TUANE FERREIRA MELO

DIAGNOSIS OF VISCERAL LEISHMANIASIS

LAVRAS - MG
2024

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Sanidade Animal e Saúde Coletiva, para obtenção do título de Doutor.

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DIAGNÓSTICO DE LEISHMANIOSE VISCERAL

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À minha família e aos animais.

Dedico.

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"Comece fazendo o que é necessário, depois o que é possível, em breve estarás fazendo o impossível." (São Francisco de Assis)

RESUMO

O objetivo da presente tese foi estabelecer as medidas de acurácia dos testes diagnósticos para leishmaniose visceral canina (LVC) e realizar o diagnóstico sorológico e molecular de cães e gatos abandonados da região Central da Colômbia, identificando a espécie circulante de *Leishmania* spp.. Por meio de uma revisão sistemática e metanálise foram selecionados estudos que calcularam sensibilidade diagnóstica (DSe) e a especificidade diagnóstica (DSp) dos testes diagnóstico de LVC. A partir desses dados, recalculou-se considerando a utilização ou não de animais assintomáticos e com reação cruzada. O teste de aglutinação direta (DAT) com antígeno bruto de *Leishmania donovani* apresentou os melhores resultados, tanto para DSe [0,9942 (IC 95%: 0,9892-0,9969)] quanto para DSp [0,9796 (IC 95%: 0,9492-0,9920) e além disso, verificou que os valores DSe diminuem conforme se adicionam animais assintomáticos e que os valores de DSp diminuem quando se acrescentam animais de reação cruzada. Os testes sorológicos são os mais utilizados e o DAT- *L. donovani* apresentou melhores resultados. Já o diagnóstico LVC de cães abandonados da Colômbia ocorreu em um abrigo de Girardot e realizou-se o exame clínico, exames laboratoriais, diagnóstico sorológico, diagnóstico molecular e sequenciamento genômico. A soropositividade foi de 25,3% e 37,5% em ambas as técnicas sorológicas, teste imunocromatográfico rKDDR-plus (ICT-rKDDR-plus) e imunoenzimático rKDDR-plus (ELISA-rkDDR-plus). Na reação em cadeia polimerase (PCR) usando o gene RV1-RV2, 10,5% dos animais testaram positivo e na PCR multiplex, 11,8% dos animais testaram positivos. A amostra sequenciada apresentou 99,0% de similaridade com *L. infantum.* Confirmou-se pela primeira vez a presença de *L. infantum* na região Central da Colômbia, o que enfatiza a necessidade de desenvolver estratégias para controle e prevenção dessa zoonose na região. O diagnóstico sorológico e molecular de leishmaniose de gatos abandonados na Colômbia ocorreu no mesmo abrigo em Girardot. Encontrou-se 100,00% de soropositividade no ELISA-rKDDR-plus e 37,5% de soropositividade no ICT-rKDDR-plus e na PCR com gene HSP-70 foi 33,0%. Portanto, encontrou-se felinos positivos sorologicamente e molecularmente para leishmaniose, sugerindo o envolvimento dessa espécie no ciclo de transmissão dessa doença. Essa parceria entre o Brasil e a Colômbia permitiu compreender que *L. infantum* está presente em ambos os territórios, além da possibilidade de compartilhar conhecimento com novas percepções e avançar nas pesquisas em relação a temática.

Palavras-chave: Zoonoses; Doença negligenciada; Flebotomíneos; América do Sul.

ABSTRACT

The aim of this thesis was to establish the accuracy measures of diagnostic tests for canine visceral leishmaniasis (CVL) and to carry out serological and molecular diagnosis of abandoned dogs and cats in the Central region of Colombia, identifying the circulating species of Leishmania spp. Through a systematic review and meta-analysis, studies were selected that calculated the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of CVL diagnostic tests. These data were then recalculated taking into account whether or not asymptomatic and cross-reacting animals were used. The direct agglutination test (DAT) with crude Leishmania donovani antigen showed the best results, both for DSe [0.9942 (95% CI: 0.9892-0.9969)] and DSp [0.9796 (95% CI: 0.9492-0.9920)] and it was also found that DSe values decrease as asymptomatic animals are added and that DSp values decrease when cross-reacting animals are added. Serological tests are the most commonly used and the DAT- L. donovani showed the best results. The CVL diagnosis of abandoned dogs in Colombia took place in a shelter in Girardot and involved a clinical examination, laboratory tests, serological diagnosis, molecular diagnosis and genomic sequencing. Seropositivity was 25.3% and 37.5% in both serological techniques, the rKDDR-plus immunochromatographic test (ICT-rKDDR-plus) and the rKDDR-plus enzyme-linked immunosorbent assay (ELISA-rkDDR-plus). In the polymerase chain reaction (PCR) using the RV1-RV2 gene, 10.5 per cent of the animals tested positive and in the multiplex PCR, 11.8 per cent of the animals tested positive. The sequenced sample showed 99.0% similarity to L. infantum. The presence of L. infantum in the Central region of Colombia was confirmed for the first time, which emphasises the need to develop strategies to control and prevent this zoonosis in the region. The serological and molecular diagnosis of leishmaniasis in abandoned cats in Colombia took place at the same shelter in Girardot. It found 100.00% seropositivity in the ELISA-rKDDR-plus and 37.5% seropositivity in the ICT-rKDDR-plus and in the PCR with the HSP-70 gene it was 33.0%. Therefore, felines were found to be serologically and molecularly positive for leishmaniasis, suggesting the involvement of this species in the transmission cycle of this disease. This partnership between Brazil and Colombia has made it possible to realise that L. infantum is present in both territories, as well as the possibility of sharing knowledge with new insights and advancing research on the subject.

Keywords: Zoonoses; Neglected disease; Phlebotomines; South America.

INIDICADORES DE IMPACTO

A tese “DIAGNOSIS OF VISCERAL LEISHMANIASIS” apresenta como tema central a leishmaniose, uma doença negligenciada e zoonótica que afeta diversos países. A pesquisa objetivou estabelecer as medidas de acurácia dos testes diagnósticos para leishmaniose visceral canina (LVC) e realizar o diagnóstico sorológico e molecular de cães e gatos abandonados da região Central da Colômbia, identificando a espécie circulante de Leishmania spp.. A partir de uma revisão sistemática e metanálise foram selecionados estudos que calcularam sensibilidade diagnóstica (DSe) e a especificidade diagnóstica (DSp) dos testes diagnóstico de LVC. O teste de aglutinação direta (DAT) com antígeno bruto de Leishmania donovani apresentou os melhores resultados, tanto para DSe [0,9942 (IC 95%: 0,9892-0,9969)] quanto para DSp [0,9796 (IC 95%: 0,9492-0,9920). O diagnóstico LVC de cães abandonados da Colômbia ocorreu em um abrigo de Girardot e realizou-se o exame clínico, exames laboratoriais, diagnóstico sorológico, diagnóstico molecular e sequenciamento genômico. A amostra sequenciada apresentou 99,0% de similaridade com L. infantum, confirmando pela primeira vez a presença de L. infantum na região Central da Colômbia. Na oportunidade, realizou-se um diagnóstico sorológico e molecular de leishmaniose de gatos abandonados no mesmo abrigo em Girardot, encontrando felinos positivos sorologicamente e molecularmente para leishmaniose, sugerindo o envolvimento dessa espécie no ciclo de transmissão dessa doença. A pesquisa realizada contou com a colaboração do departamento de Medicina Veterinária da Universidade Federal de Lavras e departamento de Parasitologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, ambos no Brasil, bem como da Universidad de Ciencias Aplicadas y Ambientales de Bogotá, na Colômbia. A parceria internacional tem um impacto social e na área de saúde relevante, uma vez que a leishmaniose é classificada como uma doença negligenciada e a identificação de leishmaniose visceral canina (LVC) em cães e gatos no território colombiano permite a elaboração de políticas públicas direcionadas à informação da população e no combate mais assertivo dessa zoonose. A pesquisa ainda ressalta, de acordo com a Política Nacional de Extensão, o compromisso social das instituições de ensino envolvidas na formação integral do estudante, com a sociedade e na promoção de iniciativas no campo da saúde. Com relação aos Objetivos de Desenvolvimento Sustentável (ODS) da Organização das Nações Unidas (ONU) para que o Brasil cumpra a Agenda 2030, a pesquisa fornece dados relevantes para que sejam criadas estratégias para os objetivos de saúde e bem estar, mais especificamente, de acordo com o item 3.3, que tem como meta, até 2030, acabar com as epidemias de AIDS, tuberculose, malária e doenças tropicais negligenciadas (aqui no caso a leishmaniose), e combater a hepatite, doenças transmitidas pela água, e outras doenças transmissíveis, bem como o item 3.d, em reforçar a capacidade de todos os países, particularmente os países em desenvolvimento, para o alerta precoce, redução de riscos e gerenciamento de riscos nacionais e globais de saúde.

IMPACT INDICATORS

The thesis “DIAGNOSIS OF VISCERAL LEISHMANIASIS” presents central theme leishmaniasis, a neglected and zoonotic disease that affects several countries. The research aimed to establish accuracy measures for diagnostic tests for canine visceral leishmaniasis (CVL) and perform the serological and molecular diagnosis of abandoned dogs and cats from the Central region of Colombia, identifying the circulating species of Leishmania spp.. Based on a review Systematic and meta-analysis studies were selected that calculated diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of CVL diagnostic tests. The direct agglutination test (DAT) with Leishmania donovani crude antigen showed the best results, both for DSe [0.9942 (95% CI: 0.9892-0.9969)] and for DSp [0.9796 (IC 95 %: 0.9492-0.9920). The LVC diagnosis of abandoned dogs in Colombia occurred in a shelter in Girardot and a clinical examination, laboratory tests, serological diagnosis, molecular diagnosis and genomic sequencing were carried out. The sequenced sample showed 99.0% similarity with L. infantum, confirming for the first time the presence of L. infantum in the Central region of Colombia. On that occasion, a serological and molecular diagnosis of leishmaniasis was carried out in cats abandoned in the same shelter in Girardot, finding felines that were serologically and molecularly positive for leishmaniasis, suggesting the involvement of this species in the transmission cycle of this disease. The research carried out included the collaboration of the departments of Veterinary Medicine at the Federal University of Lavras and Parasitology at the Institute of Biological Sciences at the Federal University of Minas Gerais, as well as the Universidad de Ciencias Aplicadas y Ambientales de Bogotá, in Colombia. The international partnership has a relevant social and economic impact, since the identification of canine visceral leishmaniasis (CVL) in dogs and cats in Colombia allows the development of public policies aimed at informing the population and combating this zoonosis more assertively, reducing their costs through the use of more sensitive tests. The research also highlights, in accordance with the National Extension Policy, the social commitment of educational institutions involved in the comprehensive training of students, with society and in the promotion of initiatives in the field of health. In relation to the Sustainable Development Goals (SDGs) of the United Nations (UN) for Brazil to comply with the 2030 Agenda, the research provides relevant data so that strategies can be created for health and well-being objectives, more specifically, of in accordance with item 3.3, which aims, by 2030, to end the epidemics of AIDS, tuberculosis, malaria and neglected tropical diseases (in this case, leishmaniasis), and combat hepatitis, waterborne diseases, and other communicable diseases , as well as item 3.d, in strengthening the capacity of all countries, particularly developing countries, for early warning, risk reduction and management of national and global health risks.

