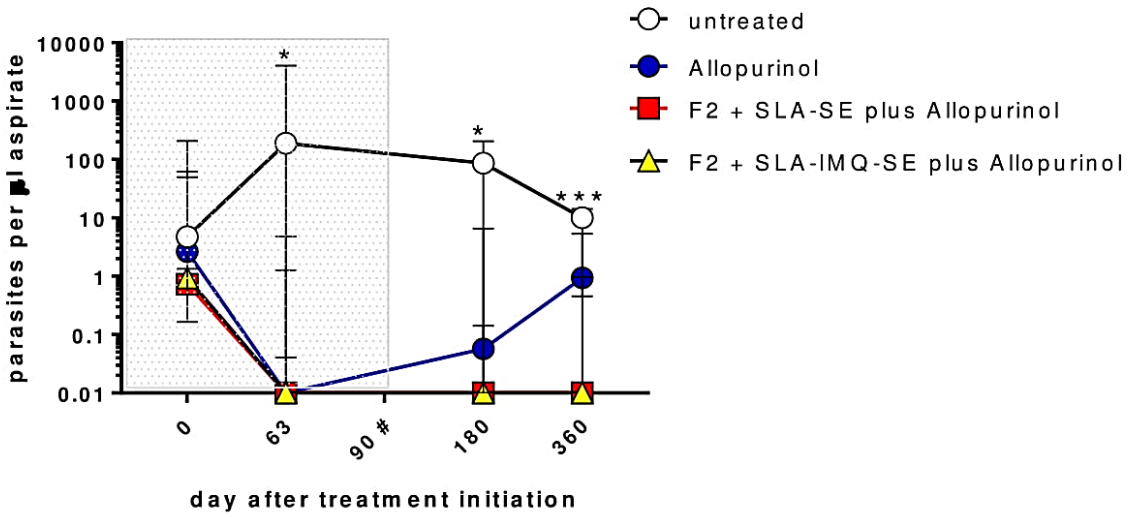


B

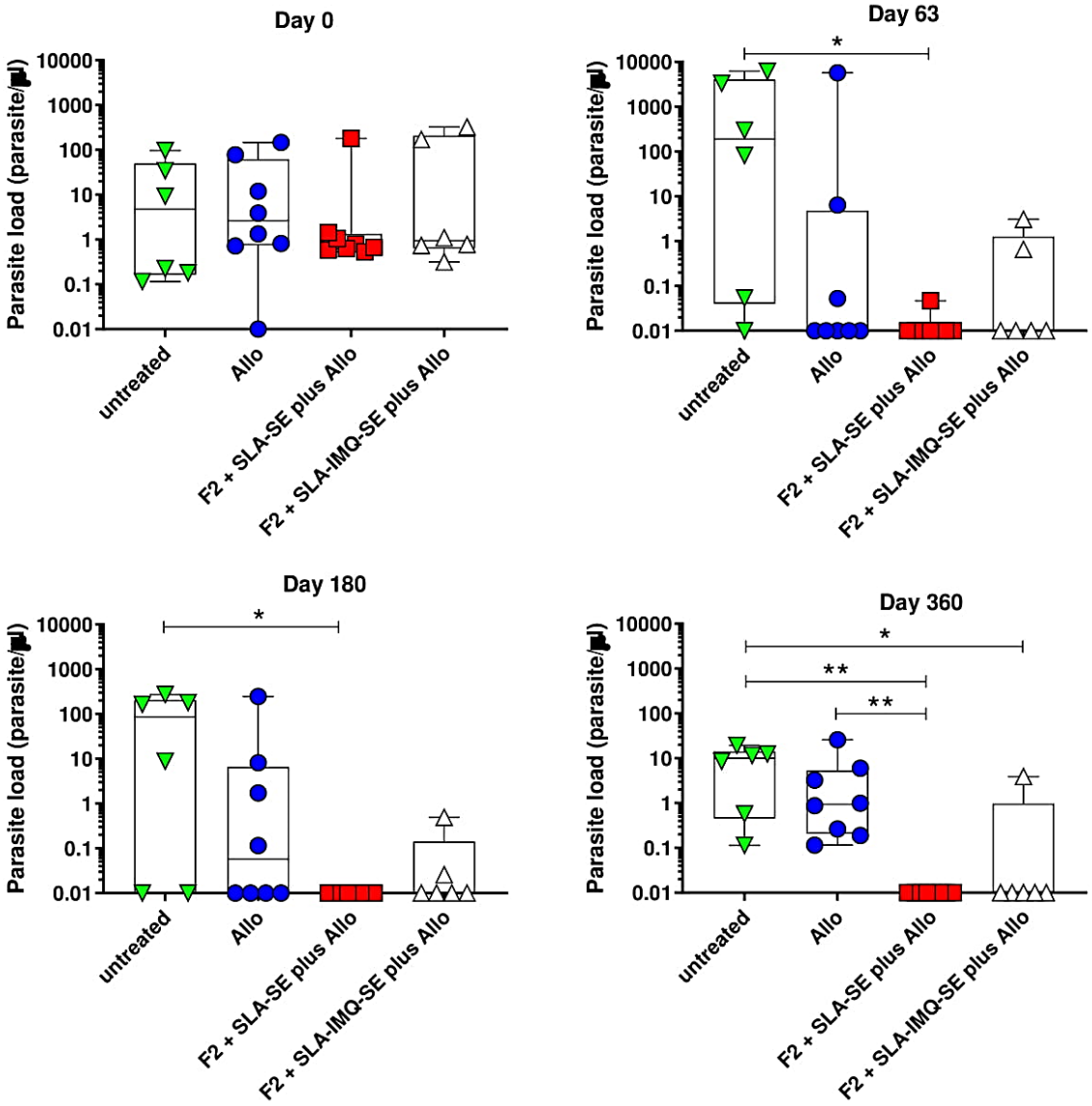


Nascimento et al, Figure 2

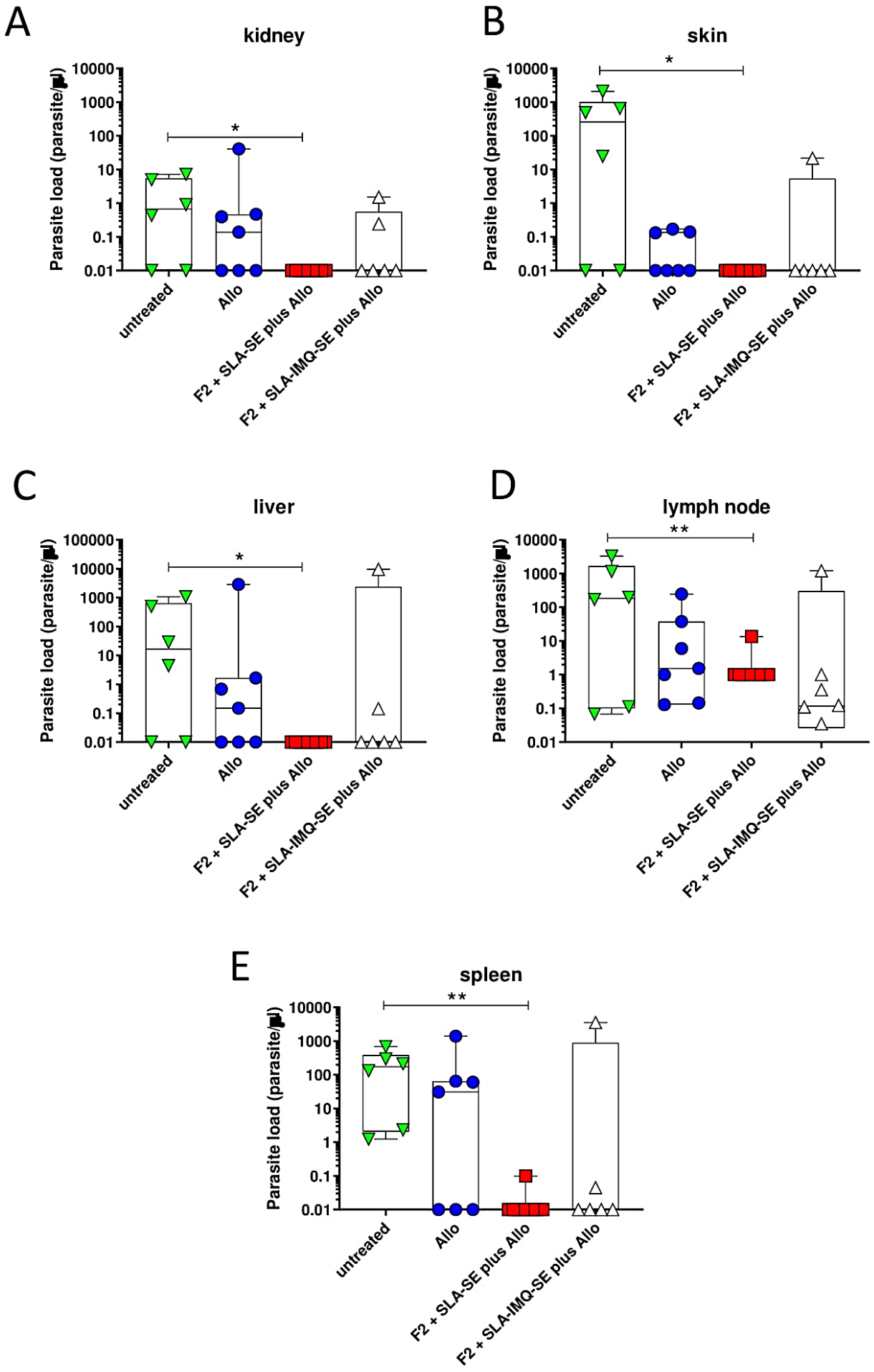
A



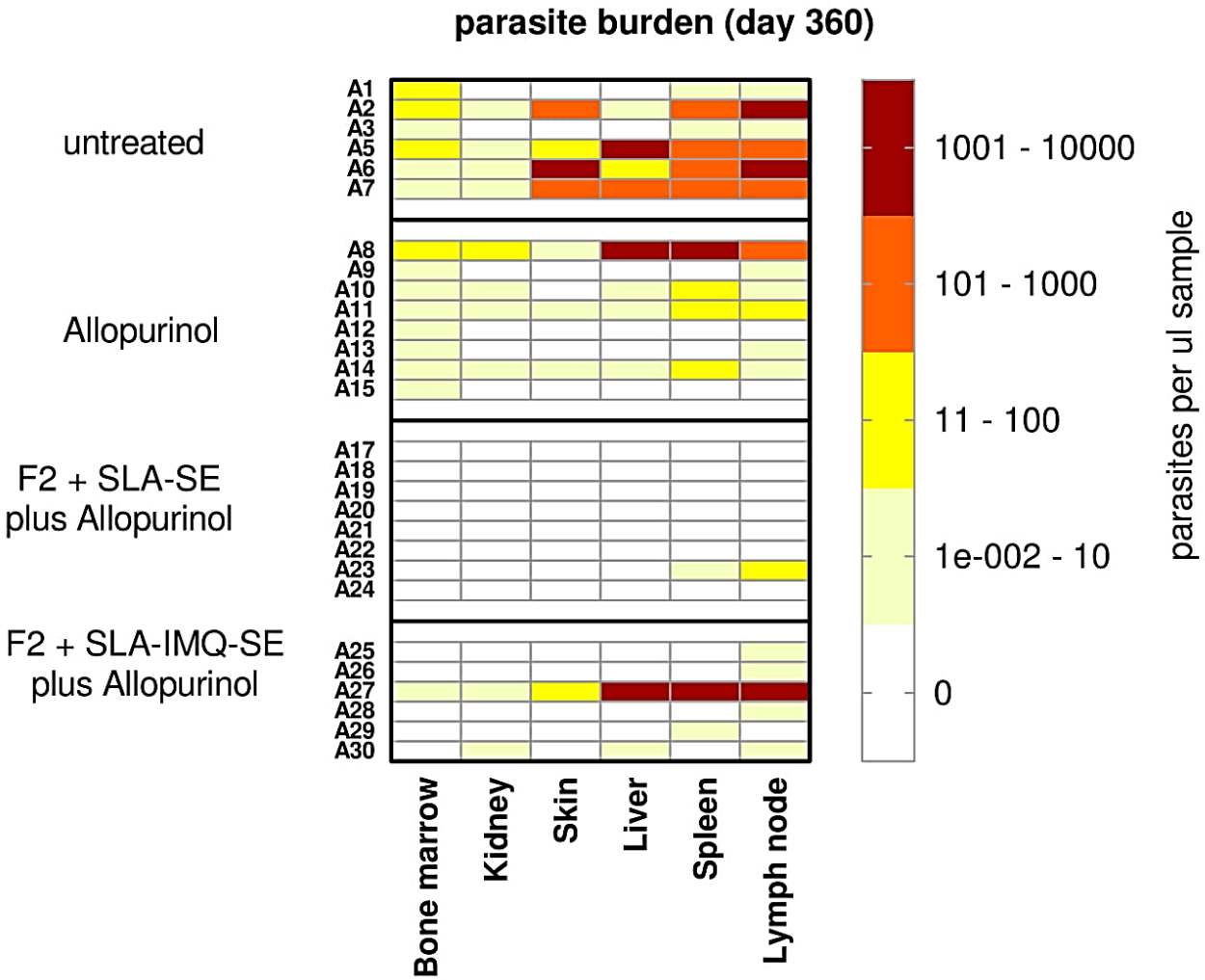
B



Nascimento et al, Figure 3



Nascimento et al, Figure 4



CAPÍTULO II*

*Publicada na Revista Memórias do Instituto Oswaldo Cruz.

Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. *112*(1): 53-62, January 2017 **53**

Scoring clinical signs can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil

Kleverton Ribeiro da Silva[1], Vitor Rosa Ramos de Mendonça[1], Kellen Matuzzy Silva[2], Leopoldo Fabrício Marçal do Nascimento[2], Antonio Ferreira Mendes-Sousa[3], Flaviane Alves de Pinho[2], Manoel Barral-Netto[1,4,5], Aldina Maria Prado Barral[1,4,5], Maria do Socorro Pires e Cruz[2]/+

[1]Fundação Oswaldo Cruz-Fiocruz, Centro de Pesquisas Gonçalo Moniz, Salvador, BA, Brasil
[2]Universidade Federal do Piauí, Departamento de Morfofisiologia Veterinária, Teresina, PI, Brasil
[3]Universidade Federal do Piauí, Picos, PI, Brasil
[4]Universidade Federal da Bahia, Faculdade de Medicina, Salvador, BA, Brasil
[5]Instituto Nacional de Ciência e Tecnologia, Instituto de Investigação em Imunologia, São Paulo, SP, Brasil

Canine visceral leishmaniasis (CVL) diagnosis is still a challenge in endemic areas with limited diagnostic resources. This study proposes a score with the potential to distinguish positive CVL cases from negative ones. We studied 265 dogs that tested positive for CVL on ELISA and parasitological tests. A score ranging between 0 and 19 was recorded on the basis of clinical signs. Dogs with CVL had an overall higher positivity of the majority of clinical signs than did dogs without CVL or with ehrlichiosis. Clinical signs such as enlarged lymph nodes (83.93%), muzzle/ear lesions (55.36%), nutritional status (51.79%), bristle condition (57.14%), pale mucosal colour (48.21%), onychogryphosis (58.93%), skin lesion (39.28%), bleeding (12.50%), muzzle depigmentation (41.07%), alopecia (39.29%), blepharitis (21.43%), and keratoconjunctivitis (42.86%) were more frequent in dogs with CVL than in dogs with ehrlichiosis or without CVL. Moreover, the clinical score increased according to the positivity of all diagnostic tests (ELISA, $p < 0.001$; parasite culture, $p = 0.0021$; and smear, $p = 0.0003$). Onychogryphosis (long nails) [odds ratio (OR): 3.529; 95% confidence interval (CI): 1.832-6.796; $p < 0.001$], muzzle depigmentation (OR: 4.651; 95% CI: 2.218-9.750; $p < 0.001$), and keratoconjunctivitis (OR: 5.400; 95% CI: 2.549-11.441; $p < 0.001$) were highly associated with CVL. Interestingly, a score cut-off value ≥ 6 had an area under the curve of 0.717 ($p < 0.0001$), sensitivity of 60.71%, and specificity of 73.64% for CVL diagnosis. The clinical sign-based score for CVL diagnosis suggested herein can help veterinarians reliably identify dogs with CVL in endemic areas with limited diagnostic resources.

Key words: canine visceral leishmaniasis - clinical score - diagnosis - clinical signs

American visceral leishmaniasis is a chronic parasitic zoonosis widespread in Latin America, with 90% of the cases occurring in Brazil, where it affects more than 3,300 individuals per year (Alvar et al. 2012). It is caused by a protozoan parasite *Leishmania infantum* (syn *L. chagasi*) transmitted by the bite of infected female sand flies of the genus *Lutzomyia*. This disease mainly affects malnourished children under 10 years of age, and it is commonly fatal if not treated early (Alvar et al. 2012). In endemic urban areas, domestic dogs are important hosts of the parasite, acting as an easy source of *Leishmania* infection for sand flies because of intense cutaneous parasitism (Dantas-Torres 2007). In addition, canine visceral leishmaniasis (CVL) is highly associated with cases of human disease (de Araújo et al. 2013).

Infected dogs can present different clinical features ranging from apparently healthy (asymptomatic dogs) to several characteristic signs (symptomatic dogs) such as lymphadenopathy, onychogryphosis, cutaneous lesions, alopecia, apathy, vomiting, fever, diarrhoea, polyuria, polydipsia, splenomegaly, and pale mucous membranes (Mancianti et al. 1988). Many studies have shown that the clinical signs of infected dogs are related to haematological and biochemical alterations as well as to antibody titres, parasite load, and infectivity to the sand fly vector (da Costa-Val et al. 2007, Manna et al. 2009, de Freitas et al. 2012, Manzillo et al. 2013, Ribeiro et al. 2013).

Diagnosis of CVL is a difficult task for veterinarians because of the following factors. First, the clinical signs are highly variable and are similar to those of other pathologies, such as ehrlichiosis (Tafuri et al. 2001, de Castro et al. 2004). Second, the histopathologic analysis is invasive, time-consuming, and expensive. Third, developing a 100% specific and sensitive diagnostic test is not possible (Ferrer 1999). In endemic regions with limited resources, the diagnostic procedure is even worse because serological or molecular methods are difficult to perform. In this context, a reliable clinical score based on clinical signs is needed to help CVL diagnosis and management in endemic regions. In Italy, a severity score based on CVL signs was proposed; however, it was not compared with the scores of CVL-negative dogs to predict its diagnostic capacity (Manna et al. 2009).

doi: 10.1590/0074-02760160305
Financial support: CAPES.
KRS and VRRM contributed equally to this work.
+ Corresponding author: mspcruz@gmail.com
Received 6 July 2016
Accepted 20 September 2016

In this study, we propose a new scoring system based on frequently observed clinical signs in order to help diagnose CVL in regions with limited resources. In order to verify the efficacy of this clinical score, we applied it in a large sample of sick dogs (including CVL-positive and CVL-negative animals) that were brought to a reference veterinary hospital in a highly endemic, low-resource area in Brazil.

MATERIALS AND METHODS

Study design and dogs - All dogs that were brought to the reference veterinary hospital of the Federal University of Piauí (UFPI) in Teresina, a city in the northeast of Brazil, between 2011 and 2012 underwent a careful clinical examination by trained veterinarians. The dogs were usually brought in when they showed clinical signs, and when the veterinarians from communities and small villages suspected them of having CVL. Moreover, sick, stray dogs collected by the Zoonosis Control Center following the Brazilian Zoonosis Control Program were also included. These animals were recruited because they were homeless dogs, and many of them were sick. The veterinary hospital at the UFPI is a regional reference centre for CVL, and all dogs showing clinical signs similar to leishmaniasis underwent routine clinical and laboratory tests for this pathology.

Fourteen different signs were evaluated in the dogs by observing the presence of signs attributable to *Leishmania* infection by using the following criteria:

Systemic signs - Attitude: active (0), apathetic (1); ectoparasites: absence (0), fleas (1), fleas and ticks (2); nutritional status: normal (0), thin (1), cachectic (2); lymph nodes: normal (0), enlarged (1); mucosal colour: normal (0), pale (1); bleeding: absence (0), presence (1).

Cutaneous signs - Bristles: good (0), regular (1), bad/opaque (2); muzzle/ear lesion: absence (0), presence (1); nails: normal (0), long/onychogryphosis (1); skin lesion: absence (0), presence (1), ulcer (2); muzzle depigmentation: absence (0), presence (1); alopecia: absence (0), presence (1).

Ocular signs - Blepharitis: absence (0), presence (1); keratoconjunctivitis: absence (0), serous (1), mucopurulent (2).

The clinical assessment was performed by three veterinarians who scored the signs according to the above criteria. In addition, all the individual scores were added to produce a total sign-based score ranging between 0 and 19, adapted from Manna et al. (2009).

In total, 443 mixed-breed adult male and female dogs of different ages (range, six months to 13 years) and breeds naturally infected with CVL were studied. The remaining dogs (sick dogs) were considered positive for CVL following a positive serological test and at least one positive parasitological test (CVL positive). Dogs with negative results on all three diagnostic tests were considered negative for CVL (CVL negative). CVL-positive dogs co-infected with *Ehrlichia canis* and/or *Babesia canis* were excluded from this study. In addition, a group of dogs infected by *E. canis* (and negative for CVL, n = 22) underwent clinical examination (based on the proposed score) and was included in this study.

Diagnostic tests - Blood was collected using jugular venipuncture, and samples of bone marrow were obtained using sternal puncture and lymph node aspiration (both tests for all dogs). Scrapings of skin lesions suggestive of CVL were also collected. The smears from these tissues were prepared and stained with Giemsa (Sigma Aldrich, St. Louis, MO, USA) stain and examined microscopically under a 100× objective for the presence of amastigotes. Bone marrow and lymph node aspirate was transferred to NNN-Schneider's culture medium (Sigma-Aldrich), which was maintained at 25ºC and examined weekly for 30 days. Soluble *Leishmania* antigen was obtained from *Leishmania* spp. cultivation using 1×10^{10} promastigotes (Souza et al. 2013) to detect IgG anti-*Leishmania* antibodies by using an ELISA (Badaró et al. 1986). The serum titration used was 1:400, and the cut-off was calculated for each plate as the mean of three negative sera ± 2 standard deviations. Diagnostic CVL tests, including ELISA, parasite culture, or smear, were performed for all dogs. Dogs were considered positive for CVL following a positive ELISA (highly sensitive method) and positive parasite culture or smear (highly specific methods). Dogs considered negative for CVL were those with negative results for all three diagnostic tests.

E. canis and *B. canis* infections were diagnosed through nested polymerase chain reaction (PCR) and PCR, respectively, via protocols routinely used in the Animal Pathology Laboratory of the UFPI by using DNA from peripheral blood (Wen et al. 1997, Jefferies et al. 2007). Detection of *E. canis* was based on the 16S rRNA gene and *B. canis* was based on the 18S rRNA gene.

Ethics statement - This study was approved by the Institutional Review Board of the UFPI, under protocol number 021/12. In addition, the animal care protocols used in this study adhered to the guidelines regulated by the Brazilian College of Animal Experimentation.

Data analysis - In the exploratory analysis of the data, frequency tables were constructed and the Chi-square or Fisher's exact tests were applied to evaluate the association between the qualitative variables (sex and breed). The severity of clinical signs was treated as an ordinal variable. The quantitative variables (total score and score based on statistically different signs) were tested for Gaussian distribution within the total sample by using the D'Agostino-Pearson omnibus normality test. None of the variables showed a normal distribution ($p < 0.0001$ for all; thus, they did not pass the normality test), and hence, non-parametric tests were used. In this context, the Mann-Whitney test was used to assess the differences between two groups. Heat maps and hierarchical cluster analyses using Ward's method were performed to better understand the association of signs in all sick dogs and according to CVL status. Univariate logistic regression analysis was performed to test the associations between the presence or absence of each clinical sign and clinical score cut-off values with CVL positivity. Receiver operator characteristic (ROC) curves with C-statistics were used to establish the threshold value of clinical scores that could discriminate between positive and negative CVL.

In order to understand how the signs associate with each other, network analyses based on clinical signs and represented by statistically significant correlations were performed for all sick and CVL-positive dogs. Correlations between all quantitative variables were analysed with the Spearman test by using the JMP 11.0 software (SAS, Cary, NC, USA), and these correlations were used to build the networks represented by the association between signs. The links between the signs represent only the statistically significant correlations ($p < 0.05$). The complexity of the networks was estimated from the density of interactions of each network, which could be measured using the following equation: density (D) = L/(N (N-1)/2), where L is the number of observed edges (*i.e.*, Spearman correlations with $p < 0.05$) and N is the total number of nodes in the network; the density could range between 0 (no edges in the network) and 1 (all possible edges present). Network figures were customised using the Pathways Analysis software (Ingenuity Systems, Redwood City, CA, USA) and Adobe Illustrator (Adobe Systems Inc., Seattle, WA, USA) The statistical analyses were performed using the programs GraphPad Prism 6.0 (GraphPad Software Inc., USA), IBM SPSS Statistics for Windows/Macintosh, Version 22.0 (IBM Corp., Armonk, NY, USA), and JMP 11.0 (SAS). A p value lower than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics - Of the 443 dogs screened, 178 co-infected with *L. infantum* and *E. canis* or *B. canis* were excluded from this study (n = 120 and 58, respectively). Finally, 265 sick dogs irrespective of sex and breed were recruited; 89 (33.58%) were originally from the veterinary hospital and 176 (66.42%) from the Brazilian Zoonosis Control Program. The percentage of male and female animals was very similar (47.55% males; Table I). No differences were seen in the sex or breed of dogs relative to the presence or absence of CVL ($p = 1.000$ and $p = 0.539$, respectively). Reproductive status was not recorded in this study. The distribution of breeds among all the sick dogs and according to CVL status is described in detail in Table I.

The frequency of all the clinical signs recorded in the score in the different study groups (CVL-positive, CVL-negative, and *E. canis*-infected dogs) are described in Table II. As expected, the dogs with CVL had an overall higher positivity for the majority of clinical signs than did dogs without CVL or with ehrlichiosis (Table II). Clinical signs such as enlarged lymph nodes ($p < 0.0001$), muzzle/ear lesions ($p < 0.0001$), nutritional status ($p < 0.0001$), bristle condition ($p = 0.0001$), pale mucosal colour ($p = 0.0202$), onychogryphosis ($p < 0.0001$), muzzle depigmentation ($p = 0.0002$), alopecia ($p = 0.0129$), blepharitis ($p = 0.0159$), and keratoconjunctivitis ($p = 0.0009$) were more frequent in dogs with CVL than in those with ehrlichiosis (Table II). To confirm whether these clinical signs were more important in CVL cases, the signs were compared with those of sick dogs without CVL and with ehrlichiosis. Thus, it was seen that dogs with ehrlichiosis had less enlarged lymph nodes ($p < 0.0001$), less muzzle/ear lesions ($p = 0.0022$), less onychogryphosis ($p = 0.0274$), and better nutritional status ($p = 0.0274$) and bristle condition ($p = 0.0035$) (Table II).

Diagnostic tests and clinical signs - ELISA, cultures, and smears were performed in all dogs. The positivity for each and the association of tests are described in Fig. 1A. Of the 265 dogs, 129 (48.68%) tested negative for all tests and were considered negative for CVL. Fifty-six dogs (21.13%) had positive ELISA and positive parasite culture results (n = 46) or/and positive parasite smear results (n = 35), and were considered positive for CVL (Fig. 1A). The next objective was to determine whether the total score value (range, 0-19), which combined all the signs, differed according to the results of the diagnostic tests (Fig.

TABLE I

Baseline characteristics of sick dogs recruited in this study

	CVL positive n = 56	CVL negative n = 129	All dogs n = 265	p value*
Male n(%)	26 (46.43)	60 (46.51)	126 (47.55)	1.000**
Breed n(%)				
Dalmatian	0 (0.00)	1 (0.77)	2 (0.75)	0.539***
Brazilian mastiff	0 (0.00)	1 (0.77)	3 (1.13)	
German Shepherd	1 (1.79)	2 (1.55)	5 (1.89)	
Pinscher	2 (3.57)	3 (2.33)	7 (2.64)	
Pit bull	2 (3.57)	4 (3.10)	9 (3.40)	
Poodle	2 (3.57)	9 (6.98)	14 (5.28)	
Rottweiler	1 (1.79)	3 (2.33)	5 (1.89)	
Yorkshire	0 (0.00)	0 (0.00)	2 (0.75)	
Mongrel	46 (82.14)	94 (72.87)	201 (75.85)	
Others	2 (3.57)	12 (9.30)	17 (6.42)	

*: p value was calculated by comparing the canine visceral leishmaniasis (CVL)-positive and CVL-negative subjects; **: Fisher's exact test was used; ***: Chi-square test was used, and breeds with less than two dogs in each group were excluded.

TABLE II

Frequency of clinical signs in dogs with canine visceral leishmaniasis (CVL), ehrlichiosis, and without CVL

Clinical signs n(%)	1 VL positive n = 56	2 VL negative n = 129	3 *E. canis* n = 22	p value 1 vs. 2	p value 1 vs. 3	p value 2 vs. 3
Apathy*	3 (5.36)	4 (3.10)	1 (4.54)	0.4341	1.000	0.5502
Nutritional status**						
Thin	26 (46.43)	35 (27.13)	1 (4.54)	0.0023	< 0.0001	0.0274
Cachectic	3 (5.36)	0 (0.00)	0 (0.00)			
Enlarged lymph nodes*	47 (83.93)	88 (68.22)	5 (22.73)	0.0308	< 0.0001	< 0.0001
Pale*	27 (48.21)	46 (35.66)	4 (18.18)	0.1404	0.0202	0.1425
Bleeding*	7 (12.50)	4 (3.10)	0 (0.00)	0.0194	0.1815	1.000
Bristles**						
Regular	23 (41.07)	43 (33.33)	1 (4.54)	0.1100	0.0001	0.0035
Bad/opaque	9 (16.07)	11 (8.53)	0 (0.00)			
Muzzle/ear lesion*	31 (55.36)	36 (27.91)	0 (0.00)	0.0005	<0.0001	0.0022
Onychogryphosis*	33 (58.93)	37 (28.68)	2 (9.09)	0.0001	<0.0001	0.0650
Skin lesion***						
Presence	16 (28.57)	19 (14.73)	3 (13.64)	0.0162	0.0695	0.5727
Ulcer	6 (10.71)	6 (4.65)	0 (0.00)			
Muzzle depigmentation*	23 (41.07)	17 (13.18)	0 (0.00)	< 0.0001	0.0002	0.1352
Alopecia*	22 (39.29)	29 (22.48)	2 (9.09)	0.0308	0.0129	0.2511
Blepharitis*	12 (21.43)	8 (6.20)	0 (0.00)	0.0039	0.0159	0.6039
Keratoconjunctivitis**						
Serous	21 (37.50)	12 (9.30)	1 (4.54)	<0.0001	0.0009	0.4693
Mucopurulent	3 (5.36)	4 (3.10)	0 (0.00)			

*: Fisher's exact test was used; **: positive signals were combined and analysed using Fisher's exact test; ***: Chi-square test was used.

1B). The sign-based score was higher for the positive results of all three individual diagnostic tests (ELISA, $p < 0.001$; parasite culture, $p = 0.0021$; and smear, $p = 0.0003$) and their combined results (ELISA and parasite culture, $p < 0.0001$; ELISA and parasite smear, $p < 0.0001$) than for the negative results of these tests (Fig. 1B).

A heat map analysis was performed for identifying clusters based on the signs of sick dogs (Fig. 2). Overall, the presence of some signs was more frequent in sick dogs with CVL, as illustrated by lymph node enlargement, presence of ectoparasites, alterations in the bristles, and muzzle/ear lesions (Fig. 2). In addition, organ-related signs from the proposed score (systemic, cutaneous, and ocular groups) had a general trend for clustering. Clusters were observed between the ocular signs (keratoconjunctivitis and blepharitis) and between the cutaneous signs (skin lesion and muzzle/ear lesion, for example), demonstrating that these signs were characteristic of sick dogs from this endemic area (Fig. 2).

Networking the clinical signs according to CVL status - A higher complexity, measured by the network density ($D = 0.346$), was observed in all dogs when compared with the CVL-positive dogs ($D = 0.165$) (Fig. 3A, 3C, respectively); however, this could be attributed to the different sample sizes. The majority of signs (except lymph node size and ectoparasite presence) had a high level of association in all dogs brought into the reference veterinary hospital (Fig. 3A). The percentage of positivity for each sign for all dogs is illustrated in Fig. 3B. In the CVL-positive dogs, half of the signs (keratoconjunctivitis, mucosal colour, muzzle depigmentation, nutritional status, nails, bristles, and blepharitis) had the highest number of connections, suggesting their important role in the final clinical profile. The only significant negative correlation was between the lymph node size and nutritional status variables (Fig. 3C). The percentage of positivity for each sign in the CVL-positive dogs is demonstrated in Fig. 3D. The results showed that the overall positivity for the clinical signs was slightly more expressed in CVL-positive dogs than in all the sick dogs, and that the relative distributions of some signs differed between the two groups, as illustrated by serous or mucopurulent keratoconjunctivitis (42.86% and 20.65%, $p = 0.001$, Fig. 3D, B, respectively).

Heat map analyses according to the different signs were performed for CVL-negative and CVL-positive dogs (Fig. 4A-B, respectively). The results showed that the majority of signs were expressed more highly in the CVL-positive dogs than in the CVL-negative dogs. Moreover, the signs with the highest connectivity demonstrated in Fig. 3C also clustered in the CVL-positive heat map (highlighted in Fig. 4B) with smaller clusters between the following pairs of signs: mucosal colour and muzzle depigmentation; nutritional status and nails; and bristles and blepharitis (Fig. 4B).

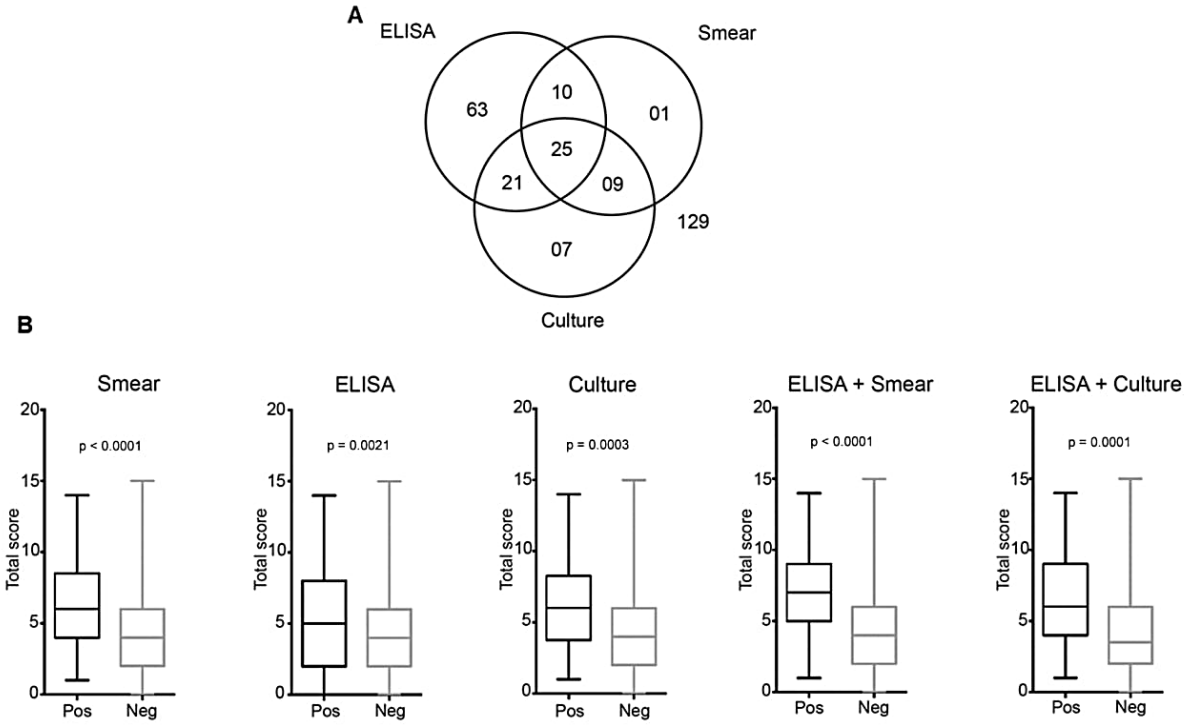


Fig. 1: canine visceral leishmaniasis (CVL) diagnostic tests and clinical signs. ELISA, smear, and culture tests for CVL were performed for all dogs brought to a reference veterinary hospital in the endemic area (A). Total clinical scores (range, 0-19) were higher in dogs with positive results on ELISA, culture, and smear tests and for the combinations of ELISA + culture or ELISA + smear tests (B). Data were compared using the Mann-Whitney test (B).

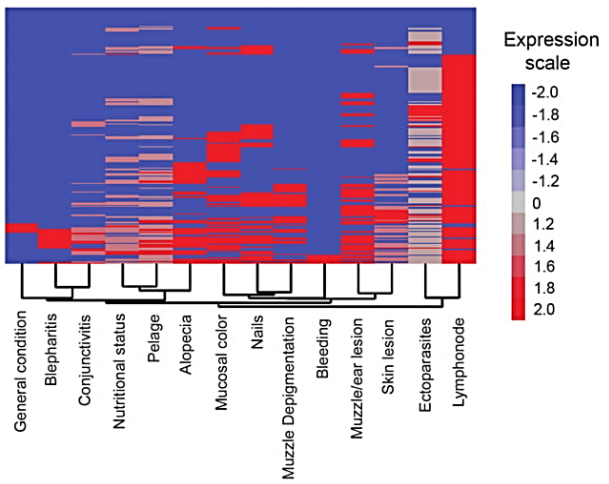


Fig. 2: heat map analysis of all sick dogs. Fourteen different signs were evaluated according to the proposed clinical score, and a two-way hierarchical cluster analysis (Ward's method) was performed to identify patterns of associations between different signs among all the dogs. The colours show the fold variation from the median values (log transformed) calculated for each sign as represented by the expression scale.

Association between clinical profile and CVL status - Ten of the 14 signs seemed to favour CVL, with special influences of onychogryphosis (long nails) (OR: 3.529; 95% CI: 1.832-6.796; $p < 0.001$), muzzle depigmentation (OR: 4.651; 95% CI: 2.218-9.750; $p < 0.001$), and keratoconjunctivitis (OR: 5.400; 95% CI: 2.549-11.441; $p < 0.001$) (Fig. 5A). Apathy (attitude), presence of ectoparasites, pale mucosal colour, and lymph node enlargement did not show any influence on CVL ($p = 0.433$, $p = 0.609$, $p = 0.097$, and $p = 0.068$, respectively; Fig. 5A). The cut-off values (≥ 6, ≥ 9, and ≥ 12) for the total clinical score (range, 0-19) established by using the ROC curve and C-statistics analyses (Fig. 5B) were also imputed in the regression model. The cut-off values ≥ 6 (OR: 4.151; 95% CI: 2.141-8.047; $p < 0.001$) and ≥ 9 (OR: 5.812; 95% CI: 2.399-14.083; $p < 0.001$) had almost similar performance in distinguishing between the CVL-positive and CVL-negative animals, and the cut-off value ≥ 12 had a lower precision (with a higher CI) than did the other described cut-off values (OR: 5.040; 95% CI: 1.213-20.20937; $p = 0.026$) (Fig. 5A).

The total clinical score (14 signs; range, 0-19) and a score based on the statistically different signs (10 signs; range, 0-14) shown in Fig. 5A had considerable power to distinguish between the CVL-positive and the CVL-negative dogs (Fig. 5B). The total score had an area under the curve (AUC) of 0.717 ($p < 0.0001$), and the cut-off value of ≥ 6 was the one with the best sensitivity for association (60.71%) and specificity (73.64%) to discriminate the CVL-positive and CVL-negative dogs (Fig. 5B). With the higher cut-off values (≥ 9 and ≥ 12), the specificity increased (93.02% and 97.67%, respectively) despite the decrease in sensitivity (30.36% and 10.71%, respectively). Furthermore, the score based on statisti-

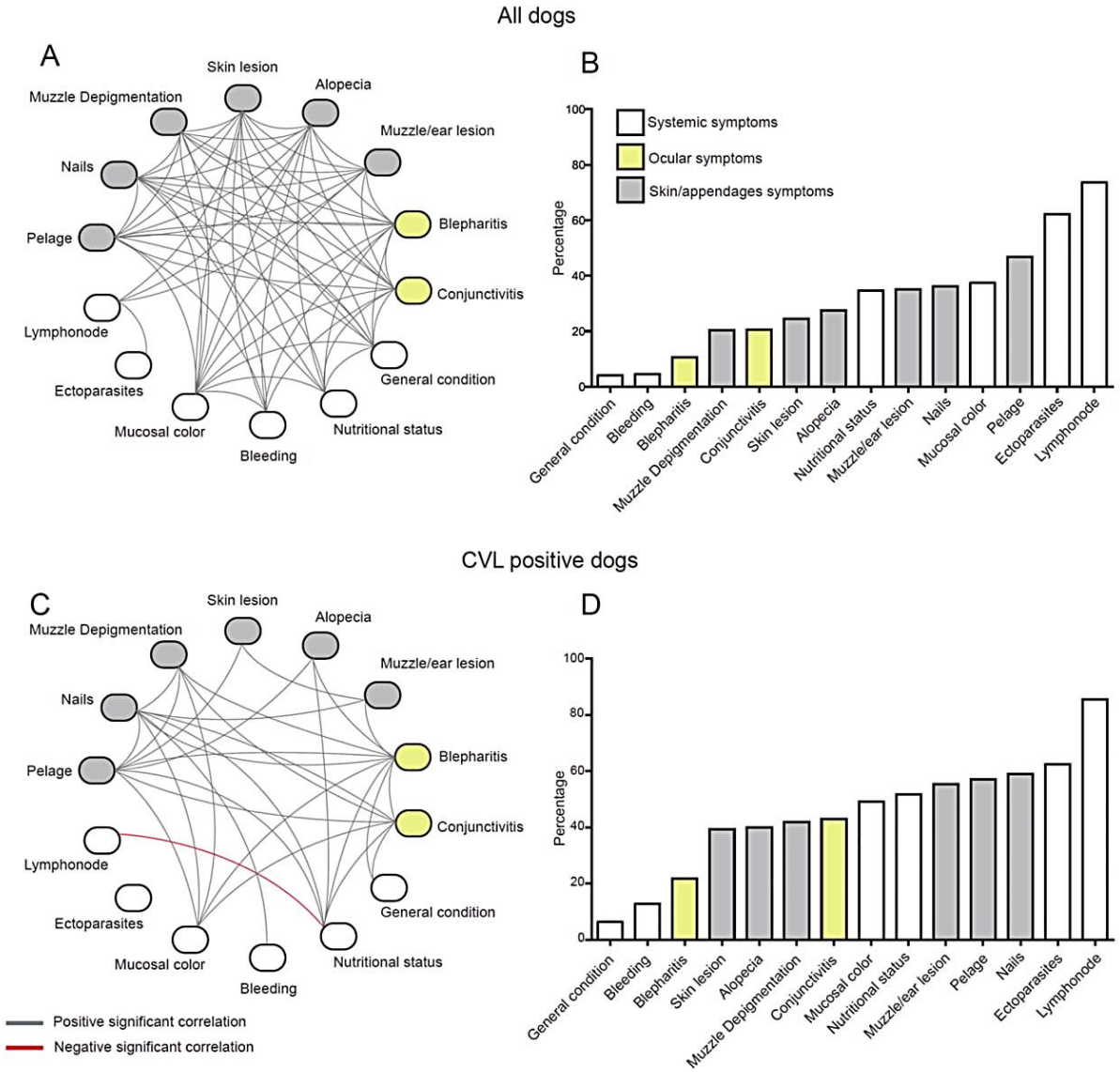


Fig. 3: networking the clinical signs of dogs with canine visceral leishmaniasis (CVL). The network analysis (interactome) shows statistically significant correlations ($p < 0.05$) between all the signs recorded for all sick dogs (A) and CVL-positive dogs (C). Data were analysed using Spearman rank tests. Yellow-coloured items are ocular signs, grey icons are skin/appendage signs, and white entries are systemic signs. Grey and red lines represent significant positive and negative correlations, respectively. The percentages of positivity for each sign are illustrated for all dogs (B) and CVL-positive dogs (D).

cally different signs had an AUC of 0.727 ($p < 0.0001$), and the cut-off value ≥ 3 had a sensitivity and specificity of 73.21% and 62.02%, respectively, to discriminate animals according to CVL status (Fig. 5B).