SUMÁRIO

PRIMEIRA PARTE –
REFERENCIAL TEÓRICO

1 INTRODUÇÃO

Embora a leishmaniose seja classificada como uma doença negligenciada, representa um problema global. Segundo a Organização Mundial da Saúde, em 2018, mais de 1 bilhão de pessoas viviam em áreas endêmicas para leishmaniose, estando sob risco de infecção. Essa infeção abrange mais de 98 países, com aproximadamente 350 milhões de pessoas em risco e aproximadamente 12 milhões de casos, sendo que mais de 90% dos casos globais de Leishmaniose Visceral (LV) ocorrem no Afeganistão, Irã, Arábia Saudita, Síria, Argélia, Tunísia, Bangladesh, Índia, Nepal, Etiópia, Brasil, Quênia e Sudão (Akhoundi *et al.*, 2017; Alvar *et al.*, 2012).

No Brasil, a LV apresenta um grande número de casos espalhados por todo país, com registros de 53.715 casos humanos no período compreendido entre os anos de 2001 a 2017 (Azevedo; Lorenz; Chiaravalloti-Neto, 2019). A Colômbia, país localizado no extremo norte da América do Sul e com extensão de 1644,2 km de fronteira com o Brasil, está entre os dez países com maior número de leishmaniose cutânea (LC) (Alvar *et al.*, 2012). Contudo, as informações sobre a LV na Colômbia são poucas, limitando-se a relatos de casos e alguns estudos (Sánchez *et al.*, 2020). Nesse contexto, percebe-se a necessidade de estudos sobre LV em cães e gatos da Colômbia para contribuir com estratégias diagnósticas, definição epidemiológica do território e auxiliar profissionais Médicos Veterinários na tomada de decisão. Dessa forma, como o Brasil apresenta uma diversidade de estudos sobre LV e com extensa área de limite territorial, além da existência da possibilidade de interações filogenéticas com espécies da Colômbia, a colaboração entre pesquisadores dos dois países visa compreender a distribuição desse protozoário em ambos os territórios e permitiu um compartilhamento de conhecimento com novas percepções e parcerias estabelecidas.

Essa enfermidade é uma infecção tropical, porém os cenários epidemiológicos sofrem alterações associadas à modificação humana dos ecossistemas, bem como às suas mudanças climáticas (Purse *et al.*, 2017). Tanto a LC quanto a LV são doenças em que possuem a participação de vetores na transmissão do protozoário, através da picada da fêmea do inseto da família *Psychodidae*, subfamília *Phlebotominae*, sendo os principais gêneros *Phlebotomus* e *Lutzomya* (Telleria *et al.*, 2018). Antes, essas doenças que envolve a transmissão por vetores como a leishmaniose, afetavam apenas países tropicais em desenvolvimento. Porém, os impactos do uso da terra e das mudanças

climáticas tem alterado o padrão de transmissão dessas enfermidades. Outro fator que tem impacto positivo na presença de leishmaniose é a biodiversidade de mamíferos, uma vez que eles atuam como hospedeiros das espécies do gênero de *Leishmania* spp. (Purse *et al.*, 2017).

A alteração nos cenários epidemiológicos é relatada em um estudo na Colômbia no qual se identificou que o aumento da temperatura global e as mudanças nos padrões de precipitação junto às altas taxas de desmatamento podem levar à domiciliação do vetor e, consequentemente, a urbanização da leishmaniose, que anteriormente era considerada uma enfermidade rural (González; Paz; Ferro, 2014). O desmatamento, assim como a maior proximidade do homem com grandes matas tem causado maior adaptação das espécies de vetores em áreas urbanas e periurbanas (Sánchez *et al.*, 2020). Contudo, é importante ter os reservatórios infectados para estabelecer a circulação do parasito e isso pode ocorrer por meio da migração de cães domésticos de áreas endêmicas para áreas não endêmicas, pois os cães são considerados os principais reservatórios de *Leishmania infantum,* participando da manutenção do ciclo desse protozoário no ambiente (González; Paz; Ferro, 2014).

Para compreender a dinâmica de transmissão do protozoário, estabelecer medidas para minimizar a transmissão e consequentemente, reduzir o aparecimento da doença, é fundamental conhecer os reservatórios (Lago *et al.*, 2019). Atualmente, também foi determinada a participação dos felinos domésticos na manutenção do ciclo do patógeno, como competentes hospedeiros das *Leishmania* spp. (Metzdorf *et al.*, 2017; Nascimento *et al.*, 2022). Dessa forma, os cães e gatos permitem a manutenção do ciclo urbano devido ao contato íntimo que esses animais apresentam com os humanos (Nascimento *et al.*, 2022). Existe um aumento do número de estudos relacionados a leishmaniose felina, porém há uma escassez de evidências científicas relacionadas a sintomatologia, achados laboratoriais e diagnóstico dessa enfermidade em felinos (Abramo *et al.*, 2021). Ademais, sabe-se que a infecciosidade é maior em cães com a infecção sintomática por possuírem maior carga parasitária, porém o mesmo precisa ser avaliado em nível populacional em felinos para confirmar seu papel de reservatório (Quinnell; Courtenay, 2009).

Um diagnóstico rápido e preciso torna-se uma das estratégias fundamentais para que as medidas de prevenção, controle e tratamento da leishmaniose sejam tomadas de maneira eficaz. Para isso, o quadro clínico em pacientes sintomáticos apresentado pelo

indivíduo pode ser utilizado como ponto inicial, sendo confirmado por meio de diversos testes laboratoriais, que incluem pesquisa de anticorpos, métodos moleculares e pesquisa parasitária (Freire *et al.*, 2019). Os testes diagnósticos imunocromatográficos rápidos (ICT) têm se apresentado como uma ferramenta valiosa, devido a sua facilidade de manuseio, baixos custos e resultados práticos, sendo úteis na triagem epidemiológica (Herrera *et al.*, 2019). No entanto, as técnicas diagnósticas precisam identificar e quantificar a infecção independentemente da presença de sinais clínicos, pois os animais assintomáticos representam uma dificuldade ao diagnóstico (Duthie; Lison; Courtenay, 2018).

Os ICT para LV canina, baseados no antígeno rK39 podem gerar resultados falsos negativos em animais assintomáticos e falsos positivos quando ocorre reações cruzadas com outras infecções parasitárias. Dessa forma, o uso da proteína antigênica denominada rKDDR-plus tem como objetivo, melhorar a precisão desse diagnóstico apresentando maior acurácia tanto em soros humanos quanto em soros caninos, quando comparada a utilização dos ICTs que utilizam rK39 e rKDDR (Siqueira *et al.*, 2021). Assim, a partir da sua testagem e utilização na Colômbia, o teste rKDDR-plus foi avaliado considerando outra situação epidemiológica. Além disso, o teste rKDDR-plus ainda não tinha sido utilizado em soros de outras espécies animais. No entanto o teste rKDDR que apresenta muitas semelhanças com o rKDDR-plus foi utilizado em felinos e canídeos selvagens, obtendo bom potencial para uso como teste de triagem para LV em animais silvestres (Tolentino *et al.*, 2019).

Dessa forma, a presente tese está estruturada em duas partes: a primeira parte apresenta um referencial teórico sobre a leishmaniose e seu contexto histórico, bem como a caracterização do agente etiológico, sua abrangência territorial, formas clínicas e medidas de controle e prevenção. A segunda parte é composta por três artigos científicos: (I) acurácia dos testes sorológicos, moleculares e parasitológicos para diagnóstico da leishmaniose visceral canina: a revisão sistemática e metanálise; (II) primeiro relato de leishmaniose visceral em cães abandonados da região Central da Colômbia; (III) primeiro levantamento de leishmaniose em gatos da região Central da Colômbia.

2 REFERENCIAL TEÓRICO

2.1 Contexto histórico e o agente etiológico

Estima-se que a leishmaniose é uma doença bastante antiga e diversas teorias foram montadas para tentar explicar a sua disseminação pelo mundo. Em 1571, Pedro Pizzaro relatou lesões que afetavam lábios e narizes de pessoas na Cordilheira dos Andes (Lainson, 2010). O mesmo ocorreu em 1756, em Aleppo, quando Alexander Russell descreveu a presença de feridas úmidas causadas provavelmente por *Leishmania major* e, secas comumente causadas por *Leishmania tropica*, em indivíduos que se recuperavam das lesões em um período de oito meses a um ano (Steverding, 2017).

Em 1901, o pesquisador de sobrenome Leishman identificou como tripanossomos os organismos encontrados no baço de um paciente indiano que faleceu de "febre dum-dum". No mesmo ano, Donovan também identificou os organismos, que foram denominados Leishman-Donovan. Em 1903, o pesquisador Ross propôs que fossem chamados de *Leishmania donovani,* com diversas descobertas importantes ao longo dos anos. Em 1936, Carlos Chagas descreveu a leishmaniose visceral no Brasil, isolando no ano seguinte a espécie *Leishmania chagasi* (Akhoundi *et al.*, 2017).

Existem cinquenta e três espécies pertencentes ao gênero *Leishmania*, sendo que vinte delas podem infectar seres humanos e animais, estando presentes em 98 países, com aproximadamente 335 milhões de pessoas em áreas de risco (de Souza *et al.*, 2018; Pagliano *et al.*, 2016). As espécies *L. infantum, L. chagasi* e *L. donovani* estão comumente relacionadas à forma visceral da doença e em 2015 afetou 12 países das Américas, sendo o Brasil o mais afetado com 96% dos casos, principalmente na região nordeste. As espécies *L. tropica, L. major, L. amazonensis* e *L. braziliensis,* geralmente são responsáveis pela forma cutânea da enfermidade, com 70% dos casos registrados no Peru em 2015. Dos 69% infectados por leishmaniose cutânea nas Américas são indivíduos do sexo masculino e 12,7% crianças com menos de dez anos de idade (de Souza *et al.*, 2018; Goncalves *et al.*, 2019).

A LV é considerada a forma mais grave da doença e possui sinais clínicos caracterizados por longos períodos de febre, perda de peso, hepatoesplenomegalia e anemia, lesões severas que podem resultar em óbito (Bi *et al*., 2018). A LV pode ser caracterizada por úlceras localizadas ou múltiplas e lesões nodulares no tecido cutâneo,

que podem ou não envolver mucosas, sendo a forma mais prevalente da enfermidade (de Vries; Reedijk; Schallig, 2015).

O gênero *Leishmania* pertence ao filo Sarcomastigophora, ordem Kinetoplastida e família Trypanosomatidae, é classificado em subgêneros *Leishmania* e *Viannia*, identificadas como *L. major, L. infantum, L. donovani, L.amazonensis, L. guyanensis,* entre outras (Fraga *et al*., 2010). Considerado um parasito intracelular obrigatório, com ciclo de vida envolvendo um hospedeiro mamífero e um inseto vetor, o flebotomíneo, pertencente à família *Psychodidae*, subfamília *Phlebotomina*. Os principais gêneros transmissores são *Phlebotomus* e *Lutzomyia,* sendo *Lutzomiya longipalpis* a principal espécie transmissora no Brasil. A fêmea do flebotomíneo participa do ciclo de transmissão do protozoário ao realizar o repasto sanguíneo em indivíduos que possuem o parasito na pele, como os cães e, ao se infectar, consegue transmiti-lo a um outro hospedeiro (Telleria *et al.*, 2018).

A alta incidência da leishmaniose está associada a diversidade das condições ambientais, climáticas e socioeconômicas. Existe a necessidade do contato humano com áreas de vegetação com a presença dos reservatórios e vetores. A passagem da doença de área rural para áreas periurbanas e urbanas aconteceu devido ao desmatamento e o desenvolvimento de assentamentos rurais. Além disso, a população humana tem ficado mais próximas de áreas de vegetação e essas áreas abrigam vetores flebotomíneos e reservatórios (Valero; Uriarte, 2019).

As espécies de flebotomíneos, antes encontradas em ambientes selvagens ou rurais, têm se adaptado muito bem à ambientes urbanos em busca de alimento. Uma vez que as fêmeas do flebotomíneo necessitam de sangue para a maturação de seus ovos e por isso são atraídas pela presença de animais como cães, galinhas, porcos, cavalos e outros, com preferência bastante grande por humanos (Barata *et al.*, 2005). Além disso, vivem em locais com acúmulo de matéria orgânica, condições comumente encontradas em periferias de grandes centros urbanos de diversos estados brasileiros (Prestes-Carneiro *et al.*, 2019).