The score proposed herein was applied in dogs with ehrlichiosis (but without CVL) in order to assess the ability of this score to test the specificity to CVL diagnosis. The clinical signs observed in the subjects with ehrlichiosis are described in Table II. With a cut-off value ≥ 3, the total score had an AUC of 0.9391 ($p < 0.0001$), and a sensitivity and specificity of 85.71% and 86.36%, respectively, to distinguish the CVL-positive dogs from those with ehrlichiosis.

DISCUSSION

The serological and molecular diagnostic methods for CVL may be unavailable in endemic areas with limited resources, and in such areas, the diagnosis may be based on only clinical signs. In the present study, dogs that tested positive for CVL with different diagnostic methods (ELISA, parasite smear, or culture) had a higher clinical score than did CVL-negative sick dogs. The cut-off value ≥ 6 obtained from the clinical score proposed herein performed well in distinguishing CVL-positive dogs from CVL-negative sick dogs with 60.71% and 73.64% sensitivity and specificity, respectively.

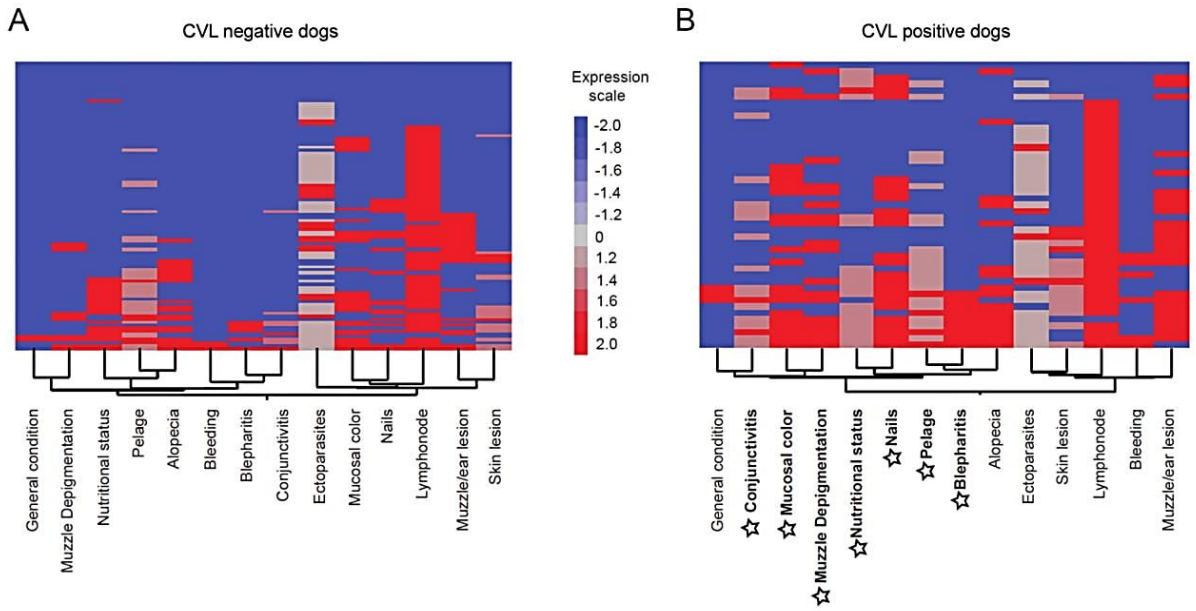


Fig. 4: heat map analysis of the clinical signs of dogs according to canine visceral leishmaniasis (CVL) status. Two-way hierarchical cluster analysis (Ward's method) was performed to identify patterns of associations between different signs for CVL-negative dogs (A) and CVL-positive dogs (B). The colours show the fold variation from the median values (log transformed) calculated for each sign. Highlighted in bold and with star symbols are those signs with the highest association observed in CVL-positive dogs in Fig. 3C.

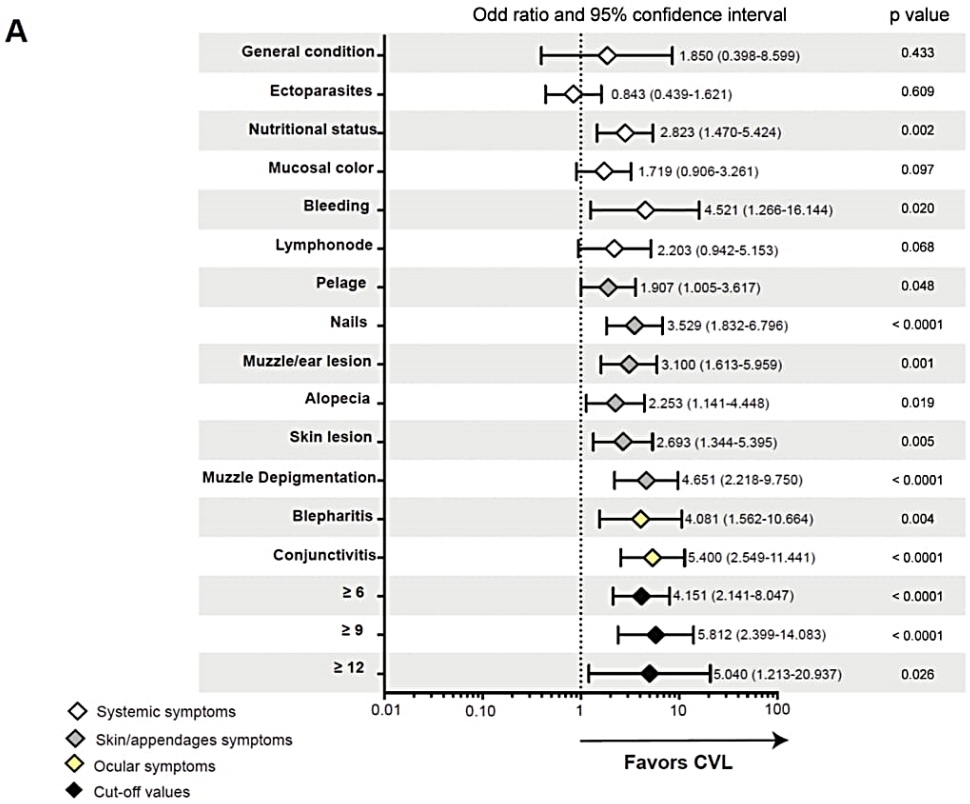
Gouvêa et al. (2016), who conducted a study in the same area, found a cut-off value ≥ 2 for a model based on the clinical score only (sensitivity = 75.3%; specificity = 65.9%) and a cut-off value ≥ 9 for an association of IFAT + clinical score (sensitivity = 86.5%; specificity = 70%), thereby showing it was the best cut-off value for detecting the most infectious dogs and for disease control.

The clinical signs of CVL are important for diagnosis, and the clinical score based on sick dogs' clinical signs proposed herein was higher in subjects who tested positive for CVL on different diagnostic tests, suggesting it can help in diagnosis depending on the cut-off value chosen. A group of clinical signs of *Leishmania* infection (keratoconjunctivitis, mucosal colour, muzzle depigmentation, nutritional status, nails, bristles, and blepharitis) was highly associated and clustered in the heat map results. Furthermore, the sole presence of onychogryphosis, muzzle depigmentation, or keratoconjunctivitis correlated highly with leishmaniasis. Manna et al. (2009) also created a severity clinical score that was associated with parasite load in CVL; however, this severity score was not compared with those of sick dogs without CVL to establish its diagnostic power. Other studies have demonstrated a model based on a scoring system combining the clinical signs and serological (IFAT) results as a tool to help in CVL control strategies (Proverbio et al. 2014, Gouvêa et al. 2016).

CVL diagnosis is still a challenge for veterinarians because of the lack of a perfect diagnostic test for this condition (Solano-Gallego et al. 2011). In the present study, CVL-positive dogs were diagnosed by both a positive serological test (ELISA) and a positive parasitological test (parasite culture or smear) to confirm CVL diagnosis by using high-sensitivity and high-specificity methods, respectively. CVL diagnoses based only on anti-*Leishmania* antibody detection are not satisfactory as they do not discriminate between the disease and an asymptomatic condition; moreover, the antibodies cross-react with other pathogens (Sarkari et al. 2005, Zanette et al. 2014). For example, the conventional serological methods using crude antigen to detect antibodies against *Leishmania* spp. show cross-reactivity with *Trypanosoma cruzi* because both parasites share similar antigenic epitopes owing to their phylogenetic similarity (Zanette et al. 2014). Moreover, dogs infected with *T. cruzi* have not yet been detected in Teresina, PI. These aspects may have contributed to the different sensitivity and specificity results obtained in the studies that used serological methods for detecting CVL (de Arruda et al. 2016).

In both sick and CVL-positive dogs, lymph node enlargement was the most common sign. In several case series, lymphadenomegaly was reported as the most frequent clinical sign in dogs with CVL (Ciaramella et al. 1997, Moreno & Alvar 2002, Amusategui et al. 2003, Manna et al. 2009). However, lymph node enlargement is a frequent response to infectious agents, and it is not specific to CVL (Mylonakis et al. 2011, Rodríguez-Morales et al. 2011, Salem & Farag 2014).

Keratoconjunctivitis and ocular signs in general are commonly reported in several case series with similar frequencies (Ferrer 1999, Amusategui et al. 2003, Manna et al. 2009, de Freitas et al. 2012, Manzillo et al. 2013). In the present report, 42.86% and 21.82% of dogs with CVL had keratoconjunctivitis and blepharitis,



	AUC	p value	Cut-off	Sensitivity (%)	Specificity (%)
Scored based on statistically different symptoms 10 symptoms; range 0-14	0.727	< 0.0001	≥ 3	73.21	62.02
Total score 14 symptoms; range 0-19	0.717	< 0.0001	≥ 6	60.71	73.64
			≥ 9	30.36	93.02
			≥ 12	10.71	97.67

Fig. 5: association of clinical signs and clinical scores with canine visceral leishmaniasis (CVL). Univariate logistic regression analyses were conducted to estimate the association of each sign [based on median values or cut-off values (≥ 6, ≥ 9, and ≥ 12)] with CVL (A). Yellow-coloured items are ocular signs, grey icons are skin/appendage signs, white items are systemic signs, and black ones represent random cut-off values for the total clinical score (range, 0-19). Receiver operator characteristic (ROC) curves of the total score (red line) and the score based on statistically significant different signs (based on the analysis in A; blue line) for discriminating between dogs with and without CVL are shown in B. AUC, area under the curve. The cut-off values for the scores were established using C-statistics and are shown in the table (B). The odds ratios (OR), respective 95% confidence intervals (95% CIs), and p values are shown in each panel.

respectively. Intriguingly, the clinical spectrum of CVL may vary according to the phase of the disease, the dog's immunity status, and previous specific therapies; thus, these features should be considered during CVL diagnosis (Ciaramella et al. 1997, Manzillo et al. 2013).

In the network analysis, we observed that some clinical signs (keratoconjunctivitis, mucosal colour, muzzle depigmentation, nutritional status, nails, bristles, and blepharitis) were more associated with CVL positivity than were other signs. Moreover, these signs clustered in the heat map analysis, indicating their important role in predicting CVL status. Ocular signs (*i.e.*, blepharitis and keratoconjunctivitis), cutaneous signs (*i.e.*, onychogryphosis and opaque bristles), and systemic signs (*i.e.*, pale mucous membrane and weight loss) have been extensively described in CVL clinical studies (Ciaramella et al. 1997, Amusategui et al. 2003, Manna et al. 2009, de Freitas et al. 2012, Manzillo et al. 2013). Cutaneous findings are very common in dogs with CVL, and the presence of skin lesions (including ulcers), onychogryphosis,

alterations in the bristles, dermatitis, and alopecia should be carefully considered in sick dogs from endemic areas (Ciaramella et al. 1997, Ferrer 1999).

Among the 14 different clinical signs that comprise the clinical score proposed in this study, many could be used to differentiate CVL-positive dogs from other sick dogs, especially the presence of keratoconjunctivitis, onychogryphosis, and muzzle depigmentation in the CVL-positive dogs. Mancianti et al. (1988) proposed classifying dogs with CVL into asymptomatic, oligosymptomatic, and symptomatic cases, with the presence of onychogryphosis, keratoconjunctivitis, and cutaneous alterations being the clinical signs expected in symptomatic dogs. Interestingly, chronic cutaneous changes and ocular lesions were also signs that characterised dogs with severe CVL (Ciaramella et al. 1997). In addition, the cut-off values for the clinical score proposed herein performed well (AUCs higher than 70%) in distinguishing CVL-positive dogs from other sick dogs (with different diseases). Higher cut-off values had better specificity for CVL diagnosis because dogs with elevated clinical scores had more signs of this disease; although high cut-off values lacked sensitivity, they could be used with good reliability for confirming CVL in severely sick dogs in endemic areas.

In this study, 17 dogs had positive smear and/or culture test results and negative ELISA results for CVL (Fig. 1A). Notably, when the clinical scores of the CVL-positive dogs (based on this study criteria) were compared to those with only positive smear and/or culture test results, the latter were found to have a lower score ($p = 0.0057$). This could probably be because these dogs were in the initial stages of infection and had not yet had a clinical response to leishmaniasis.

In this study, we had excluded CVL-positive dogs co-infected with *E. canis* and *B. canis*, which can produce clinical findings similar to CVL (Ciaramella et al. 1997). Some studies have demonstrated that dogs with co-infection presented more severe clinico-pathological abnormalities and were frequently misdiagnosed in routine veterinary practices (Andrade et al. 2014). Thus, we have applied our clinical model only in dogs with CVL and ehrlichiosis. When the clinical score was applied in dogs infected with *E. canis*, we observed a great discriminatory power to distinguish dogs with CVL from those with ehrlichiosis, suggesting that the ability of this score to detect CVL when *E. canis* infection was not previously excluded. Dogs with other diseases requiring potential differential diagnosis for CVL were also included in the present study. Dogs with trypanosomiasis caused by *T. cruzi* have a general lymphadenopathy (Barr et al. 1991), which is a usual clinical sign found in all dogs in this study. Furthermore, severe seborrhoea, focal or diffuse alopecia, pustules, and blemishes are frequently observed in 12% to 23% of dogs with canine atopic dermatitis and could be found in the CVL-negative sick dogs (Hensel et al. 2015). Similarly, bacterial skin diseases, superficial and deep folliculitis, and keratinisation disorders may also be confused with clinical signs of CVL and should be considered in the CVL-negative dogs (Cardoso et al. 2011) .

This study proposes a clinical sign-based score for CVL diagnosis that can help veterinarians reliably identify dogs with CVL. Although there is no evidence supporting dog culling for CVL control, culling is still practiced in many endemic areas; this makes determining a correct diagnosis of CVL even more important (Costa 2011). The clinical score proposed herein is a guide for veterinarians mainly from highly endemic areas with limited diagnostic resources. Moreover, this clinical score can help confirm the accuracy of some diagnostic test results (*i.e.*, serological methods). Nevertheless, diagnostic tests are still the best way to confirm CVL in sick dogs and should not be totally replaced by diagnosis based only on clinical signs.

Appropriate CVL diagnosis is a big challenge in endemic areas with limited diagnostic resources. We report here that a score based on the clinical signs of sick dogs in a highly endemic region can help CVL diagnosis. Overall, the clinical score proposed had a good performance in discriminating CVL-positive dogs from other sick dogs. Furthermore, some clinical signs were more correlated with CVL positivity, particularly the ocular and cutaneous signs. The results of this study may help veterinarians correctly diagnose CVL in sick dogs; however, further studies with larger samples are needed to confirm and validate this clinical score because this study only examined dogs brought to the veterinary hospital or collected by the Zoonosis Control Center. The higher prevalence of CVL among these dogs could have influenced the positive predictive values of the diagnostic tests.

ACKNOWLEDGEMENTS

To all employees of the veterinary hospital of the Federal University of Piauí, Teresina, Brazil; our team at FIOCRUZ-Bahia, Brazil, Jorge Tolentino, Adorielze Leite, Nataly Alexandrino, and Andrezza Kariny, for help with logistics; and Global Science Editing, UK, for English language corrections.

REFERENCES

Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012; 7(5): e35671.

Amusategui I, Sainz A, Rodriguez F, Tesouro MA. Distribution and relationships between clinical and biopathological parameters in canine leishmaniasis. Eur J Epidemiol. 2003; 18(2): 147-56.

Andrade GB, Barreto WT, Santos LL, Ribeiro LR, Macedo GC, Sousa KC, et al. Pathology of dogs in Campo Grande, MS, Brazil naturally co-infected with *Leishmania infantum* and *Ehrlichia canis*. Rev Bras Parasitol Vet. 2014; 23: 509-15.

Badaró R, Reed SG, Barral A, Orge G, Jones TC. Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis: antigen selection for detection of infection-specific responses. Am J Trop Med Hyg. 1986; 35(1): 72-8.

Barr SC, Gossett KA, Klei TR. Clinical, clinicopathologic, and parasitologic observations of trypanosomiasis in dogs infected with North American *Trypanosoma cruzi* isolates. Am J Vet Res. 1991; 52(6): 954-60.

Cardoso MJL, Machado LHA, Melussi M, Zamarian TP, Carnielli CM, Ferreira Jr JCM. Skin diseases in dogs: a review of 257 cases. Arch Vet Sci. 2011; 16(2): 66-74.

Ciaramella P, Oliva G, Luna RD, Gradoni L, Ambrosio R, Cortese L,

et al. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. Vet Rec. 1997; 141(21): 539-43.

Costa CH. How effective is dog culling in controlling zoonotic visceral leishmaniasis? A critical evaluation of the science, politics and ethics behind this public health policy. Rev Soc Bras Med Trop. 2011; 44(2): 232-42.

da Costa-Val AP, Cavalcanti RR, Gontijo NF, Michalik MS, Alexander B, Williams P, et al. Canine visceral leishmaniasis: relationships between clinical status, humoral immune response, haematology and *Lutzomyia (Lutzomyia) longipalpis* infectivity. Vet J. 2007; 174(3): 636-43.

Dantas-Torres F. The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania (Leishmania) infantum* and *Leishmania (Viannia) braziliensis*. Vet Parasitol. 2007; 149(3-4): 139-46.

de Araújo VEM, Pinheiro LC, Almeida MCM, Menezes FC, Morais MHF, Reis IA, et al. Relative risk of visceral leishmaniasis in Brazil: a spatial analysis in urban area. PLoS Negl Trop Dis. 2013; 7(11): e2540.

de Arruda MM, Figueiredo FB, Marcelino AP, Barbosa JR, Werneck GL, Noronha EF, et al. Sensitivity and specificity of parallel or serial serological testing for detection of canine *Leishmania* infection. Mem Inst Oswaldo Cruz. 2016; 111(3): 168-73.

de Castro MB, Machado RZ, de Aquino LP, Alessi AC, Costa MT. Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings. Vet Parasitol. 2004; 119(1): 73-86.

de Freitas JC, Lopes-Neto BE, de Abreu CR, Coura-Vital W, Braga SL, Reis AB, et al. Profile of anti-*Leishmania* antibodies related to clinical picture in canine visceral leishmaniasis. Res Vet Sci. 2012; 93(2): 705-9.

Ferrer L. Clinical aspects of canine leishmaniasis. In: R Killick-Kendrick, editor. Canine leishmaniasis: an updat. Proceedings of a Canine Leishmaniasis Forum. Barcelona: Sitges; 1999. pp. 6-10.

Gouvêa MV, Mendonça IL, Cruz MSP, Costa CHN, Braga JU, Werneck GL. Predictive factors for *Leishmania infantum* infection in dogs examined at a veterinary teaching hospital in Teresina, state of Piauí, Brazil. Rev Soc Bras Med Trop. 2016; 49(1): 107-11.

Hensel P, Santoro D, Favrot C, Hill P, Griffin C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. BMC Vet Res. 2015; 11: 196.

Jefferies R, Ryan UM, Irwin PJ. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Vet Parasitol. 2007; 144(1-2): 20-7.

Mancianti F, Gramiccia M, Gradoni L, Pieri S. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. Trans R Soc Trop Med Hyg. 1988; 82(4).

Manna L, Reale S, Vitale F, Gravino AE. Evidence for a relationship between *Leishmania* load and clinical manifestations. Res Vet Sci. 2009; 87(1).

Manzillo VF, Muccio TD, Cappiello S, Scalone A, Paparcone R, Fiorentino E, et al. Prospective study on the incidence and progression of clinical signs in naïve dogs naturally infected by *Leishmania infantum*. PLoS Negl Trop Dis. 2013; 7(5): e2225.

Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol. 2002; 18(9).

Mylonakis ME, Borjesson DL, Leontides L, Siarkou VI, Theodorou K, Koutinas AF. Cytologic patterns of lymphadenopathy in canine monocytic ehrlichiosis. Vet Clin Pathol. 2011; 40(1): 78-83.

Proverbio D, Spada E, de Giorgi GB, Perego R, Valena E. Relationship between *Leishmania* IFAT titer and clinicopathological manifestations (clinical score) in dogs. BioMed Res Int. 2014; 2014: 412808.

Ribeiro RR, da Silva SM, Fulgêncio GO, Michalick MS, Frezard FJ. Relationship between clinical and pathological signs and severity of canine leishmaniasis. Rev Bras Parasitol Vet. 2013; 22(3): 373-8.

Rodríguez-Morales O, Ballinas-Verdugo MA, Alejandre-Aguilar R, Reyes PA, Arce-Fonseca M. *Trypanosoma cruzi* connatal transmission in dogs with Chagas disease: experimental case report. Vector Borne Zoonotic Dis. 2011; 11(10): 1365-70.

Salem NY, Farag HS. Clinical, hematologic, and molecular findings in naturally occurring *Babesia canis vogeli* in Egyptian dogs. Vet Med Int. 2014; 2014: 270345.

Sarkari B, Michael C, Hommel M. A capture ELISA for the diagnosis of visceral leishmaniasis using a monoclonal antibody against a leishmanial urinary antigen. Iran Biomed J. 2005; 9: 117-22.

Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, et al. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors. 2011; 4: 86.

Souza AP, Soto M, Costa JML, Boaventura VS, Oliveira CI, Cristal JR, et al. Towards a more precise serological diagnosis of human tegumentary leishmaniasis using *Leishmania* recombinant proteins. PLoS ONE. 2013; 8(6): e66110.

Tafuri WL, de Oliveira MR, Melo MN. Canine visceral leishmaniosis: a remarkable histopathological picture of one case reported from Brazil. Vet Parasitol. 2001; 96(3): 203-12.

Wen B, Rikihisa Y, Mott JM, Greene R, Kim HY, Zhi N, et al. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J Clin Microbiol. 1997; 35(7): 1852-5.

Zanette MF, Lima VF, Laurenti MD, Rossi CN, Vides JP, Vieira RFC, et al. Serological cross-reactivity of *Trypanosoma cruzi*, *Ehrlichia canis*, *Toxoplasma gondii*, *Neospora caninum* and *Babesia canis* to *Leishmania infantum chagasi* tests in dogs. Rev Soc Bras Med Trop. 2014; 47: 105-7.

CONSIDERAÇÕES FINAIS

Esta pesquisa traz informações altamente relevantes sobre a utilização de sinais clínicos capazes de auxiliar médicos veterinários no diagnóstico da LVC principalmente em áreas endêmicas com recursos diagnósticos limitados, bem como a utilização de proteínas recombinantes e adjuvantes combinada com o alopurinol na imunoquimioterapia de cães naturalmente infectados com LVC.

O escore clínico aqui proposto teve um bom desempenho na discriminação de cães positivos para LVC de cães com outras patologias. Além disso alguns sinais clínicos foram mais correlacionados com a positividade da LVC em particular os sinais oculares e cutâneos.

A quimioterapia aqui avaliada teve resultados promissores indicando que o uso de uma abordagem imunoquimioterapica combinada, envolvendo o tratamento com alopurinol em conjunto com a formulação da vacina Leish-F2 + SLA-SE, para o tratamento de LVC aliviou os sintomas e reduziu as cargas de *L. infantum* tanto a curto como a longo prazo.

Todos os resultados aqui apresentados representam um grande avanço no diagnóstico, tratamento e manejo da LVC, contribuindo dessa forma no manejo da infecção por *L. infantum* e potencial transmissão para causar doença humana.

REFERÊNCIAS DA INTRODUÇÃO

ABBEHUSEN, M. M. C. et al. Clinical and immunopathological findings during long term follow-up in Leishmania infantum experimentally infected dogs. **Scientific Reports**, v. 7, n. 1, p. 15914, 21 dez. 2017.

ABDIAN, N. et al. Evaluation of DNA/DNA and prime-boost vaccination using LPG3 against Leishmania major infection in susceptible BALB/c mice and its antigenic properties in human leishmaniasis. **Experimental Parasitology**, v. 127, n. 3, p. 627–636, 1 mar. 2011.

AKHOUNDI, M. et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. **PLoS Neglected Tropical Diseases**, v. 10, n. 3, p. 1–40, 3 mar. 2016.

AKHOUNDI, M. et al. Leishmania infections: Molecular targets and diagnosis. **Molecular Aspects of Medicine**, v. 57, p. 1–29, 1 out. 2017.

ALVAR, J. et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. **PLoS ONE**, v. 7, n. 5, p. e35671, 31 maio 2012.

ALVAR, J. et al. Case study for a vaccine against leishmaniasis. **Vaccine**, v. 31, n. SUPPL2, p. B244–B249, 2013.

ALVING, C. R. et al. Adjuvants for human vaccines. **Current Opinion in Immunology**, v. 24, n. 3, p. 310–315, 1 jun. 2012.

ANDERSON, R. C. et al. Physicochemical characterization and biological activity of synthetic TLR4 agonist formulations. **Colloids and Surfaces B: Biointerfaces**, v. 75, n. 1, p. 123–132, 2010.

ANDRADE, H. M. et al. Evaluation of miltefosine for the treatment of dogs naturally infected with L. infantum (=L. chagasi) in Brazil. **Veterinary Parasitology**, v. 181, n. 2–4, p. 83–90, 27 set. 2011.

ANTINORI, S.; SCHIFANELLA, L.; CORBELLINO, M. Leishmaniasis: New insights from an old and neglected disease. **European Journal of Clinical Microbiology and Infectious Diseases**, v. 31, n. 2, p. 109–118, 1 fev. 2012.

ARAÚJO, M. S. S. et al. Immunological changes in canine peripheral blood leukocytes triggered by immunization with first or second generation vaccines against canine visceral leishmaniasis. **Veterinary Immunology and Immunopathology**, v. 141, n. 1–2, p. 64–75, 15 maio 2011.

BALDRIDGE, J. R.; CRANE, R. T. Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines. **Methods (San Diego, Calif.)**, v. 19, n. 1, p. 103–7, set. 1999.