Há diferenças na carga parasitária dos hospedeiros para infectar diferentes espécies de flebotomíneos, por exemplo, *Phebotomus pernicious* precisa de uma carga parasitária menor para ser infectado do que *L. longipalpis* (Telleria *et al.*, 2018). Os vetores flebotomíneos são capazes de prosperar nas variações climáticas das regiões

tropicais, mas eles conseguem também se adaptar a temperaturas entre 19 a 30ºC (Prestes-Carneiro *et al.*, 2019).

Os fatores socioeconômicos como baixo nível de educação e pobreza são frequentemente associados a desnutrição, habitação precária e problemas sanitários o que aumenta o risco de leishmaniose. Isso promove condições do vetor se desenvolver adequadamente e também existe a presença de animais abandonados ou de rua (Valero; Uriarte, 2019).

As espécies de *Leishmania* sp. possuem forma bem estabelecida por meio de microtúbulos, importantes no processo de divisão celular, uma organela denominada cinetoplasto. Essa organela possui uma massa de DNA mitocondrial, anterior ao núcleo e diretamente ligada ao flagelo e essa parte está presente nas promastigotas. As promastigotas são ovais e encontradas no aparelho digestivo do flebotomíneo e há duas formas: imatura ou madura. A forma imatura conhecida como promastigota procíclica é passível de multiplicação. Já a forma madura conhecida como promastigota metacíclica é infectante para hospedeiros vertebrados, altamente móvel, e ao encontrar as células do sistema fagocítico mononuclear, sofre diversas mudanças bioquímicas e metabólicas e passa à forma amastigota. A forma amastigota possui tamanho menor, esférica, sem mobilidade e com grande capacidade de multiplicação (Sunter; Gull, 2017), que irá infectar inicialmente macrófagos e células dendríticas dos mamíferos, sendo encontradas em neutrófilos, fibroblastos e outras células, persistindo por dias ou semanas, a depender das relações estabelecidas entre protozoário e sistema imune do hospedeiro (Kima, 2007).

O ciclo de transmissão é estabelecido quando a fêmea do flebotomíneo realiza o repasto sanguíneo em mamíferos por meio de seu aparelho bucal em forma de serra. Assim, produzirá uma pequena lesão cutânea para que ela tenha acesso ao sangue advindo de capilares sanguíneos da pele, onde estão as células infectadas com as formas amastigotas do protozoário. Quando essas formas estão em órgãos internos como baço, fígado, dentre outros, não ficam acessíveis ao inseto (Bates, 2018).

O protozoário fica localizado na extremidade posterior do intestino médio do flebotomíneo, no entanto, ainda não têm capacidade de infectar outro hospedeiro, o que vai ocorrer somente após a maturação do parasito. Isso acontece uma ou duas semanas depois do repasto sanguíneo, em que ele vai se transformar em promastigota próciclica, se replicar e se diferenciar em promastigota metacíclica, com capacidade de infectar as

células do indivíduo vertebrado em um próximo repasto sanguíneo (da Costa *et al*., 2019). As promastigotas infectam as células do indivíduo, em especial macrófagos, se diferenciando em formas amastigotas, que vão se replicar e invadir outras células, podendo causar os sinais clínicos comuns à enfermidade caso o sistema imune do hospedeiro não consiga montar uma resposta adequada (Conceicao-Silva; Morgado, 2019).

2.2 Distribuição mundial

A leishmaniose está distribuída por mais de 98 países, principalmente em áreas tropicais e subtropicais e aproximadamente 90% dos casos ocorrem no Afeganistão, Irã, Arábia Saudita, Síria, Argélia, Tunísia, Bangladesh, Índia, Nepal, Etiópia, Brasil, Quênia e Sudão. A forma cutânea é de ocorrência mais comum, com incidência anual de 0,7 a 1,2 milhões de casos, já a forma mais grave é a visceral, com 0,2 a 0,4 milhões de casos todos os anos (Akhoundi *et al.*, 2017; Alvar *et al.*, 2012). No Brasil, existe dificuldade no controle da LV, sendo que as regiões Nordeste, Sudeste e Centro-Oeste apresentam maior risco de transmissão e é considerado o país da América com maior número de casos (Azevedo; Lorenz; Chiaravalloti-Neto, 2019).

Na Colômbia, país que tem fronteiras com o Brasil, há duas áreas endêmicas de LV, uma na Costa Caribenha e outra no médio Vale do Rio Magdalena, porém a prevalência de LV ainda é desconhecida. Sabe-se que as espécies do tripassomideos, vetores na Colômbia, são *Lutzmonia evansi* na região da Costa do Caribe e *Lutzomia longipalpis* no restante do país (Castillo-Castañeda *et al*., 2021; Arbelaez *et al.*, 2020; Sánchez *et al.*, 2020).

A cidade de Cali, localizada no sudoeste da Colômbia, no Valle del Cauca, teve seu primeiro registro de um paciente canino diagnosticado com LVC. Esse paciente da raça buldogue francês nunca morou em área endêmica e viajou para lugares próximos, onde também casos humanos e caninos não foram documentados. Além disso, não foi registrado a presença do vetor na região (Arbelaez *et al.*, 2020). Também foi realizado um estudo na Área Metropolitana de Bucaramanga, no Vale do Rio Oro, mostrando a circulação de cães com LV (Jaimes-Dueñez *et al.*, 2023). Essas informações mostram a necessidade de estratégias de vigilância com uma busca ativa para adequar à realidade

do país, pois há registro da doença sem registro do vetor e também tem registro de locais em que não há a doença, mas existe a presença do vetor (Sánchez *et al.*, 2020).

A leishmaniose é considerada uma enfermidade dinâmica e representa uma ameaça a países nos quais ela é endêmica e também em locais onde não há casos registrados, pois o parasito pode ser introduzido por meio de atividades de ecoturismo, operações militares e imigração. Geralmente, ocorre uma introdução insidiosa (por não ser reconhecida) do parasito, com sua consequente adaptação aos reservatórios e vetores locais. Para reduzir as chances de disseminação e também para controle em áreas onde o agente está presente, é fundamental uma vigilância adequada, que visa identificar novas espécies/genótipos de *Leishmania* que podem ser introduzidos e potencialmente transmitidos por vetores flebotomíneos presentes nos locais (Di Muccio *et al.*, 2015).

2.3 Filogenética da *Leishmania* spp.

Leishmania spp. se adaptam e sofrem expansão epidêmica após mudanças ambientais, além disso, a recombinação genética permite uma alta diversidade fenotípica. Com o sequenciamento genômico de espécies/genótipos de *Leishmania* é possível realizar a caracterização genética e desempenhar um papel importante no diagnóstico, tratamento e controle de doenças infectocontagiosas, como na produção de vacinas, pois é possível identificar novos alvos (de Almeida et al., 2011; Schallig; Oskam, 2002).

Para diagnóstico de leishmaniose é muito utilizado técnicas que visualiza por meio de microscópio o parasita, são chamadas de técnicas parasitológicas, porém as espécies de *Leishmania* são semelhantes morfologicamente e para a identificação das espécies é feito por meio técnicas altamente sensíveis (de Almeida *et al.*, 2011). Um exemplo de técnica utilizada para identificação de espécies de *Leishmania* é a reação em cadeia polimerase (PCR) universal visando a região do espaçador 1 transcrito interno (ITS1) seguido de polimorfismo de comprimento de fragmento de restrição (RFLP), essa técnica se mostrou muito confiável na identificação de *L. infantum* e *L. tropica* na Turquia (Toz *et al.*, 2009). Outra abordagem para discriminação das espécies acessível é baseada em duas PCR em real time (RT-PCR), que permiti distinguir os subgêneros *Leishmania* e *Viannia* por meio da RT-PCR-ML e diferenciar *L.* (*L.*) *infantum* e *L.* (*L.*) *amazonensis* por meio RT-PCR-ama (Diotallevi *et al.*, 2020).

Ao analisar a evolução do gênero *Leishmania* é possível compreender os aspectos básicos da sua biologia, como as relações parasito-hospedeiro ou parasito-vetor, biogeografia ou epidemiologia. Estudos filogenéticos permitiram concluir que o parasito tripanossomatídeo *L. infantum* foi introduzido nas Américas durante a era colonial europeia, no qual estabeleceu ciclos de transmissão generalizado usando vetores alternativos (Schwabl *et al.*, 2021). Além disso, o avanço da leishmaniose visceral para regiões não endêmicas representa um alerta para a saúde humana e animal, dessa forma ao avaliar a frequência e a circulação do parasito é possível estabelecer medidas para seu controle e prevenção (Marcili *et al.*, 2020).

Segundo Abdelhaleem *et al.* (2021), é necessário que os estudos sobre LV sejam direcionados para identificar as espécies, reservatórios e vetores causadores da leishmaniose. Essa discriminação de espécies é possível por meio do sequenciamento genômico e ao determinar a espécie de *Leishmania*, possibilita inferir se é uma espécie já existente ou se é uma espécie nova. Ao identificar uma nova espécie é necessário analisar se há vetores compatíveis e assim, é possível definir estratégias de controle para evitar a sua disseminação (Wall *et al.*, 2012). Além disso, diferenciar as espécies desse protozoário é importante para definir melhor prognóstico e adequar o tratamento, pois há espécies de *Leishmania* que são refratárias ao tratamento (Blum; Hatz, 2009).

As inferências filogenéticas permitem constatar a identidade completa entre os isolados comparando com sequências de isolados de outras origens geográficas. Isso possibilita verificar se o perfil de identidade é mantido mostrando que os isolados pertencem a determinada espécie e também é possível confirmar a ausência de variações intraespecíficas. Como exemplo, houve um estudo realizado na região metropolitana de São Paulo, considerada a quarta maior cidade do mundo em número de habitantes, com legislação que regulamenta a posse de cães e gatos por indivíduo, no qual evidenciou o parasito circulando e não houve diferenças entre os isolados com os de outras regiões geográficas do Brasil. Assim é possível perceber a inexistência de variabilidade intraespecífica entre os isolados de *L. infantum* circulantes em São Paulo (Marcili *et al.*, 2020).

Existe uma segregação entre *L. infantum* e *L. infantum chagasi* durante o processo de colonização europeia na América e o caráter conservado de suas sequências. No Brasil, apesar de ser um país ecologicamente diverso, com diferentes reservatórios e populações de vetores, todas as cepas brasileiras pertencem a mesma

população de *L. infantum*. O mesmo ocorre no Paraguai, no qual a estrutura populacional é muito homogênea a do Brasil, consistindo exclusivamente de cepas com genótipo MON-1 e uma estrutura populacional mista com cepas MON-1 e não-MON-1 na região do Caribe. Já no território colombiano, esse estudo sugere que as cepas colombianas formam uma população separada (Kuhls *et al.*, 2011).

Na Argentina, duas variantes do citocromo b da espécie *L.* (*L.*) *infantum* foram identificadas como agentes causais de leishmaniose visceral canina (LVC) em uma área na qual a enfermidade está se espalhando. Essa espécie tem componentes silvestres e domésticos participando do seu ciclo. Acredita-se que o ciclo silvestre se iniciou durante e após a colonização da América e uma invasão de hospedeiros acidentais devido ao desmatamento levou a urbanização dessa enfermidade. Isso mostra a conexão existente entre ciclo silvestre e ciclo urbano, o que pode dificultar o controle enquanto apenas medidas em animais domésticos forem tomadas (Barrio *et al.*, 2012; Barroso *et al.*, 2015).