BALDWIN, S. L. et al. Synthetic TLR4 agonists enhance functional antibodies and CD4+ T-cell responses against the Plasmodium falciparum GMZ2.6C multi-stage vaccine antigen. **Vaccine**, v. 34, n. 19, p. 2207–2215, 2016.

BELO, V. S. et al. Factors Associated with Visceral Leishmaniasis in the Americas: A Systematic Review and Meta-Analysis. **PLoS Neglected Tropical Diseases**, v. 7, n. 4, p. e2182, 25 abr. 2013a.

BELO, V. S. et al. A systematic review and meta-analysis of the factors associated with Leishmania infantum infection in dogs in Brazil. **Veterinary Parasitology**, v. 195, n. 1–2, p. 1–13, jul. 2013b.

BETHONY, J. M. et al. Vaccines to combat the neglected tropical diseases. **Immunological Reviews**, v. 239, n. 1, p. 237–270, 2011.

BORGES, M. M. et al. Potent Stimulation of the Innate Immune System by a Leishmania brasiliensis Recombinant Protein. **Infection and Immunity**, v. 69, n. 9, p. 5270–5277, 1 set. 2001.

BORJA-CABRERA, G. P. et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante, RN). **Vaccine**, v. 20, n. 27–28, p. 3277–84, 10 set. 2002.
BORJA-CABRERA, G. P. et al. Immunotherapy with the saponin enriched-Leishmune®vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis. **Vaccine**, v. 28, n. 3, p. 597–603, 2010.
BRASIL. **Manual de Vigilância e Controle da Leishmaniose Visceral**. [s.l: s.n.].
BRASIL. **Manual de vigilância, prevenção e controle de zoonoses**. [s.l: s.n.].
BRAZ, P. H. et al. Perfil hematológico de cães naturalmente infectados por leishmania spp. **Acta Veterinaria Brasilica**, v. 9, n. 1, p. 87–90, 2015.
BRITO, V. N. DE et al. Phlebotomine fauna, natural infection rate and feeding habits of Lutzomyia cruzi in Jaciara, state of Mato Grosso, Brazil. **Memórias do Instituto Oswaldo Cruz**, v. 109, n. 7, p. 899–904, nov. 2014.
BRITO, F. L. DA C. et al. Uveitis associated to the infection by Leishmania chagasi in dog from the Olinda city, Pernambuco, Brazil. **Ciência Rural**, v. 34, n. 3, p. 925–929, jun. 2004.
BUSH, B. M. **Interpretação de resultados laboratoriais para clínicos de pequenos animais**. São Paulo: Roca, 2004.
CAMPOS-NETO, A. et al. Protection against Cutaneous Leishmaniasis Induced by Recombinant Antigens in Murine and Nonhuman Primate Models of the Human Disease. **Infection and Immunity**, v. 69, n. 6, p. 4103–4108, 1 jun. 2001.
CANTOS-BARREDA, A. et al. Changes in serum anti- Leishmania antibody concentrations measured by time-resolved immunofluorometric assays in dogs with leishmaniosis after treatment. **Veterinary Immunology and Immunopathology**, v. 198, n. January, p. 65–69, abr. 2018.
CARTER, D. et al. A structure-function approach to optimizing TLR4 ligands for human vaccines. **Clinical & Translational Immunology**, v. 5, n. 11, p. e108, 2 nov. 2016.
CARVALHO, A. G. DE et al. High seroprevalence and peripheral spatial distribution of visceral leishmaniasis among domestic dogs in an emerging urban focus in Central Brazil: a cross-sectional study. **Pathogens and Global Health**, p. 1–8, 20 fev. 2018.
CHAUHAN, P. et al. Redundant and regulatory roles for Toll-like receptors in Leishmania infection. **Clinical & Experimental Immunology**, v. 190, n. 2, p. 167–186, nov. 2017.
CIARAMELLA, P. et al. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by Leishmania infantum. **Vet Rec**, v. 141, n. 21, p. 539–543, 22 nov. 1997.
COELHO, E. A. F. et al. Immune Responses Induced by the Leishmania (Leishmania) donovani A2 Antigen, but Not by the LACK Antigen, Are Protective against Experimental Leishmania (Leishmania) amazonensis Infection. **Infection and Immunity**, v. 71, n. 7, p. 3988–3994, 1 jul. 2003.
COLER, R. N. et al. Leish-111f, a Recombinant Polyprotein Vaccine That Protects against Visceral Leishmaniasis by Elicitation of CD4+ T Cells. **Infection and Immunity**, v. 75, n. 9, p. 4648–4654, 1 set. 2007.
COLER, R. N. et al. Development and Characterization of Synthetic Glucopyranosyl Lipid Adjuvant System as a Vaccine Adjuvant. **PLoS ONE**, v. 6, n. 1, p. e16333, 26 jan. 2011.
COLER, R. N.; REED, S. G. Second-generation vaccines against leishmaniasis. **Trends in parasitology**, v. 21, n. 5, p. 244–9, maio 2005.
CONNELL, N. D. et al. Effective immunization against cutaneous leishmaniasis with

recombinant bacille Calmette-Guérin expressing the Leishmania surface proteinase gp63. **Proceedings of the National Academy of Sciences of the United States of America**, v. 90, n. 24, p. 11473–7, 15 dez. 1993.
COSTA, C. H. N.; PEREIRA, H. F.; ARAÚJO, M. V. Epidemia de leishmaniose visceral no Estado do Piauí, Brasil, 1980-1986. **Revista de Saúde Pública**, v. 24, n. 5, p. 361–372, out. 1990.
COSTA, D. N. C. C. et al. Culling Dogs in Scenarios of Imperfect Control: Realistic Impact on the Prevalence of Canine Visceral Leishmaniasis. **PLoS Neglected Tropical Diseases**, v. 7, n. 8, p. e2355, 8 ago. 2013.
COURA-VITAL, W. et al. Evaluation of change in canine diagnosis protocol adopted by the visceral leishmaniasis control program in Brazil and a new proposal for diagnosis. **PLoS ONE**, v. 9, n. 3, p. 1–6, 2014.
CROFT, S. L.; COOMBS, G. H. Leishmaniasis– current chemotherapy and recent advances in the search for novel drugs. **Trends in Parasitology**, v. 19, n. 11, p. 502–508, 1 nov. 2003.
CUSCÓ, A. et al. Non-synonymous genetic variation in exonic regions of canine Toll-like receptors. **Canine Genetics and Epidemiology**, v. 1, n. 1, p. 11, 2014.
DAVID, J. R. et al. Deltamethrin-impregnated dog collars have a potent anti-feeding and insecticidal effect on Lutzomyia longipalpis and Lutzomyia migonei. **Memórias do Instituto Oswaldo Cruz**, v. 96, n. 6, p. 839–847, ago. 2001.
DAY, M. J. Immunoglobulin G subclass distribution in canine leishmaniosis: A review and analysis of pitfalls in interpretation. **Veterinary Parasitology**, v. 147, n. 1–2, p. 2–8, 20 jun. 2007.
DE AMORIM, I. F. G. et al. Humoral immunological profile and parasitological statuses of Leishmune® vaccinated and visceral leishmaniasis infected dogs from an endemic area. **Veterinary Parasitology**, v. 173, n. 1–2, p. 55–63, 11 out. 2010.
DE LUCA, P. M. et al. A randomized double-blind placebo-controlled trial to evaluate the immunogenicity of a candidate vaccine against American tegumentary leishmaniasis. **Acta tropica**, v. 80, n. 3, p. 251–60, 21 dez. 2001.
DE MENDONÇA, L. Z. et al. Multicomponent LBSap vaccine displays immunological and parasitological profiles similar to those of Leish-Tec® and Leishmune® vaccines against visceral leishmaniasis. **Parasites and Vectors**, v. 9, n. 1, p. 1–12, 2016.
DE QUEIROZ, N. M. G. P. et al. Detection of Leishmania (L.) chagasi in canine skin. **Veterinary Parasitology**, v. 178, n. 1–2, p. 1–8, 2011.
DE VASCONCELOS, T. C. B. et al. Canine susceptibility to visceral leishmaniasis: A systematic review upon genetic aspects, considering breed factors and immunological concepts. **Infection, Genetics and Evolution**, 5 out. 2017.
DIEBOLD, S. S. et al. Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. **Science**, v. 303, n. 5663, p. 1529–1531, 2004.
DORLO, T. P. C. et al. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. **Journal of Antimicrobial Chemotherapy**, v. 67, n. July 2012, p. 2576–2597, 2012.
DRUMOND, O.; COSTA, F. A. L. Quarenta anos de leishmaniose visceral no estado do piauí: Revisão. **Revista do Instituto de Medicina Tropical de Sao Paulo**, v. 53, n. 1, p. 3–11, 2011.
EVANS, J. T. et al. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. **Expert review of vaccines**, v. 2, n. 2, p. 219–29, abr. 2003.
FALCÃO DE OLIVEIRA, E. et al. Experimental infection and transmission of Leishmania by Lutzomyia cruzi (Diptera: Psychodidae): Aspects of the ecology of

parasite-vector interactions. **PLOS Neglected Tropical Diseases**, v. 11, n. 2, p. e0005401, 24 fev. 2017.
FERNANDES, A. P. et al. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. **Vaccine**, v. 26, n. 46, p. 5888–5895, 29 out. 2008.
FERNANDES, C. B. et al. Comparison of two commercial vaccines against visceral leishmaniasis in dogs from endemic areas: IgG, and subclasses, parasitism, and parasite transmission by xenodiagnosis. **Vaccine**, v. 32, n. 11, p. 1287–1295, 2014.
FOGLIA MANZILLO, V. et al. Deltamethrin-impregnated collars for the control of canine leishmaniasis: Evaluation of the protective effect and influence on the clinical outcome of Leishmania infection in kennelled stray dogs. **Veterinary Parasitology**, v. 142, n. 1–2, p. 142–145, 2006.
FOX, C. B. et al. Current Status of Toll-Like Receptor 4 Ligand Vaccine Adjuvants. In: **Immunopotentiators in Modern Vaccines**. [s.l.] Elsevier, 2017. p. 105–127.
FREITAS, J. C. C. DE et al. Clinical and laboratory alterations in dogs naturally infected by Leishmania chagasi. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 45, n. 1, p. 24–29, fev. 2012.
FUKATA, M.; VAMADEVAN, A. S.; ABREU, M. T. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders. **Seminars in Immunology**, v. 21, n. 4, p. 242–253, ago. 2009.
GARCÍA-ALONSO, M. et al. Immunopathology of the uveitis in canine leishmaniasis. **Parasite immunology**, v. 18, n. 12, p. 617–23, dez. 1996.
GHARBI, M. et al. Leishmaniosis (Leishmania infantum infection) in dogs. **Revue Scientifique et Technique de l'OIE**, v. 34, n. 2, p. 613–626, 2015.
GIUNCHETTI, R. C. et al. Histopathological and immunohistochemical investigations of the hepatic compartment associated with parasitism and serum biochemical changes in canine visceral leishmaniasis. **Research in Veterinary Science**, v. 84, n. 2, p. 269–277, 2008.
GOMES, R. et al. KSAC, a Defined Leishmania Antigen, plus Adjuvant Protects against the Virulence of L. major Transmitted by Its Natural Vector Phlebotomus duboscqi. **PLoS Neglected Tropical Diseases**, v. 6, n. 4, p. e1610, 3 abr. 2012.
GOMES, Y. M. et al. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. **Veterinary Journal**, v. 175, n. 1, p. 45–52, 2008.
GÓMEZ-OCHOA, P. et al. Use of domperidone in the treatment of canine visceral leishmaniasis: A clinical trial. **The Veterinary Journal**, v. 179, n. 2, p. 259–263, 1 fev. 2009.
GONTIJO, C. M. F.; MELO, M. N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. **Revista Brasileira de Epidemiologia**, v. 7, n. 3, p. 338–349, set. 2004.
GRADONI, L. et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from Leishmania infantum infection and to prevent disease progression in infected animals. **Vaccine**, v. 23, n. 45, p. 5245–5251, 1 nov. 2005.
GRADONI, L. Canine Leishmania vaccines: Still a long way to go. **Veterinary Parasitology**, v. 208, n. 1–2, p. 94–100, 28 fev. 2015.
GRAMICCIA, M. Recent advances in leishmaniosis in pet animals: Epidemiology, diagnostics and anti-vectorial prophylaxis. **Veterinary Parasitology**, v. 181, n. 1, p. 23–30, 2011.
GRIMALDI, G. et al. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. **Transactions of the Royal Society of Tropical Medicine and**

Hygiene, v. 106, n. 1, p. 54–59, 1 jan. 2012.
GRIMALDI, G. et al. Field trial of efficacy of the Leish-tec® vaccine against canine leishmaniasis caused by Leishmania infantum in an endemic area with high transmission rates. **PLOS ONE**, v. 12, n. 9, p. e0185438, 27 set. 2017.
GUARGA, J. L. et al. Evaluation of a specific immunochemotherapy for the treatment of canine visceral leishmaniasis. **Veterinary Immunology and Immunopathology**, v. 88, n. 1–2, p. 13–20, 6 set. 2002.
HANDMAN, E. et al. Passive transfer of Leishmania lipopolysaccharide confers parasite survival in macrophages. **Journal of immunology (Baltimore, Md. : 1950)**, v. 137, n. 11, p. 3608–13, 1 dez. 1986.
HARHAY, M. O. et al. Urban parasitology: visceral leishmaniasis in Brazil. **Trends in Parasitology**, v. 27, n. 9, p. 403–409, set. 2011.
HEIL, F. et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. **Science**, v. 303, n. 5663, p. 1526–1529, 2004.
HEMMI, H. et al. Small-antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. **Nature Immunology**, v. 3, n. 2, p. 196–200, 2002.
HERWALDT, B. L. Leishmaniasis. **Lancet**, v. 354, n. 0140-6736 (Print), p. 1191–1199, 1999.
HOSEIN, S.; BLAKE, D. P.; SOLANO-GALLEGO, L. Insights on adaptive and innate immunity in canine leishmaniosis. **Parasitology**, v. 144, n. 01, p. 95–115, 20 jan. 2017.
HUSSEIN, W. M. et al. Toll-like receptor agonists: a patent review (2011 - 2013). **Expert opinion on therapeutic patents**, v. 24, n. 4, p. 453–70, 2014.
IKEDA-GARCIA, F. A. et al. Evaluation of renal and hepatic functions in dogs naturally infected by visceral leishmaniasis submitted to treatment with meglumine antimoniate. **Research in Veterinary Science**, v. 83, n. 1, p. 105–108, 2007.
INIESTA, L. et al. Immunoglobulin G and E responses in various stages of canine leishmaniosis. **Veterinary Immunology and Immunopathology**, v. 103, n. 1–2, p. 77–81, 10 jan. 2005.
IRETON, G. C.; REED, S. G. Adjuvants containing natural and synthetic Toll-like receptor 4 ligands. **Expert Review of Vaccines**, v. 12, n. 7, p. 793–807, 9 jul. 2013.
JAIN, K.; JAIN, N. K. K. Vaccines for visceral leishmaniasis: A review. **Journal of Immunological Methods**, v. 422, p. 1–12, jul. 2015.
JARDIM, A. et al. The Leishmania donovani lipophosphoglycan T lymphocyte-reactive component is a tightly associated protein complex. **Journal of immunology (Baltimore, Md. : 1950)**, v. 147, n. 10, p. 3538–44, 15 nov. 1991.
JOHNSTON, D.; BYSTRYN, J. C. Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine. **Vaccine**, v. 24, n. 11, p. 1958–1965, 2006.
JURK, M. et al. Modulating responsiveness of human TLR7 and 8 to small molecule ligands with T-rich phosphorothiate oligodeoxynucleotides. **European Journal of Immunology**, v. 36, n. 7, p. 1815–1826, 2006.
KASZAK, I.; PLANELLAS, M.; DWORECKA-KASZAK, B. Canine leishmaniosis - an emerging disease. **Annals of parasitology**, v. 61, n. 2, p. 69–76, 2015.
KAWAI, T.; AKIRA, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. **Nature Immunology**, v. 11, n. 5, p. 373–384, 20 maio 2010.
KAYE, P. M.; AEBISCHER, T. Visceral leishmaniasis: immunology and prospects for a vaccine. **Clinical Microbiology and Infection**, v. 17, n. 10, p. 1462–1470, 1 out. 2011.
KAYE, P.; SCOTT, P. Leishmaniasis: complexity at the host–pathogen interface.