A estrutura populacional e as mudanças rápidas na composição genética da *Leishmania* sp. podem ocorrer e são de grande preocupação para a saúde pública, uma vez que a variação genética intraespecífica dentro deste gênero está associada a alterações na patogenicidade, na resistência aos medicamentos e outras características epidemiológicas. Houve deleção de quatro genes em chr31 de isolados de *L. infantum* no sudeste, leste e nordeste do Brasil. No Estado do Mato Grosso do Sul não houve essa deleção. Esse cenário evidencia extensa troca genética com efeito direto na hibridização no fenótipo com maior fator de virulência (Schwabl *et al.*, 2021).

Frequentemente, os casos caninos de LV precedem os casos humanos. Um estudo realizado na região de Namangan, no Uzbequistão, mostrou que os cães abrigam o mesmo grupo único de cepas *L. infantum* MON-1 identificado em crianças infectadas dessa região (Alam *et al.*, 2009; Kovalenko *et al.*, 2011). Reforça-se a necessidade da prevenção e controle de cães com LV. Porém o controle da LVC é uma ação trabalhosa, pois muitas vezes os cães apresentam resultados sorológicos divergentes e são mantidos nas casas sem tratamento, atuando como reservatórios (de Carvalho *et al.*, 2018).

Para prevenir surtos de outras formas de leishmaniose é importante a identificação de espécies dos parasitos. O Brasil, por exemplo, delimita ações de prevenção e controle para LV contra a *L. infantum*. No entanto, existem alguns relatos de cães infectados por *L. amazonensis*, sendo responsáveis por casos humanos da

doença. Outra questão é que na Região Metropolitana de Belo Horizonte, no Estado de Minas Gerais, o flebotomíneo mais abundante é *Lutzomia whitimani*, que transmite principalmente *L. amazonensis* e posteriormente, *L. braziliensis.* Já a *L. infantum* também pode ser transmitida por outros flebotomíneos, mas é principalmente transmitida por *Lutzmonia longipalpis* (Souza *et al.*, 2019).

A presença de espécies comumente associadas a LC provocam sinais clínicos de LV, como perda de peso, linfadenopatia, conjuntivite, queratite, anemia, úlceras, alopecia, dermatite, onicrogrifose e vasculite. Um exemplo foi a identificação de *L.* (*L.*) *amazonensis* em cães por de meio de análise filogenética na região de Governador Valadares em Minas Gerais (Brasil) com clínica de LV, que já tinha sido identificada na cidade de Araçatuba (Estado de São Paulo) e na cidade de Paracatu (Minas Gerais). A região de Governador Valadares é endêmica para casos de *L.* (*L.*) *infantum,* por isso, o controle é voltado para essa espécie, no entanto, é importante identificar outras espécies circulantes para o desenvolvimento de ações adequadas a elas. Dessa forma, deve-se considerar o potencial de envolvimento da *L.* (*L.*) *amazonensis* na causa de LVC, já que, o contato próximo entre humanos e cães pode aumentar o risco de transmissão dessa espécie às pessoas, favorecendo seu processo de urbanização (Valdivia *et al.*, 2017).

Há lacunas sobre o entendimento da relação genótipo do parasita e a manifestação clínica, pois o estudo de Valdivia *et al.* (2017), mostra *L.* (*L.*) *amazonensis* causando manifestações clínicas diferentes do habitual. Isso pode estar relacionado à expansão de genes promotores de alterações na produção de moléculas do parasito e às características imunológicas do próprio hospedeiro (Silva *et al.*, 2021)

No Brasil, o Programa de Vigilância e Controle da Leishmaniose Visceral (PVCLV) baseia o diagnóstico em técnicas sorológicas que podem detectar, sem diferenciação de espécies, animais infectados por *L. amazonensis, L. braziliensis* e *L. infantum*, por meio do soro (Brasil, 2014b). Por esse motivo, esses resultados podem estar superestimando a prevalência *L. infantum*, mostrando a necessidade do aprimoramento dos métodos diagnósticos que visam a discriminação de espécies de *Leishmania* (Souza *et al.*, 2019; Valdivia *et al.*, 2017). Além disso, a ocorrência de infecção mista pelos três agentes, *L. infantum*, *L. braziliensis* e *L. amazonensis*, gera a necessidade de compreender o significado dessa infecção com o intuito de atualizar as abordagens de controle e prevenção da LVC (Souza *et al.*, 2019). Dessa forma, compreender a variabilidade genética das espécies de *Leishmania* circulantes permite

fortalecer as redes de vigilância epidemiológica e estabelecer medidas de controle adequadas (Herrera *et al.*, 2019).

O sequenciamento genômico é uma técnica que ao longo dos anos tem se tornado cada vez mais acessível. Portanto, ao utilizá-la no diagnóstico da leishmaniose permite melhor compreensão dos mecanismos de transmissão e consequentemente, contribuem para o controle e prevenção dessa enfermidade (Di Muccio *et al.*, 2015).

2.4 Formas clínicas da leishmaniose

Para que o quadro clínico da leishmaniose seja estabelecido, fatores inerentes ao parasito e aspectos ambientais são de grande importância, bem como as características da resposta imune do hospedeiro. Como exemplo, a resistência do indivíduo ao protozoário está ligada à formação do perfil imune do tipo Th1, com efetiva ação dos componentes celulares do sistema imunológico, como os linfócitos TCD4+, TCD8+ e células NK, bem como o tipo de citocinas do ambiente, em que o INF-γ ea IL-12, são fundamentais, havendo uma melhor performance de macrófagos e outras células no combate ao protozoário. O mesmo não ocorre quando há alta concentração de citocinas como IL-4, IL-5, IL-10 e IL-13, além do TGF-β produzido pelas células T regulatórias CD4+CD25+Foxp3+, substâncias que induzem o perfil imune do tipo Th2 ou T regulatório, e à redução da capacidade fagocítica de macrófagos M1, com consequente diminuição da resistência do indivíduo à infecção, havendo o desenvolvimento das formas clínicas da enfermidade (Khaden; Uzoona, 2014)

Essas formas clínicas consistem em síndromes que podem ser classificadas em cutânea (LC), muco-cutânea (MLC), visceral (LV), também chamada de kala-azar e a dérmica pós-kala-azar (PKDL), causadas por diferentes espécies de *Leishmania* (Bi *et al.*, 2018). A LV possui grande impacto em indivíduos imunocomprometidos, como os portadores do vírus da imunodeficiência humana (HIV), que em países como o Brasil, chegam a representar 8,5% dos casos e 35% em países como a Etiópia (Pagliano *et al.*, 2016).

O período de incubação da leishmaniose visceral é de dois a seis meses, podendo variar de semanas a anos, apresentando como sintomas febre, perda de peso e hepatoesplenomegalia. Isso pode culminar em hemorragia do baço à palpação, linfadenopatia, podendo haver escurecimento da pele, o que ocorre principalmente no

sul da Ásia e por isso recebeu o nome de kala-azar. Além disso, pode haver aplasia medular, anemia, trombocitopenia, neutropenia, hiperglobulinemia, aumento de enzimas hepáticas, caquexia, hipoalbuminemia epistaxe, sangramento gengival, disfunção hepática, ascite e outras complicações que podem surgir de infecções concomitantes, podendo levar à morte caso não haja tratamento adequado (Chappuis *et al.*, 2007).

A forma cutânea da leishmaniose é causada geralmente por *L. major, L. tropica, L. braziliensis* e *L. mexicana* e, é caracterizada por pequenas pápulas avermelhadas. Essas pápulas medem 5 a 10 mm, podendo progredir para nódulos eritematosos, placas endurecidas ou escamosas e úlceras, que podem ser secas ou exsudativas, em um intervalo de 1 a 3 meses. Além disso, pode ter linfoadenopatias locais, deixando cicatrizes retraídas e despigmentadas ou ainda evoluindo para a forma muco-cutânea da enfermidade (David; Craft, 2009). Quando há o envolvimento das mucosas, com lesões ulcerativas e purulentas, ocorrendo geralmente em indivíduos imunocomprometidos, como os pacientes portadores de HIV, sendo que é necessário o diagnóstico diferencial entre LC, MLC e outras doenças de pele com as mesmas características clínicas, como psoríase, lesões por *Mycobacterium*, carcinoma epidermoide, entre outras (Garrido-Jareño *et al.*, 2020).

2.5 Diagnóstico

Para reduzir a disseminação da LV uma alternativa é monitorar e controlar os casos de LVC por meio de um diagnóstico preciso, pois os cães representam a principal fonte de infecção devido ao parasitismo cutâneo intenso (Brasil, 2014b). No entanto, novos hospedeiros também têm sido reservatórios potenciais, como é o caso dos felinos. Ainda que não esteja bem esclarecido o papel na transmissão de *Leishmania* spp. tem sido considerados reservatórios secundários ou acidentais (Cardoso *et al*., 2021). Existe uma variedade de testes disponíveis para o diagnóstico de LVC, desde métodos sorológicos, parasitológicos e moleculares (Brasil, 2014b). No entanto, o diagnóstico ainda é um desafio devido à ausência de um protocolo sensível frente a prevalência diversa (de Carvalho *et al.*, 2018).

O diagnóstico é feito inicialmente com base no quadro clínico em pacientes sintomáticos, sendo confirmado por métodos laboratoriais. Os métodos parasitológicos pesquisam-se a presença da amastigota no organismo por meio de análise microscópica

de tecidos como medula óssea, baço ou linfonodos. São técnicas altamente específicas por demonstração direta do parasito, no entanto, com sensibilidade variada conforme o grau de parasitismo, porque pode produzir resultados falsos negativos em animais com carga parasitária baixa ou amostras hemodiluídas. Além disso, é uma técnica mais invasiva, que pode levar o paciente à morte por riscos como hemorragia no baço e necessita de um indivíduo treinado para realizar o exame, que pode ser demorado e oneroso (Pessoa-e-Silva *et al.*, 2019; Srividya *et al*., 2012). As técnicas parasitológicas utilizadas podem ser microscopia, histopatologia e cultura parasitária. Além disso, pode-se coletar as amostras por biopsia, raspagem ou *imprintig* (de Vries; Reedijk; Schallig, 2015).

As técnicas moleculares como a reação em cadeia de polimerase (PCR) são consideradas métodos com alta especificidade e sensibilidade, que pode ser realizada com diversos tipos de material biológico (Srividya *et al.*, 2012). Porém, as espécies de *Leishmania* spp. têm preferência por parasitar linfonodos, baço e medula óssea, e o paciente pode apresentar baixa parasitemia, e por isso comumente a PCR realizada com amostras sanguíneas possuem resultados insatisfatórios. Pode-se utilizar PCR qualitativo ou quantitativo. A PCR quantitativa além de ser útil no diagnóstico da LVC, também facilita o monitoramento dos níveis do parasito durante a terapia por permitir a quantificação precisa do DNA molde presente no início da reação permitindo uma estimativa relativa de parasitos. Já a PCR qualitativa é importante quando necessita de um diagnóstico mais imediato e sensível especialmente em casos com resultados sorológicos questionáveis (Bezerra *et al.*, 2019).

A RT-PCR possui resultado superior à PCR convencional, pois permite o monitoramento contínuo do acúmulo de produtos de PCR durante a reação de amplificação. Além disso, tem maior homogeneidade entre grupos sintomáticos e assintomáticos quando comparados ao DPP e ao ensaio de imunoabsorção enzimática (ELISA), pois permite identificar DNA de *Leishmania* spp. em cães assintomáticos com sorologia negativa. Dessa forma, para identificar animais assintomáticos em regiões não endêmicas é fundamental aplicar técnicas moleculares para confirmação de LVC (de Carvalho *et al.*, 2018). Os animais assintomáticos apresentam baixa carga parasitária e possui exames parasitológicos negativos, como esfregaços e cultura do parasita, por isso precisam ser avaliados por métodos moleculares (de Carvalho *et al.*, 2018; Riboldi *et al.*, 2018). No entanto, o diagnóstico molecular também possui limitações

principalmente gerando resultados falsos negativos quando tem armazenamento incorreto ou inibidores da *Taq*-polimerase (Pessoa-e-Silva *et al.*, 2019).