Nature Reviews Microbiology, v. 9, n. 8, p. 604–615, 11 jul. 2011.
KILLICK-KENDRICK, R. et al. Protection of dogs from bites of phlebotomine sandflies by deltamethrin collars for control of canine leishmaniasis. **Medical and veterinary entomology**, v. 11, n. 2, p. 105–11, abr. 1997.
KILLICK-KENDRICK, R. The biology and control of Phlebotomine sand flies. **Clinics in Dermatology**, v. 17, n. 3, p. 279–289, 1 maio 1999.
KOUTINAS, A. F. et al. A randomised, blinded, placebo-controlled clinical trial with allopurinol in canine leishmaniosis. **Veterinary Parasitology**, v. 98, n. 4, p. 247–261, 27 jul. 2001.
KOUTINAS, A. F.; KOUTINAS, C. K. Pathologic Mechanisms Underlying the Clinical Findings in Canine Leishmaniosis due to Leishmaniainfantum/chagasi. **Veterinary Pathology**, v. 51, n. 2, p. 527–538, 2014.
KUMAR, R.; ENGWERDA, C. Vaccines to prevent leishmaniasis. **Clinical & Translational Immunology**, v. 3, n. 3, p. e13, mar. 2014.
LAINSON, R.; RYAN, L.; SHAW, J. J. Infective stages of Leishmania in the sandfly vector and some observations on the mechanism of transmission. **Memórias do Instituto Oswaldo Cruz**, v. 82, n. 3, p. 421–424, set. 1987.
LARANJEIRA, D. F. et al. Serological and infection statuses of dogs from a visceral leishmaniasis-endemic area. **Revista de Saude Publica**, v. 48, n. 4, p. 563–570, ago. 2014.
LAURENTI, M. D. et al. Asymptomatic dogs are highly competent to transmit Leishmania (Leishmania) infantum chagasi to the natural vector. **Veterinary Parasitology**, v. 196, n. 3–4, p. 296–300, set. 2013.
LAURENTI, M. D. et al. Comparative evaluation of the DPP®CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. **Veterinary Parasitology**, v. 205, n. 3–4, p. 444–450, 15 out. 2014.
LEE, J. et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. **Proceedings of the National Academy of Sciences of the United States of America**, v. 100, n. 11, p. 6646–51, 27 maio 2003.
LIEW, F. Y.; O'DONNELL, C. A. Immunology of Leishmaniasis. **Advances in Parasitology**, v. 32, p. 161–259, 1 jan. 1993.
LIMA, L. V. DO R. et al. Further evidence associating IgG1, but not IgG2, with susceptibility to canine visceral leishmaniasis caused by Leishmania (L.) infantum chagasi -infection. **Parasite**, v. 24, p. 37, 13 out. 2017.
LLANOS-CUENTAS, A. et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with sodium stibogluconate for the treatment of mucosal leishmaniasis. **Vaccine**, v. 28, n. 46, p. 7427–7435, 28 out. 2010.
LOUSADA-DIETRICH, S. et al. A synthetic TLR4 agonist formulated in an emulsion enhances humoral and Type 1 cellular immune responses against GMZ2 - A GLURP-MSP3 fusion protein malaria vaccine candidate. **Vaccine**, v. 29, n. 17, p. 3284–3292, 2011.
MACHADO-COELHO, G. L. L. et al. American cutaneous leishmaniasis in Southeast Brazil: Space-time clustering. **International Journal of Epidemiology**, v. 28, n. 5, p. 982–989, 1999.
MACHADO, C. J. S.; SILVA, E. G.; VILANI, R. M. Use of an instrument of controversial public health policy: Euthanasia of dogs contaminated by leishmaniasis in Brazil. **Saude e Sociedade**, v. 25, n. 1, p. 247–258, 2016.
MAIA-ELKHOURY, A. N. S. et al. Visceral leishmaniasis in Brazil: trends and challenges. **Cad. Saúde Pública**, v. 24, n. 12, p. 2941–2947, dez. 2008.

MAIA, C.; CAMPINO, L. Methods for diagnosis of canine leishmaniasis and immune response to infection. **Veterinary Parasitology**, v. 158, n. 4, p. 274–287, 20 dez. 2008.
MANNA, L. et al. Study of efficacy of miltefosine and allopurinol in dogs with leishmaniosis. **The Veterinary Journal**, v. 182, n. 3, p. 441–445, dez. 2009.
MANNA, L. et al. Long-term follow-up of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol. **Parasites & Vectors**, v. 8, n. 1, p. 289, 28 dez. 2015.
MAROLI, M. et al. Evidence for an impact on the incidence of canine leishmaniasis by the mass use of deltamethrin-impregnated dog collars in southern Italy. **Medical and Veterinary Entomology**, v. 15, n. 4, p. 358–363, 1 dez. 2001.
MAROLI, M. et al. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. **Medical and Veterinary Entomology**, v. 27, n. 2, p. 123–147, jun. 2013.
MCGWIRE, B. S.; SATOSKAR, A. R. Leishmaniasis: clinical syndromes and treatment. **QJM**, v. 107, n. 1, p. 7–14, 1 jan. 2014.
MCMAHON-PRATT, D. et al. Recombinant vaccinia viruses expressing GP46/M-2 protect against Leishmania infection. **Infection and immunity**, v. 61, n. 8, p. 3351–9, ago. 1993.
MELBY, P. C. et al. Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library. **Infection and immunity**, v. 68, n. 10, p. 5595–602, out. 2000.
MELÉNDEZ-LAZO, A. et al. Clinicopathological findings in sick dogs naturally infected with Leishmania infantum : Comparison of five different clinical classification systems. **Research in Veterinary Science**, v. 117, n. October 2017, p. 18–27, abr. 2018.
MIRET, J. et al. Evaluation of an immunochemotherapeutic protocol constituted of N-methyl meglumine antimoniate (Glucantime®) and the recombinant Leish-110f®+MPL-SE® vaccine to treat canine visceral leishmaniasis. **Vaccine**, v. 26, n. 12, p. 1585–1594, 17 mar. 2008.
MIRÓ, G. et al. Infectivity to Phlebotomus perniciosus of dogs naturally parasitized with Leishmania infantum after different treatments. **Parasites & vectors**, v. 4, p. 52, 13 abr. 2011.
MIRÓ, G. et al. Novel Areas for Prevention and Control of Canine Leishmaniosis. **Trends in Parasitology**, v. 33, n. 9, p. 718–730, set. 2017.
MIRÓ, G.; LÓPEZ-VÉLEZ, R. Clinical management of canine leishmaniosis versus human leishmaniasis due to Leishmania infantum : Putting “One Health” principles into practice. **Veterinary Parasitology**, v. 254, n. January, p. 151–159, abr. 2018.
MIYAKE, K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. **Seminars in Immunology**, v. 19, n. 1, p. 3–10, fev. 2007.
MIZEL, S. B.; BATES, J. T. Flagellin as an Adjuvant: Cellular Mechanisms and Potential. **The Journal of Immunology**, v. 185, n. 10, p. 5677–5682, 15 nov. 2010.
MOGENSEN, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. **Clinical Microbiology Reviews**, v. 22, n. 2, p. 240–273, 2009.
MONDELAERS, A. et al. Genomic and Molecular Characterization of Miltefosine Resistance in Leishmania infantum Strains with Either Natural or Acquired Resistance through Experimental Selection of Intracellular Amastigotes. **PloS one**, v. 11, n. 4, p. e0154101, 28 abr. 2016.
MORENO, J. et al. Immunization with H1, HASPB1 and MML Leishmania proteins in a vaccine trial against experimental canine leishmaniasis. **Vaccine**, v. 25, n. 29, p. 5290–5300, 20 jul. 2007.

MORENO, J.; ALVAR, J. Canine leishmaniasis: epidemiological risk and the experimental model. **Trends in Parasitology**, v. 18, n. 9, p. 399–405, set. 2002.
MUKHERJEE, A. K. et al. Miltefosine triggers a strong proinflammatory cytokine response during visceral leishmaniasis: Role of TLR4 and TLR9. **International Immunopharmacology**, v. 12, n. 4, p. 565–572, 2012.
MURRAY, H. W. et al. Advances in leishmaniasis. **The Lancet**, v. 366, n. 9496, p. 1561–1577, 29 out. 2005.
NAGILL, R.; KAUR, S. Vaccine candidates for leishmaniasis: A review. **International Immunopharmacology**, v. 11, n. 10, p. 1464–1488, out. 2011.
NASCIMENTO, E. et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with meglumine antimoniate for the treatment of cutaneous leishmaniasis. **Vaccine**, v. 28, n. 40, p. 6581–6587, 14 set. 2010.
NETEA, M. G. et al. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. **Journal of Leukocyte Biology**, v. 75, n. 5, p. 749–755, 2004.
NO, J. H. Visceral leishmaniasis: Revisiting current treatments and approaches for future discoveries. **Acta Tropica**, v. 155, p. 113–123, mar. 2016.
NOLI, C.; AUXILIA, S. T. Treatment of canine Old World visceral leishmaniasis: a systematic review. **Veterinary Dermatology**, v. 16, n. 4, p. 213–232, ago. 2005.
NUNES, C. M. et al. Relationship between dog culling and incidence of human visceral leishmaniasis in an endemic area. **Veterinary Parasitology**, v. 170, n. 1–2, p. 131–133, 28 maio 2010.
OLIVA, G. et al. Guidelines for treatment of leishmaniasis in dogs. **Journal of the American Veterinary Medical Association**, v. 236, n. 11, p. 1192–1198, 1 jun. 2010.
OLIVEIRA, C. D. L.; MORAIS, M. H. F.; MACHADO-COELHO, G. L. L. Visceral leishmaniasis in large Brazilian cities: challenges for control. **Cadernos de Saúde Pública**, v. 24, n. 12, p. 2953–2958, dez. 2008.
OSPELT, C.; GAY, S. TLRs and chronic inflammation. **The International Journal of Biochemistry & Cell Biology**, v. 42, n. 4, p. 495–505, abr. 2010.
PAES, W. et al. Recombinant polymorphic membrane protein D in combination with a novel, second-generation lipid adjuvant protects against intra-vaginal Chlamydia trachomatis infection in mice. **Vaccine**, v. 34, n. 35, p. 4123–4131, 2016.
PAIVA-CAVALCANTI, M.; REGIS-DA-SILVA, C.; GOMES, Y. Comparison of real-time PCR and conventional PCR for detection of Leishmania (Leishmania) infantum infection: a mini-review. **Journal of Venomous Animals and Toxins including Tropical Diseases**, v. 16, n. 4, p. 537–542, 2010.
PALATNIK-DE-SOUSA, C. B. et al. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune® in Brazilian endemic areas. **Vaccine**, v. 27, n. 27, p. 3505–3512, 2 jun. 2009.
PALATNIK-DE-SOUSA, C. B.; DAY, M. J. One Health: The global challenge of epidemic and endemic leishmaniasis. **Parasites and Vectors**, v. 4, n. 1, p. 197, 2011.
PALATNIK, C. B. et al. Inhibition of Leishmania donovani promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate ligands. **Infection and immunity**, v. 57, n. 3, p. 754–63, mar. 1989.
PALTRINIERI, S. et al. Laboratory tests for diagnosing and monitoring canine leishmaniasis. **Veterinary Clinical Pathology**, v. 45, n. 4, p. 552–578, dez. 2016.
PARADIES, P. et al. Monitoring the reverse to normal of clinico-pathological findings and the disease free interval time using four different treatment protocols for canine leishmaniosis in an endemic area. **Research in Veterinary Science**, v. 93, n. 2, p. 843–

847, 2012.
PARK, B. S. et al. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. **Nature**, v. 458, n. 7242, p. 1191–1195, 2009.
PARRA, L. E. et al. Safety trial using the Leishmune® vaccine against canine visceral leishmaniasis in Brazil. **Vaccine**, v. 25, n. 12, p. 2180–2186, 8 mar. 2007.
PEIXOTO, H. M.; DE OLIVEIRA, M. R. F.; ROMERO, G. A. S. Serological diagnosis of canine visceral leishmaniasis in Brazil: systematic review and meta-analysis. **Tropical Medicine & International Health**, v. 20, n. 3, p. 334–352, mar. 2015.
PEREIRA-FONSECA, D. C. M. M. et al. Dog skin parasite load, TLR-2, IL-10 and TNF-α expression and infectiousness. **Parasite Immunology**, v. 39, n. 11, p. e12493, nov. 2017.
PESSOA, S.; MARTINS, A. V. Parasitologia médica. **Guanabara Koogan**, 1982.
PICCININI, A. M.; MIDWOOD, K. S. DAMPening Inflammation by Modulating TLR Signalling. **Mediators of Inflammation**, v. 2010, p. 1–21, 2010.
PINEDA, C. et al. Treatment of canine leishmaniasis with marbofloxacin in dogs with renal disease. **PLOS ONE**, v. 12, n. 10, p. e0185981, 5 out. 2017.
PROBST, P. et al. ALeishmania protein that modulates interleukin (IL)-12, IL-10 and tumor necrosis factor-α production and expression of B7-1 in human monocyte-derived antigen-presenting cells. **European Journal of Immunology**, v. 27, n. 10, p. 2634–2642, out. 1997.
PROVERBIO, D. et al. Failure of miltefosine treatment in two dogs with natural Leishmania infantum infection. **Case Reports in Veterinary Medicine**, v. 2014, p. 640151-Article ID 640151, 14 ago. 2014.
QURESHI, N. et al. Structure of the monophosphoryl lipid A moiety obtained from the lipopolysaccharide of Chlamydia trachomatis. **The Journal of biological chemistry**, v. 272, n. 16, p. 10594–600, 18 abr. 1997.
RACHAMIM, N.; JAFFE, C. L. Pure protein from Leishmania donovani protects mice against both cutaneous and visceral leishmaniasis. **Journal of immunology (Baltimore, Md. : 1950)**, v. 150, n. 6, p. 2322–31, 15 mar. 1993.
RAFATI, S. et al. Identification of Leishmania major cysteine proteinases as targets of the immune response in humans. **Molecular and biochemical parasitology**, v. 113, n. 1, p. 35–43, mar. 2001.
RAHMAN, M. T. et al. Polymerase Chain Reaction (PCR): A Short Review. **Anwer Khan Modern Medical College Journal**, v. 4, n. 1, 2013.
RAKOFF-NAHOUM, S.; MEDZHITOV, R. Toll-like receptors and cancer. **Nature Reviews Cancer**, v. 9, n. 1, p. 57–63, 2009.
READY, P. D. Biology of Phlebotomine Sand Flies as Vectors of Disease Agents. **Annual Review of Entomology**, v. 58, n. 1, p. 227–250, 7 jan. 2013.
REALE, S. et al. Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. **Journal of clinical microbiology**, v. 37, n. 9, p. 2931–5, set. 1999.
REED, S. G. et al. New horizons in adjuvants for vaccine development. **Trends in Immunology**, v. 30, n. 1, p. 23–32, jan. 2009.
REED, S. G.; BADARO, R.; LLOYD, R. M. Identification of specific and cross-reactive antigens of Leishmania donovani chagasi by human infection sera. **Journal of immunology (Baltimore, Md. : 1950)**, v. 138, n. 5, p. 1596–601, 1 mar. 1987.
REED, S. G.; COLER, R. N.; CAMPOS-NETO, A. Development of a leishmaniasis vaccine: the importance of MPL. **Expert Review of Vaccines**, v. 2, n. 2, p. 239–252, 9 abr. 2003.
REED, S. G.; ORR, M. T.; FOX, C. B. Key roles of adjuvants in modern vaccines. **Nature Medicine**, v. 19, n. 12, p. 1597–1608, 5 dez. 2013.

REGINA-SILVA, S. et al. Field randomized trial to evaluate the efficacy of the Leish-Tec ® vaccine against canine visceral leishmaniasis in an endemic area of Brazil. **Vaccine**, v. 34, n. 19, p. 2233–2239, 2016.
REGUERA, R. M. et al. Current status on prevention and treatment of canine leishmaniasis. **Veterinary Parasitology**, v. 227, p. 98–114, 30 ago. 2016.
REIS, A. B. et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. **Research in Veterinary Science**, v. 81, n. 1, p. 68–75, ago. 2006.
RIBEIRO, R. R. et al. Canine Leishmaniasis: An Overview of the Current Status and Strategies for Control. **BioMed Research International**, v. 2018, n. Cl, p. 1–12, 2018.
ROATT, B. M. et al. Immunotherapy and Immunochemotherapy in Visceral Leishmaniasis: Promising Treatments for this Neglected Disease. **Frontiers in Immunology**, v. 5, n. JUN, p. 1–12, 13 jun. 2014.
ROBERTS, W. L.; BERMAN, J. D.; RAINEY, P. M. In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. **Antimicrobial Agents and Chemotherapy**, v. 39, n. 6, p. 1234–1239, jun. 1995.
RODRIGUES, V. et al. Regulation of immunity during visceral Leishmania infection. **Parasites and Vectors**, v. 9, n. 1, p. 1–13, 1 mar. 2016.
ROSS, R. FURTHER NOTES ON LEISHMAN'S BODIES. **British medical journal**, v. 2, n. 2239, p. 1401, 28 nov. 1903.
SABATÉ, D. et al. A single-centre, open-label, controlled, randomized clinical trial to assess the preventive efficacy of a domperidone-based treatment programme against clinical canine leishmaniasis in a high prevalence area. **Preventive Veterinary Medicine**, v. 115, n. 1–2, p. 56–63, 1 jul. 2014.
SAHA, P.; MUKHOPADHYAY, D.; CHATTERJEE, M. Immunomodulation by chemotherapeutic agents against Leishmaniasis. **International Immunopharmacology**, v. 11, n. 11, p. 1668–1679, nov. 2011.
SANTIS, B. et al. Performance of DPP ™ immunochromathographic rapid test (IRT) for canine visceral leishmaniasis : comparison with other serological methods in suspected dogs from Cuiabá , Mato Grosso State , Brazil. **Braz. J. Vet. Res. Anim. Sci**, v. 50, n. 3, p. 198–205, 2013.
SARIDOMICHELAKIS, M. N. et al. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (Leishmania infantum) in symptomatic and asymptomatic dogs. **American Journal of Tropical Medicine and Hygiene**, v. 73, n. 1, p. 82–86, jul. 2005.
SARIDOMICHELAKIS, M. N. Advances in the pathogenesis of canine leishmaniosis: Epidemiologic and diagnostic implications. **Veterinary Dermatology**, v. 20, n. 5–6, p. 471–489, 2009.
SCHEIERMANN, J.; KLINMAN, D. M. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. **Vaccine**, v. 32, n. 48, p. 6377–6389, 12 nov. 2014.
SERENO, D. et al. Antimonial-mediated DNA fragmentation in Leishmania infantum amastigotes. **Antimicrobial Agents and Chemotherapy**, v. 45, n. 7, p. 2064–2069, jul. 2001.
SEVÁ, A. P. et al. Canine-Based Strategies for Prevention and Control of Visceral Leishmaniasis in Brazil. **PLOS ONE**, v. 11, n. 7, p. e0160058, 29 jul. 2016.
SHIROTA, H.; KLINMAN, D. M. Recent progress concerning CpG DNA and its use as a vaccine adjuvant. **Expert Review of Vaccines**, v. 13, n. 2, p. 299–312, 26 fev. 2014.
SILVA, K. R. DA et al. Scoring clinical signs can help diagnose canine visceral leishmaniasis in a highly endemic area in Brazil. **Memórias do Instituto Oswaldo**

Cruz, v. 112, n. 1, p. 53–63, jan. 2017.
SILVA, V. et al. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amaranto, RN). **Vaccine**, v. 19, n. 9–10, p. 1082–1092, 8 dez. 2000.
SINGH, B.; SUNDAR, S. Leishmaniasis: Vaccine candidates and perspectives. **Vaccine**, v. 30, n. 26, p. 3834–3842, 6 jun. 2012.
SKEIKY, Y. A. et al. A recombinant Leishmania antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. **The Journal of experimental medicine**, v. 181, n. 4, p. 1527–37, 1 abr. 1995a.
SKEIKY, Y. A. et al. Immune responses of leishmaniasis patients to heat shock proteins of Leishmania species and humans. **Infection and immunity**, v. 63, n. 10, p. 4105–14, out. 1995b.
SKEIKY, Y. A. et al. LeIF: a recombinant Leishmania protein that induces an IL-12-mediated Th1 cytokine profile. **Journal of immunology (Baltimore, Md. : 1950)**, v. 161, n. 11, p. 6171–9, 1 dez. 1998.
SKEIKY, Y. A. W. et al. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. **Vaccine**, v. 20, n. 27–28, p. 3292–303, 10 set. 2002.
SLAPPENDEL, R. J.; TESKE, E. The effect of intravenous or subcutaneous administration of meglumine antimonate (glucantime®) in dogs with leishmaniasis. A randomized clinical trial. **Veterinary Quarterly**, v. 19, n. 1, p. 10–13, mar. 1997.
SMYTH, A. J. et al. Rapid and sensitive detection of Leishmania kinetoplast DNA from spleen and blood samples of kala-azar patients. **Parasitology**, v. 105 (Pt 2), p. 183–92, out. 1992.
SOLANO-GALLEGO, L. et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. **Veterinary Parasitology**, v. 165, n. 1–2, p. 1–18, 2009.
SOLANO-GALLEGO, L. et al. LeishVet guidelines for the practical management of canine leishmaniosis. **Parasites & Vectors**, v. 4, n. 1, p. 86, 20 maio 2011.
SOLANO-GALLEGO, L. et al. Early reduction of Leishmania infantum-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. **Parasites & Vectors**, v. 9, n. 1, p. 235, 10 dez. 2016.
SUNDAR, S.; OLLIARO, P. L. Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. **Therapeutics and clinical risk management**, v. 3, n. 5, p. 733–40, 4 out. 2007.
SYKES, J. E.; BANETH, G.; PETERSEN, C. A. Leishmaniosis. In: **Canine and Feline Infectious Diseases**. [s.l.] Elsevier, 2014. p. 713–726.
TASSI, P. et al. Pharmacokinetics of N-methylglucamine antimoniate after intravenous, intramuscular and subcutaneous administration in the dog. **Research in Veterinary Science**, v. 56, n. 2, p. 144–150, 1 mar. 1994.
TESH, R. B. Control of zoonotic visceral leishmaniasis: is it time to change strategies? **The American journal of tropical medicine and hygiene**, v. 52, n. 3, p. 287–92, mar. 1995.
TOMAI, M. A. et al. Resiquimod and other immune response modifiers as vaccine adjuvants. **Expert Rev. Vaccines**, v. 6, n. 5, p. 835–847, 2007.
TORRECILHA, R. B. P. et al. Prediction of lymph node parasite load from clinical data in dogs with leishmaniasis: An application of radial basis artificial neural networks. **Veterinary Parasitology**, v. 234, p. 13–18, 2017.
TORRES-GUERRERO, E. et al. Leishmaniasis: a review. **F1000Research**, v. 6, n.

May, p. 750, 2017.
TORRES, M. et al. Adverse urinary effects of allopurinol in dogs with leishmaniasis. **Journal of Small Animal Practice**, v. 57, n. 6, p. 299–304, jun. 2016.
TRAVI, B. L. Ethical and epidemiological dilemmas in the treatment of dogs for visceral leishmaniasis in Latin America. **Biomédica : revista del Instituto Nacional de Salud**, v. 34, n. 1, p. 7–12, 2014.
TRIGO, J. et al. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f + MPL-SE. **Vaccine**, v. 28, n. 19, p. 3333–3340, 26 abr. 2010.
TUON, F. F. et al. Toll-like receptors and leishmaniasis. **Infection and Immunity**, v. 76, n. 3, p. 866–872, 1 mar. 2008.
ULIANA, S. R. B.; TRINCONI, C. T.; COELHO, A. C. Chemotherapy of leishmaniasis: present challenges. **Parasitology**, p. 1–17, 2017.
VALLADARES, J. E. et al. Disposition of antimony after the administration of N-methylglucamine antimoniate to dogs. **Veterinary Record**, v. 138, n. 8, p. 181–183, 24 fev. 1996.
VAN GRIENSVEN, J.; DIRO, E. Visceral Leishmaniasis. **Infectious Disease Clinics of North America**, v. 26, n. 2, p. 309–322, 2012.
VASILAKOS, J. P.; TOMAI, M. A. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. **Expert Review of Vaccines**, v. 12, n. 7, p. 809–819, 9 jul. 2013.
VEDVICK, T. et al. An Improved Manufacturing Process for a Recombinant Polyprotein Vaccine. **Biopharm International**, v. 21, 2 jan. 2008.
VÉLEZ, I. D. et al. Safety and immunogenicity of a defined vaccine for the prevention of cutaneous leishmaniasis. **Vaccine**, v. 28, n. 2, p. 329–337, 11 dez. 2009.
VERMA, N. K.; DEY, C. S. Possible mechanism of miltefosine-mediated death of Leishmania donovani. **Antimicrobial Agents and Chemotherapy**, v. 48, n. 8, p. 3010–3015, 2004.
WEBB, J. R. et al. Molecular cloning of a novel protein antigen of Leishmania major that elicits a potent immune response in experimental murine leishmaniasis. **Journal of immunology (Baltimore, Md. : 1950)**, v. 157, n. 11, p. 5034–41, 1 dez. 1996.
WEBB, J. R. et al. Molecular characterization of the heat-inducible LmSTI1 protein of Leishmania major. **Molecular and Biochemical Parasitology**, v. 89, n. 2, p. 179–193, 1 nov. 1997.
WEBB, J. R. et al. Human and murine immune responses to a novel Leishmania major recombinant protein encoded by members of a multicopy gene family. **Infection and immunity**, v. 66, n. 7, p. 3279–89, jul. 1998.
WERNECK, G. L. Visceral leishmaniasis in Brazil: rationale and concerns related to reservoir control. **Revista de Saúde Pública**, v. 48, n. 5, p. 851–856, out. 2014.
WHO. WHO | Leishmaniasis. **WHO**, 2017.
WOERLY, V. et al. Clinical efficacy and tolerance of miltefosine in the treatment of canine leishmaniosis. **Parasitology Research**, v. 105, n. 2, p. 463–469, 26 jul. 2009.
YASUR-LANDAU, D. et al. Allopurinol Resistance in Leishmania infantum from Dogs with Disease Relapse. **PLoS Neglected Tropical Diseases**, v. 10, n. 1, p. 1–13, 2016.
ZANIN, F. H. C. et al. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against Leishmania chagasi and Leishmania amazonensis experimental infections. 2007.
ZUBEN, A. P. B. VON; DONALÍSIO, M. R. Dificuldades na execução das diretrizes do Programa de Vigilância e Controle da Leishmaniose Visceral em grandes municípios brasileiros. **Cadernos de Saúde Pública**, v. 32, n. 6, p. 1–11, 2016.