A sorologia é uma alternativa aos métodos diretos, como técnicas parasitológicas e moleculares, pois requer amostras coletadas minimamente invasivas (Siqueira *et al.*, 2021). Comercialmente existem vários ensaios sorológicos, como teste de anticorpos fluorescentes indiretos (RIFI), teste de aglutinação direta (DAT), ensaio imunoenzimático (ELISA) e os testes imucromatográficos, conhecidos como teste rápido imunocromatográfico (ICT) (Gomes *et al*., 2008). As técnicas sorológicas são amplamente usadas no diagnóstico de LVC, sendo recomendado pelo Programa de Vigilância e Controle da Leishmaniose Visceral (PVCLV) do Ministério da Saúde do Brasil o uso do teste imunocromatógráfico produzido pela Biomanguinhos (FIOCRUZ), DPP como triagem e o ELISA como teste confirmatório, desde 2012. Antes de 2012, o diagnóstico era feito pelo teste imunoflorescência (IFAT) e confirmado pelo ELISA, porém a troca pelo DPP teve como objetivo melhorar a precisão do diagnóstico, já que, o DPP e ELISA detectou maior prevalência e incidência de cães infectados. Em locais onde a demanda por exames é grande recomenda-se ELISA como teste de triagem e a confirmação pelo DPP devido o diagnóstico em laboratório ter maior controle e ao menor custo do ELISA em comparação ao DPP quando realizado em larga escala (Brasil, 2014b; Coura-Vital *et al.*, 2014). Porém, essas técnicas apresentam desempenhos diferentes conforme o perfil clínico dos cães e o resultado falso positivo pode levar a eutanásia ou o tratamento inadequado ao paciente não infectado e o resultado falso negativo promove a manutenção de cães infectados na população e, consequentemente, aumento das infecções humanas em áreas não endêmicas. Dessa maneira, é fundamental o uso de ferramentas diagnósticas mais precisas como métodos moleculares, porém são técnicas caras e não totalmente viáveis para pesquisa a campo (Riboldi *et al.*, 2018).

Como nas demais técnicas sorológicas, no ICT pode usar o extrato bruto ou solúvel de *Leishmania* spp. ou proteínas recombinantes em sua fita teste (Gomes *et al.*, 2008). O uso de antígenos brutos ou parcialmente purificados produz resultados inconsistentes devido à dificuldade do diagnóstico de pacientes assintomáticos ou em estágio inicial da doença e existe a possibilidade de reações cruzadas com outros parasitos, como *Leishmania brazieliensis, Erliquia canis* e *Tripanossoma cruzi,* em razão da sensibilidade e especificidade limitada de alguns testes sorológicos (de Castro

Ferreira *et al.*, 2007). A ocorrência de resultados falsos, como falsos-positivos pode levar a uma desnecessária eutanásia de cães. Já os resultados falsos-negativos mantêm o reservatório no ambiente levando a disseminação da doença. Resultados falsos-negativos ocorrem comumente em animais assintomáticos, pois estes podem apresentar níveis de anticorpos anti-*Leishmania* não detectáveis. Em animais polissintomáticos há maior concordância do resultado sorológico, pois ocorre devido a maior carga parasitária presente no animal (Pessoa-e-Silva *et al.*, 2019)

Uma alternativa para minimizar os resultados falsos no imunodiagnóstico é o uso de peptídeos recombinantes para promover a maior acurácia no diagnóstico da LV, uma vez que antígenos que possuem motivos de repetição em *tandem* em sua sequência, geralmente são epítopos de células B altamente antigênicos (Gomes *et al.*, 2008; Siqueira *et al.*, 2021). Dessa forma, para melhorar o diagnóstico de LV, diferentes antígenos, como rK39 e rK28 têm sido aplicados nos testes sorológicos. Comumente opta-se por proteínas kinesinas para gerar os antígenos, pois é caracterizada por duas regiões distintas, uma sequência de aminoácidos sem padrão de organização (porção não repetitiva) e uma região que apresenta um padrão de organização com vários blocos que possuem aminoácidos repetidos em *tandem* (porção repetitiva). As proteínas repetitivas de parasitos são frequentemente alvo das respostas das células B do hospedeiro e isso aumenta a eficiência dos testes utilizados para diagnosticar doenças parasitárias. (Dhom-Lemos *et al.*, 2019).

O ICT baseado no antígeno recombinante rK39 é um teste rápido que facilita o diagnóstico a campo da doença, que quanto mais ativa, maior sua sororreatividade, com especificidade de 89% e sensibilidade de 98%. Contudo, existem relatos da variabilidade e inconsistência no desempenho do rK39 em regiões iguais ou diferentes. (Srividya *et al.*, 2012).

Em busca de antígenos mais sensíveis e específico, em 2014, um novo antígeno chamado rKDDR foi descrito e representa uma proteína recombinante de kinesina *L. infantum*. Essa proteína possui 1.086 pares de bases que codificam 362 aminoácidos, além disso, é composta por duas partes, sendo uma não repetitiva e outra repetitiva, com aproximadamente 6,5 motivos de 39 aminoácidos. Tem uma sequência recorrente de 8,5 blocos repetitivos de 39 repetições de aminoácidos, sendo 92% da sequência proteica exclusivamente composta por motivos repetitivos e enquanto 8% são originários da sequência plasmídica da porção não repetitiva da cinesina. A rKDDR recombinante tem

bom potencial antigênico e apresenta uma variedade de epítopos lineares previstos de células B em suas unidades de repetição, sendo que apresentou melhor desempenho no sorodiagnóstico da LV humana e canina em comparação ao antígeno rK39, esta contém apenas 60% de motivos repetitivos em sua estrutura (Dhom-Lemos *et al.*, 2019).

Uma segunda geração de antígeno, denominado rKDDR plus, foi sintetizado a partir da região repetitiva da sequência de nucleotídeos do gene da kinesina da *L. infantum.* Considerou a sequência de 39 aminoácidos que se repete 6,5 e 8,5 vezes nos antígenos rK39 e rKDDR, respectivamente, no entanto, retirou-se a porção de aminoácidos não repetitiva presente em ambos. Esse antígeno recombinante foi produzido pela empresa Gen Script (EUA) no plasmídeo de clonagem pUC57 e possui 10.830 pares de bases e codifica uma proteína com 3.609 aminoácidos. Essa proteína KDDR plus apresenta um valor ainda maior de motivos repetitivos em sua estrutura que a KDDR, sendo 97% (596 aminoácidos) da sequência proteica exclusivamente composta por motivos repetitivos e apenas os 3% restantes (19 aminoácidos) são originários da sequência plasmídica (Siqueira *et al.*, 2021).

Ao realizar ensaios comparativos com rKDDR-plus com rKDDR e rK39 com um banco de soros humanos e caninos, encontrou a mesma sensibilidade entre os testes, porém houve aumento da especificidade do ELISA-rKDDR-plus devido ao aumento dos motivos de repetição em *tandem*, dessa forma, reduziu o número de reações cruzadas (Siqueira *et al.*, 2021). Também se comparou ICT baseado na proteína rKDDR plus com ICT IT LEISH baseado na proteína rK39 em amostras humanas e encontrou maior sensibilidade e especificidade no teste ICT-rKKDR-plus (89,7% e 100,0%, respectivamente) do que no teste ICT IT LEISH (79,3% e 97,5%, respectivamente). Apesar da necessidade de mais estudos, o antígeno recombinante rKDDR-plus possui alto potencial no diagnóstico sorológico de LV humana e canina (Siqueira *et al.*, 2021).

Um estudo realizado por Gondim *et al.* (2022) utilizou o antígeno rKDDR plus no ICT e ELISA para diagnóstico de LV em cães de uma região de transmissão recente em Nepomuceno, município de Minas Gerais (Brasil) e o comparou com outras técnicas diagnósticas. O ICT-rKDDR-plus e ELISA-rKDDR-plus apresentaram baixa acurácia ao comparar ao padrão de referência. Esse padrão referência considerou animais verdadeiramente positivos quando apresentavam amastigotas do parasita no esfregaço de medula óssea ou presença de DNA de *Leishmania* sp. em PCR de medula óssea ou *swab* conjuntival, já os animais verdadeiramente negativos foram negativos em todos os

testes. Quando considerou a presença de sinais clínicos houve uma melhora dos resultados, do ICT-rKDDR-plus e ELISA-rKDDR-plus. A diferença do resultado encontrado com ELISA-rKDDR-plus realizado por Siqueira *et al.* (2021) foi a origem das amostras, pois utilizou-se amostras de soro de cães provenientes de um banco, no qual havia um conhecimento prévio do resultado das amostras. Nesse outro estudo não houve rastreamento das amostras e elas foram comparadas ao padrão de referência (Gondim *et al.*, 2022). A proteína rKDDR-plus é um antígeno novo e os achados nos estudos de Siqueira *et al.* (2021) e Gondim *et al.* (2022) mostram a necessidade de mais pesquisas quanto ao uso da rKDDR-plus no diagnóstico sorológico de LV.

Em suma, os ICT facilitam o acesso ao diagnóstico na rotina clínica e em inquéritos epidemiológicos, porém deve ser utilizado com cautela devido a possibilidade de resultados falsos. Para evitar esses resultados falsos, o aprimoramento de antígenos para aumentar a acurácia dos testes é fundamental, sendo que o antígeno rKKDR plus apresenta bons resultados quando comparada aos antígenos rK39 e rKDDR, porém precisa de mais pesquisas para determinar quando é mais viável seu uso (Gomes *et al.*, 2008; Siqueira *et al.*, 2021).

Atualmente, recomenda-se a combinação de diferentes métodos para obtenção de um diagnóstico preciso. Além disso, a escolha da técnica empregada é definida em função do objetivo da investigação (de Castro Ferreira *et al.*, 2007).

2.6 Medidas de controle e prevenção

O controle dessa enfermidade é mais complexo do que se imaginava porque a epidemiologia da leishmaniose envolve uma rede dinâmica de interações altamente complexas entre *Leishmania* spp., vetores flebotomíneos e hospedeiros suscetíveis. Esse protozoário pode infectar hospedeiros por acaso, por necessidade ou por ambos, sendo que, para maior parte de espécies de *Leishmania*, os humanos e cães são hospedeiros acidentais. No entanto, a crescente domesticação do ciclo de transmissão tem provocado mudanças drásticas, pois as mudanças nos padrões de transmissão ocorrem devido a adaptações a novos vetores e hospedeiros (Dantas-Torres *et al.*, 2019). Uma alternativa para medidas de controle mais seguras e direcionadas é determinar o diagnóstico com base na identificação das espécies envolvidas em cada caso e coexistentes na mesma região (Galluzzi *et al.*, 2018).

Para que a doença seja controlada é necessário o controle dos fatores ambientais, enfatizando aqueles relacionados ao inseto vetor e à população canina, principal reservatório da doença (González *et al.*, 2015). Também é importante que as medidas de controle sejam integradas com intervenções simultaneamente, desde o diagnóstico e prevenção do hospedeiro, quanto o controle dos vetores e vacinação (Quinnell; Courtenay, 2009).

Nesse contexto, a diminuição do contato de flebotomíneos com os indivíduos, bem como a redução da sua população pode ser feita por meio do uso de repelentes tópicos, de piretroides, que podem ser pulverizados nas paredes e em superfícies das residências, chamada de pulverização residual interna, em abrigos de animais ou ainda utilizados em redes de proteção, cortinas e lençóis (González *et al.*, 2015). Ademais, verificar a susceptibilidade dos flebotomíneos aos inseticidas ou a classe de inseticidas utilizados permite que a ação no vetor seja eficaz, sem aumentar a resistência a esses produtos (Delinger *et al.,* 2016).

O manejo ambiental nas proximidades das residências, melhorando as condições sanitárias, eliminando lixo, matéria orgânica, diminuindo a presença de roedores e a proximidade de outros animais que possam atrair o inseto, como galinhas, pássaros e patos, evita que o flebotomíneo tenha local para se proliferar e se alimentar (Laurenti *et al.*, 2013). Uma boa alternativa ainda é o uso de iscas atraentes açucaradas, um preparado de açúcar, ácido bórico e água que é pulverizado nas plantas, atrai os insetos e causa efeitos tóxicos nos flebotomíneos (Saghafipour *et al.*, 2017). Além disso, medidas individuais de proteção para os reservatórios como uso de coleiras com inseticidas, repelentes tópicos e vacinas são válidos para diminuir o número de infectados nessa população (Marcondes; Day, 2019).

Para uma melhor perspectiva em relação ao controle e prevenção da doença, as vacinas tem sido empregadas como método preventivo e terapêutico, sendo uma estratégia mais economicamente viável quando comparada ao tratamento com drogas convencionais. As vacinas visam fortalecer o sistema imunológico do hospedeiro, com formação de células de memória e outros componentes importantes ao combate do protozoário (Goncalves *et al.*, 2019).

A vacinação também é utilizada para a redução do número de infectados na população canina, resultando em maior resistência ao parasito (Ramer; Vanloubbeeck; Jones, 2006). Associada à imunização, o uso de coleiras impregnadas com deltametrina

4% também auxilia na diminuição de cães doentes, por evitar seu contato com o inseto vetor (Silva *et al.*, 2018). Medidas como essas são estudadas com o objetivo de serem aplicadas em outras populações animais, como em felinos domésticos, que têm se mostrado de grande importância na epidemiologia da doença ao serem indicados como potenciais reservatórios em países da Europa, Oriente Médio e Brasil (Maia; Campino, 2011).

Para que a vacina seja efetiva é necessário que estimule mecanismos imunes que consigam auxiliar na eliminação do parasito. É fundamental a produção de IL-12 por células apresentadoras de antígenos (APCs), diminuição da quantidade de IL-10 produzida por linfócitos T e APCs, indução de uma resposta forte e de longo prazo mediada por linfócitos TCD4+Th1, com altas concentrações de IL-2, IFN-γ e TNF-α e por linfócitos TCD8+, que também produzem IFN-Mγ e TNF-α, e a produção de anticorpos (Fernandes *et al*., 2012).

No ano de 2023, a única vacina em comercialização no Brasil foi suspensa por baixa eficácia de alguns lotes. Essa vacina estava disponível apenas para cães e era composta de uma forma recombinante do antígeno A2 de amastigota de *Leishmania* sp. A vacina auxiliava na diminuição da sobrevivência do parasito no hospedeiro mamífero, combinado ao adjuvante saponina, que estimula a produção de IFN-γ, com menores níveis de IL-10 e aumento das concentrações de anticorpos IgG e IgG2. No entanto, sua eficácia era de 42,86%, o que demonstra que a vacina proporciona proteção parcial dos animais (Fernandes *et al.*, 2008). Apesar dessa proteção parcial, ao reduzir a carga parasitária era possível reduzir a transmissão do parasita em áreas endêmicas por diminuir a circulação do agente (Travi *et al*., 2001).

As vacinas também podem ser utilizadas como métodos de prevenção eficazes em humanos, desde que estimulem no indivíduo o perfil de resposta Th1, com o perfil de citocinas adequado e a formação de células de memória específicas contra o protozoário (Goncalves *et al.*, 2019). Podem ser vacinas vivas atenuadas, inativadas, de subunidades e de DNA, tendo como exemplos, as compostas de promastigotas de *L. tarentolae* atenuado (Jain; Jain, 2015). Essa vacina é mais segura que outras que utiliza agentes patogênicos atenuados e outras das quais foram realizados ensaios clínicos em humanos, como a composta de antígeno de *L. major* junto à BCG e estibogluconato de sódio. Denominada Leish-F1 age contra a leishmaniose dérmica pós-kala-azar, a de antígeno de *L. major* e BCG, contra leishmaniose visceral e foi testada em crianças

menores de quinze anos de idade e que se mostrou segura e imunogênica. Essa vacina também se mostrou eficaz e segura em indivíduos saudáveis e a composta por antígenos de *L. major* apenas autoclavadas, porém, não ofereceu proteção significativa contra leishmaniose visceral (Jain; Jain, 2015). Há também uma vacina composta pelo antígeno LiESP/QA-21, que é capaz de induzir uma resposta do tipo Th1 em 3 semanas após a vacinação, com células T específicas contra o protozoário, altas concentrações de IFN-γ e outras modificações favoráveis à eliminação do parasito (Moreno *et al.*, 2012).

Ressalta-se também os programas sanitários que regulamentem e auxiliam na implementação de métodos que diminuam a incidência da leishmaniose. Como por exemplo, no Brasil existe o Programa de Vigilância e Controle de Leishmaniose Visceral (PVCLV), comandado pelo Ministério da Saúde do Governo Federal que instituiu o controle da população de vetores por meio de inseticidas, manejo ambiental para reduzir o contato com humanos, identificação e tratamento de casos humanos para evitar formas graves e morte e a identificação e eutanásia de cães soropositivos, como formas de diminuir a população de pessoas e animais infectados e, consequentemente, limitar o avanço da doença (Goncalves *et al.*, 2019).

Como forma de ações de prevenção é fundamental um diagnóstico seguro e rápido da LV. O desempenho diagnóstico de testes sorológicos baseados no antígeno rK39 na triagem da LV é diferente de acordo com região geográfica, dessa forma, é importante avaliar o desempenho de cada teste em cada país antes de escolher qual utilizar (Cunningham *et al.*, 2012). O teste rápido Kalazar Detect ™ Rapid Test apresentou um bom desempenho na Colômbia, no entanto, ao utilizar testes com antígenos de espécies circulantes no país permite melhorar o desempenho dos testes diagnósticos (Herrera *et al.*, 2019).

Além de saber se aquele animal é negativo ou positivo para LV, a quantificação da carga parasitária deve ser considerada, pois permite determinar o potencial de transmissão para flebotomíneos, pois apenas cães com alta carga de parasitas na pele são considerados infecciosos (Courtenay *et al*., 2014). Outro ponto importante ao se considerar o potencial de transmissão é a presença de sinais clínicos, como onicogrifose, lesões dermatológicas, conjuntivite, linfoadenopatia e perda de peso. Dessa forma, ao identificar esses cães com maior risco de transmissão é possível contribuir para o controle da transmissão da LV em áreas endêmicas (Verçosa *et al.*, 2008)

2.7 Leishmaniose Visceral Canina

Os reservatórios favorecem a manutenção e disseminação do parasito, sendo que os cães, *Canis familiaris*, devido ao contato íntimo como os humanos, considerados os reservatórios principais de *L. infantum* em especial, os cães sintomáticos (Dantas-Torres *et al.*, 2019). No entanto, existe um número relevante de animais assintomáticos, principalmente em regiões endêmicas, que também são importantes na manutenção do ciclo do parasito (Moshfe *et al.*, 2009).

Com base no contexto epidemiológico, o cão pode ser reservatório primário de *L. infantum*, mas também pode atuar como hospedeiro acidental de *L. braziliensis* e *L. amazonensis.* Porém, o papel do cão na transmissão de leishmaniose cutânea (LC) ainda não é totalmente compreendido. Dessa forma, a tipagem da espécie de *Leishmania* é importante para definir a apresentação clínica e os ciclos de transmissão que dependem, em sua maior parte, do agente envolvido e, consequentemente, determinar um diagnóstico adequado, além de entender o contexto epidemiológico e determinar medidas de controle (Barroso *et al.*, 2015; Souza *et al.*, 2019).

No estudo de Lago *et al.* (2019), na vila de Corte de Pedra na Bahia (Brasil), uma área altamente endêmica para *L. braziliensi*s, os cães apresentam lesões ulceradas típicas de LC, com a presença do protozoário e exames diagnósticos positivos para a enfermidade. Também foram encontradas semelhanças clínicas e histológicas entre a LC canina e humana, porém os cães permanecem com a doença por mais tempo. Mais estudos são necessários para caracterizar a importância desses animais na transmissão de *L. braziliensis*, porém a ocorrência de casos humanos e cães com sinais clínicos de LC na mesma casa e as semelhanças entre os parasitas isolados entre essas espécies indicam fortemente a possibilidade da participação dos cães nessa transmissão.

A leishmaniose em cães pode provocar sinais clínicos severos, caracterizados por reação inflamatória piogranulomatosa, linfoadenopatia, diminuição do apetite, letargia, alopecia, dermatite papular e nodular, onicogrifose, epistaxe, diarreia, ceratoconjuntivite, blefarite, uveíte, nefrite e glomerulonefrite, evoluindo para falência renal crônica, entre outros sinais. No entanto, a maioria dos animais são assintomáticos, em que a prevalência da doença em áreas endêmicas é baixa, apesar da porcentagem de cães infectados pode chegar a 60% dependendo dos métodos de diagnóstico, idade,

comprimento do pelo, resistência genética, aspectos sociais, econômicos, políticos e demográficos do local (Reguera *et al*., 2016).

Devido ao papel do cão como reservatório principal no ambiente urbano do agente da LV, o diagnóstico preciso para detectar cães positivos é uma medida importante para estratégias de prevenção e controle da doença. Existe o desafio em reduzir o número de cães participantes no ciclo biológico da LV e atualmente, há técnicas parasitológicas que possuem especificidade reduzida, ou seja, o resultado é confiável quando possui resultado positivo, no qual geralmente há visualização direta do parasita. Há também técnicas moleculares, como a PCR e *q*PCR, que fornece uma acurácia no diagnóstico, porém tem limitada aplicação em grande escala devido aos seus custos e aplicabilidade (de Castro Ferreira *et al.*, 2007). Os testes sorológicos são importante ferramentas no diagnóstico da LV devido a enfermidade provocar a produção elevada de anticorpos específicos contra a *Leishmania* spp. e a estimulação de células B policlonais nos hospedeiros e pela sua aplicabilidade como testes de triagem e inquéritos epidemiológicos (de Castro Ferreira *et al.*, 2007; Gomes *et al.*, 2008)

Os testes sorológicos buscam anticorpos que são produzidos em maiores quantidades quando a doença está ativa e em menores concentrações em assintomáticos ou cães expostos, mas não infectados. Exemplos de testes sorológicos são o teste de aglutinação direta (DAT), que tem sensibilidade de 91 a 100% e especificidade de 72 a 100%, o teste de imunofluorescência (IFAT), com sensibilidade e especificidade de 100% em animais sintomáticos e com menor sensibilidade em detectar assintomáticos quando comparado ao ELISA. O ELISA possui alta sensibilidade e especificidade e é considerado padrão ouro para o diagnóstico de leishmaniose (Travi *et al*., 2018). Existe também o ICT, que é um teste qualitativo que geralmente baseia-se na detecção de anticorpos em soro, plasma, sangue ou urina, através de um antígeno recombinante na fita de teste (Farahmand; Nahrevanian, 2016). Este é um método eficiente e de baixo custo para monitorar e controlar a leishmaniose, pois é simples de realizar em campo e não requer técnicos treinados (Dhom-Lemos *et al.*, 2019). Atualmente existem kits comerciais de TRI disponíveis para o diagnóstico de LVC, como o KALA-AZAR DETECT® (InBios International), teste de escolha do Ministério da Saúde do Brasil para triagem em inquéritos epidemiológicos, IT-LEISH® (Bio-Rad Laboratories), AlereTM Leishmaniose Ac Test Kit (AlereTM), entre outros (Assis *et al.*, 2008; Brasil, 2014b; Carvalho *et al*., 2003). No Brasil, o ICT baseado na proteína rK28 é

recomendado pelo Ministério da Saúde do Governo Federal como teste rápido para a detecção de leishmaniose canina, sendo necessário que se faça o ELISA como confirmatório em animais positivos no teste rápido (Borja *et al.*, 2018).

Percebe-se que quando há uma guarda responsável de cães existe menor chance desses animais terem a infecção, pois têm maior adesão as estratégias de saúde pública que minimizam o contato com os flebotomíneos, como o uso de coleiras repelentes. Além disso, ao adquirir um cão adulto é importante submetê-lo a avaliação clínica completa, incluindo exames para verificar a LVC, pois a aquisição de cães adultos é considerado um fator de risco porque geralmente não sabe-se o histórico daquele animal (Soares *et al.*, 2022).

Após confirmado o diagnóstico, é necessário que se estabeleça se o cão será eutanasiado ou tratado. Quando se opta pelo tratamento, deve-se analisar qual a melhor conduta acerca do cão infectado. O tratamento da leishmaniose visceral canina geralmente pode ser realizado com antimoniato de meglumina, aminosidina e miltefosina, geralmente combinados ao alopurinol. Porém, não se obtém a cura parasitológica, além dos extensos efeitos colaterais: vômito, diarreia, letargia, nefrotoxicidade, além da urolitíase de xantina, causada pelo alopurinol quando utilizado por longos períodos (Pineda *et al.*, 2017).

O tratamento da leishmaniose visa a diminuição dos sinais clínicos e da carga parasitária no organismo, restaurar a eficiência do sistema imune e evitar recaídas, porém, por ser uma doença crônica, se estende por longos períodos. Além disso, possui alto custo, alta toxicidade e outros efeitos indesejados (Goncalves *et al.*, 2019; Torres *et al.*, 2011). Deve considerar a escolha da terapia conforme espécie alvo e a legislação do país. O tratamento pode ser feito com antimoniatos pentavalentes, que tem eficácia clínica de 35 a 95%, dependendo da localização geográfica, com alta toxicidade para órgãos como coração, rins e fígado. Também pode utilizar a anfotericina B, com toxicidade principalmente renal e efetividade de mais de 97%, anfotericina B lipossomal, menos tóxica e com boa efetividade. Há também o uso de miltefosina que possui efeitos colaterais no trato gastrintestinal e toxicidade em rins e fígado e potencialmente teratogênica. Já o sulfato de paromomicina, possui boa eficácia e toxicidade incomum. Há também o uso de outras drogas e terapias combinadas, com intuito de melhor a eficácia e a segurança dos medicamentos (van Griensven; Diro, 2012).

No Brasil, para uso autorizado em cães, é liberado apenas o princípio ativo miltefosina pela portaria interministerial N° 1.1426, de 11 de julho de 2008, apesar de existirem outras drogas com efeitos mais satisfatórios por serem utilizadas nas terapias para leishmaniose humana (Brasil, 2016). Antes da liberação da miltefosina em 2016, a única opção legal para cães positivos era a eutanásia, o que gerava descontentamento da população e muitas vezes não havia adesão a prática (Brasil, 2014b, 2016). Em março de 2018, houve um fórum mundial realizado no Reino Unido para discutir o controle da leishmaniose canina no contexto de Saúde Única, no qual declara que o abate de cães infectados, clinicamente doentes ou assintomáticos, como uma medida de controle não reduz a incidência de LV em humanos, uma vez que existem hospedeiros reservatórios alternativos que desempenham papel na manutenção do ciclo da *L. infantum*. Além disso, geralmente, há uma substituição rápida por cães jovens que costumam ser mais susceptíveis a infecção primária e pode gerar danos psicológicos aos tutores. Também existem questões jurídicas, já que os animais são reconhecidos por lei como seres sencientes e por isso, vai contra os direitos dos mesmos em receber os cuidados necessários ao seu bem-estar (Dantas-Torres et al., 2019; Machado; Silva; Vilani, 2016). O abate de cães não é considerada um alternativa válida e econômica para instituições governamentais, sendo que o controle eficaz de *Leishmania* sp. precisa de abordagens integradas com ações no parasita e no vetor, não apenas no cão (Dantas-Torres *et al.*, 2019).

Porém, diferente do Brasil, a Colômbia tem o abate de cães ainda como única medida recomendada oficialmente. Apesar dessa medida não ter efetividade comprovada, a questão mais importante é o que fazer com os cães sem donos com leishmaniose que possuem importante papel como reservatório de *Leishmania* spp. Na Itália, o governo custeia o tratamento desses animais, medida viável em países desenvolvidos. Dessa forma, há necessidade de investimento em medidas preventivas no cão e no controle vetorial com vacinação e piretróides tópicos. Além disso, medidas que visam melhorar a qualidade de vida da sociedade, como nutrição, moradia e saneamento básico (Dantas-Torres *et al.*, 2019).

Como método de prevenção da leishmaniose visceral canina, é necessário diminuir o contato dos animais com o inseto vetor. Isso é possível por meio de manejo ambiental, com barreiras físicas como redes de malha fina em janelas e canis, evitar a exposição ao crepúsculo, limpeza de locais com acúmulo de matéria orgânica e uso de

medidas individuais de proteção, que incluem repelentes à base de piretroides sintéticos e outros, coleiras impregnadas de deltametrina (Ribeiro *et al.*, 2018).

As vacinas também são um método promissor, que podem ser utilizadas como medidas individuais de proteção contra a leishmaniose e também como terapia. Elas tornam possível que, por meio do *status* da imunidade de rebanho, haja uma diminuição considerável da população de canina infectada, afetando também o número de casos humanos da doença (Ramer; Vanloubbeeck; Jones, 2006).

2.8 Leishmaniose Visceral Felina

A infecção em gatos (*Felis catus*) por *Leishmania* spp. foi descrita em países como Itália, França, Espanha, Portugal, Grécia, Turquia, Chipre, Irã e Brasil, levantando a suspeita de que os felinos pudessem ter uma importância maior na epidemiologia da leishmaniose (Pennisi; Persichetti, 2018). Em áreas endêmicas para LVC, os felinos podem ser infectados com *Leishmania* spp. e atuarem como reservatórios secundários (Pennisi *et al.*, 2015). A leishmaniose felina têm sido cada vez mais relada em áreas endêmicas e possui muitas semelhanças com a LVC, porém ainda são necessários mais estudos para compreender as diferenças entre elas (Pennisi; Persichetti, 2018).

Os gatos podem ser infectados pelas espécies *L. mexicana, L. venezuelensis, L. braziliensis, L. amazonensis* e *L. infantum.* No entanto, a prevalência da infecção em áreas endêmicas é menor quando comparada aos cães, persistindo por um maior período de tempo de forma mais controlada (Pennisi *et al.*, 2015). A presença de gatos infectados em áreas endêmicas não deve ser desprezada, pois eles participam no ciclo biológico do patógeno (Metzdorf *et al.*, 2017).

Quando infectados por *L. infantum,* os gatos podem ser assintomáticos ou desenvolver um quadro crônico da doença, apresentando as formas infectantes para o inseto vetor em seu sangue periférico. O felino que apresenta a enfermidade, não tem boas chances de recuperação com a terapia convencional e, por essas características e por sua proximidade com humanos em áreas endêmicas, os felinos domésticos foram considerados reservatórios secundários do parasito. Esses animais contribuem ativamente para a manutenção do ciclo da leishmaniose, desde que no local também esteja presente um reservatório primário, como os cães (Coelho *et al.*, 2016).

Ainda não foi comprovado se os gatos atuam como hospedeiro reservatório como os cães. Porém, os gatos podem ser considerados hospedeiros sentinelas por abrigar o parasita com ou sem manifestações clínicas. Dessa forma, os gatos mostram situações de circulação endêmica de *L. infantum* e também podem servir como fonte de alimentação para os vetores mantendo-os presente na região. Isso mostra a necessidade de felinos serem incluídos em levantamentos epidemiológicos de leishmaniose em áreas endêmicas (Rocha *et al.*, 2019).

Os felinos podem ou não apresentar sinais clínicos. Essa manifestação clínica variável sugere que as lesões ocorrem antes da produção de anticorpos, geralmente agravada quando há coinfecções, como o vírus da imunodeficiência felina (FIV). Os sinais clínicos incluem lesões cutâneas nodulares, crostosas ou escamosas, que podem evoluir para úlceras, principalmente em cabeça, pescoço e com menor frequência em membros. A histopatologia das lesões cutâneas é caracterizada por uma dermatite granulomatosa difusa com macrófagos infectados pelas formas amastigotas do protozoário. Há também sinais não cutâneos, como aumento do volume de linfonodos, lesões oculares, gengivoestomatite, perda de apetite, hiperproteinemia, hipergamaglobulinemia, aumento de enzimas hepáticas, entre outros (Brianti *et al.*, 2019).

Quando infectados, os felinos desenvolvem a imunidade mediada por células com ativação dos macrófagos para destruição das formas amastigotas, diferente da resposta imune observada em cães infectados por *L. infantum*. Dessa forma, mesmo quando possuem altos títulos de anticorpos, não há imunidade protetora contra a doença e possuem menor positividade na PCR. Além disso, os métodos sorológicos convencionais que mostram a infecção ativa não são confiáveis em felinos com leishmaniose (Coelho *et al.*, 2016; Costa *et al.*, 2010).

Para diagnosticar a doença em gatos é comum que se utilize IFAT, ELISA, PCR e pesquisa direta de parasitos nos tecidos como métodos principais, já que são os mais utilizados para a detecção da leishmaniose canina. Para esse diagnóstico de leishmaniose felina deve considerar mais de um método de diagnóstico junto com os sinais clínicos, uma vez que baixos títulos de anticorpos ou soronegativamente podem ser resultado da resposta imune celular, que é predominante em felinos (Coelho *et al.*, 2016). Portanto, o diagnóstico de leishmaniose felina não deve ser feito apenas com base em achados sorológicos principalmente, em áreas endêmicas (Coura *et al.*, 2018).

No entanto, a técnica de Western Blot com a fração da enzima ferro-superóxido dismutase (Fe-SOD) é promissora, por possuir boa sensibilidade e especificidade (Trevisan; Lonardoni; Demarchi, 2015).

O exame parasitológico continua sendo um método simples de determinar a enfermidade e pode ser realizado através da citologia, biópsia de lesões cutâneas, linfonodo ou medula, imuno-histoquímica ou cultivo. A citologia por aspiração do linfonodo poplíteo demonstrou ser mais sensível que a citologia de outros órgãos, como medula óssea, baço e fígado (Coelho *et al.*, 2016). Ademais, não há um consenso no órgão ideal para realização da citologia ou diagnóstico molecular, alguns autores sugerem que seja realizado no linfonodo ou na pele (Coura *et al.*, 2018). O cultivo de promastigotas de *Leishmania* spp. é um exame demorado e com baixa sensibilidade. Porém, pouco se sabe sobre a carga parasitária em gatos, dessa forma, é fundamental a associação com mais testes específicos e sensíveis, como a PCR de medula óssea ou linfonodo (Coelho *et al.*, 2016).

Como ocorre em cães, comumente encontram-se gatos soropositivos com PCR de amostras de sangue negativo, isso pode ocorrer devido à ausência de parasitemia ou baixa carga parasitária no momento da coleta do sangue e pela capacidade do protozoário em parasitar medula óssea, linfonodos e baço (Bezerra *et al.*, 2019). Dessa forma, o que ocorre é que gatos infectados produzem anticorpos contra *Leishmania* spp. e podem apresentar PCR negativo quando os parasitos não estão hospedados nos locais do corpo no qual a PCR foi realizada (Coura *et al.*, 2018). Além disso, existe a possibilidade de reações cruzadas com anticorpos de outros parasitos, como outras espécies de *Leishmania* e *Trypanosoma* (Bezerra *et al.*, 2019).

Portanto, como em felinos com leishmaniose não sabe-se se os anticorpos são protetores ou se a resposta imonológica celular é eficaz para controle da infecção, é importante que os métodos diagnóstico em felinos sejam padronizados para própria espécie e também investigar o papel desses animais como reservatório de *Leishmania* spp. (Coura *et al.*, 2018). A PCR quando combinada a outros testes diagnósticos, a confirmação clínica e o animal ser de área endêmica, torna-se mais eficaz (Martin *et al.*, 2017).

O tratamento para leishmaniose felina ainda está sendo estudado, mas a utilização de alopurinol na dose de 10 a 20 mg/kg a cada 12 ou 24 horas por um longo período de tempo tem sido efetiva em animais com coinfecção por FIV. Apesar disso, a

infecção não é eliminada por completo e pode ocorrera remissão dos sinais clínicos após o término da terapia. Outro fármaco que também pode ser utilizado em conjunto com a terapia do alopurinol é o antimoniato de meglumina (Pennisi *et al.*, 2013). Além disso, é fundamental o tratamento suporte em pacientes com insuficiência hepática e doença renal crônica causada pela própria infecção e também pelos medicamentos (Coelho *et al.*, 2016)

As medidas preventivas em felinos também são o principal objetivo (Coelho *et al.*, 2016), a partir de inseticidas tópicos que previnem a picada do flebótomo, como os colares com 10% de imidacloprida e 4,5% de flumetrina, que se mostraram efetivos na proteção dos animais, apesar de poderem causar reações adversas na pele (Brianti *et al.*, 2017). Também existem no mercado os repelentes tópicos e inseticidas que não possuem base de permetrina e deltametrina, que são tóxicos à espécie (Pennisi *et al.*, 2015). As mesmas medidas de manejo ambiental e barreiras físicas recomendadas para cães, nas quais impeçam o contato da fêmea do flebotomíneo com os felinos também são válidas como métodos de proteção (Ribeiro *et al.*, 2018).

3. CONSIDERAÇÕES FINAIS

A LV é uma doença negligenciada em que o diagnóstico dessa enfermidade, apesar de muito estudado, apresenta significativos desafios devido a presença de animais assintomáticos e com reação cruzada. Além disso, muitas vezes essa enfermidade é subnotificada e não faz parte de um diagnóstico de rotina nos países em que a epidemiologia não está bem definida, como é o caso da Colômbia. Portanto, mais investimentos e continuar os estudos se fazem necessários para construir uma nova realidade nas áreas atingidas, com menos sofrimento, consequências e melhor qualidade de vida.

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SEGUNDA PARTE – ARTIGOS*

*****_Preparado de acordo com os padrões das revistas_

ARTIGO 1

This paper has been prepared in accordance with the guidelines of the journal *Preventive Medicine Veterinary* (JCR 2.6), to which it has been submitted. The journal's editorial board may suggest changes to suit its style.

Accuracy of serological, parasitological and molecular tests for canine visceral leishmaniasis: A systematic review and meta-analysis

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Abstract

The objective of this systematic review and meta-analysis was to recalculate the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of diagnostic tests for canine visceral leishmaniasis (CVL). The protocol for this systematic review was registered in PROSPERO (CRD42022322495). The CABI, Cochrane, PubMed, Scielo, Scopus and Web of Science databases were searched, and 14,034 articles were retrieved. Based on previously established inclusion and exclusion criteria, 67 articles were ultimately included in the systematic review, and 63 articles were included in the meta-analysis. Across these 67 studies, there were 402 trials, of which 86.07% (346/402) were indirect diagnostic tests and 13.93% (56/402) were direct diagnostic tests. For the

meta-analysis, only indirect diagnostic tests were considered (46 studies and 143 tests), as it was necessary to have a quality reference standard and to have more than one test using the same antigen. Thus, 18 different serological techniques were grouped and used in the calculation of DSe and DSp. For the DSe meta-analysis, the clinical condition of the animals was considered in the calculation. Therefore, the average DSe when the clinical condition was not reported was 98.10%; for symptomatic animals, the average DSe was 90.80%; for symptomatic animals plus asymptomatic animals, the average DSe was 89.10%; and for asymptomatic animals, the average DSe was 79.00%. The DSe values were overestimated when asymptomatic animals were not used. For the DSp meta-analysis, the categories were based on cross-reaction. The average DSp when using negative animals was 98.10%; for negative animals plus cross-reaction, the average DSp was 83.40%; and for cross-reactive animals, the average DSp was 64. 30%. DSp values were also overestimated when animals with cross-reactivity were not added. When calculating DSe and DSp with each test and its antigen, it was found that the order of DSe and DSp values was the same regardless of the category used (clinical condition and cross-reaction). Furthermore, the DAT (direct agglutination test) with *L. donovani* crude antigen presented the best results for both DSe [0.9942 (95% CI: 0.9892-0.9969)] and DSp [0.9796 (95% CI: 0.9492-0.9920)], with DSe values decreasing as asymptomatic animals were added and DSp values decreasing as cross-reactive animals were added. The results of the meta-analysis revealed that the DSe and DSp values of the 18 serological techniques were overestimated according to the clinical condition and the presence/absence of cross-reactive animals, respectively. In short, serological tests are the most commonly used, and the DAT-*L. donovani* test presented the best DSe and DSp results.

Keywords: Sensitivity; Specificity; *Leishmania* spp.; Diagnosis; Validation; Indirect methods; Direct methods.

1. Introduction

Leishmaniasis is a neglected parasitic disease and is considered a global health problem, as more than 1 billion people live in an endemic area (WHO, 2006). One form of leishmaniasis is visceral leishmaniasis (VL). In South America, Central America, Central Asia, the Middle East and the Mediterranean Basin, VL is caused by the protozoan *Leishmania* spp.; in Africa and the Mediterranean, VL is caused by the

species *Leishmania infantum*; and in other areas of Asia, VL is caused by the species *Leishmania donovani* (Alvar et al., 2012). The disease is transmitted by the bite of sandflies of the *Lutzomia* sp., with dogs being the main reservoirs of the disease; thus, canine infections usually occur prior to human infections (WHO, 2006). When this disease is not properly treated, it can be fatal, leading to serious clinical changes and putting human health at risk (Dantas-Torres et al., 2019).

As dogs play an important role in maintaining the urban cycle of the protozoan, it is important to monitor and control cases of canine visceral leishmaniasis (CVL), which is possible through an accurate diagnosis (WHO, 2006). In some countries, euthanasia of CVL-positive dogs is considered a preventive measure, but its effectiveness is very questionable due to several factors – for example, although there are many techniques for the diagnosis of leishmaniasis, the serological techniques that are used in these control programs have limited accuracy (Dantas-Torres et al., 2019).

Thus, the search for a reliable method for diagnosing CVL is essential for avoiding unnecessary euthanasia of dogs and controlling or eradicating this (Fraga et al., 2016; Dantas-Torres et al., 2019). CVL can be diagnosed via serological, parasitological, or molecular methods (Maurelli et al., 2020). The optimal technique should be selected based on the following factors: the epidemiology of the disease in the population; the presence or absence of clinical signs; the diagnostic sensitivity (DSe), which is the ability of the test to correctly identify an animal with the disease; and the specificity (DSp) of the test, which is the ability of the test to correctly identify animals without the disease (Morales-Yuste et al., 2012; Pessoa et al., 2019). Despite the wide variety of tests available for the diagnosis of VL, no test is considered to have an accuracy of 100%.

For a more accurate diagnosis of CVL, it is usually necessary to use more than one diagnostic technique (Maurelli et al., 2020). In addition, it is necessary to use techniques with high DSe and DSp. However, the use of an unreliable reference standard leads to nonreal DSe and DSp values, thus enabling dogs with false-negative results to maintain the protozoan in the environment and leading to the unnecessary euthanasia of dogs with false-positive results (Fraga et al., 2016). In this context, the objective of this systematic review was to evaluate serological, parasitological, and molecular tests for the diagnosis of CVL. Furthermore, meta-analysis was performed to

recalculate the accuracy (i.e., DSe and DSp) of serological tests for diagnosing CVL worldwide to identify the most reliable tests for diagnosing the disease.

2. Material and methods

This review was performed in accordance with the Preferred Reported Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021), as shown in Supplementary Table S1. In addition, the systematic review was registered in PROSPERO under number CRD42022322495.

2.1 Strategy of search

The systematic review was started on September 9, 2020. Initially, the population, intervention, comparison and outcome (PICO) were defined as follows: canine population (population); diagnosis of visceral leishmaniasis (intervention); serological, parasitological and molecular tests (comparison); and validation, sensitivity, specificity, optimization and evaluation (outcomes). There were no restrictions on when or where the studies were published. The selected keywords were searched in all sections of the articles (title, abstract and full texts) in the following databases: CABI, Cochrane, PubMed, Scielo, Scopus and Web of Science (Supplementary Table S2).

2.2 Selection of the studies

In the first step, the studies were added to the reference manager software databases (EndNoteTM, ClarivateTM, USA). Then, the studies were screened based on their titles by two reviewers (TFM and RSA). Subsequently, two reviewers (TFM and JAMP) independently evaluated the abstracts of potentially eligible studies. Then, the same authors evaluated the full texts of the potentially eligible studies based on the inclusion and exclusion criteria. Disagreements between the two authors were resolved by consulting a third author (EMSD).

2.3 Inclusion and exclusion criteria

This review included articles that evaluated the DSe and DSp of index tests designed to diagnose CVL by means of serological or direct tests. The exclusion criteria were as follows: (i) articles that did not assess DSe and DSp; (ii) articles that focused on

evaluating the response to vaccination; (iii) articles that focused on diseases other than CVL or animal species other than dog; and (iv) studies written in languages other than English, Spanish, French or Portuguese. The detailed inclusion and exclusion criteria used in this review are provided in Supplementary Table S3.

2.4 Types of studies

Case series reports, case reports, literature reviews, conference proceedings, book chapters and books were excluded from this systematic review and meta-analysis. Original articles with a cohort, case–control and cross-sectional study design were included.

2.5 Quality assessment

After selecting the studies based on previously defined criteria, we evaluated their quality. First, the included studies were categorized according to the statistical approach used to calculate the accuracy (DSe and DSp) of the diagnostic test, i.e., frequentist or Bayesian inference. Then, studies that used the frequentist statistics were evaluated for quality based on the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool (Whiting et al., 2011) using RevMan 5.4 software (Review Manager 5.4, Cochrane, USA). The QUADAS-2 tool verifies the diagnostic accuracy of the tests because it leads to transparency of bias and applicability of primary studies of diagnostic accuracy. This tool assesses quality across four domains: patient selection, index test, reference standard, and flow and timing. The first three domains were also evaluated for their applicability (Whiting et al., 2011). As shown in Supplementary Table S4, the QUADAS-2 criteria were adjusted according to the needs of this systematic review. Studies that used Bayesian statistics were also assessed using the QUADAS tool. The assessment of study quality was carried out jointly by two reviewers (TFM and RSA), and disagreements were resolved by consulting a third reviewer (EMSD). For the quality criteria evaluated, the use of a reference standard considered to be adequate by the authors was essential for classifying the study as a high-quality study. Negative samples should have at least two different negative tests to be considered an adequate reference standard. However, truly negative samples were those with cross-reacting animals, even when tests for the diagnosis of CVL were not

156 performed. On the other hand, positive samples needed to be positive in a direct test or
157 in two different serological tests to be considered an adequate reference standard.

158 *2.6 Data extraction*

159 Data were extracted from the articles by one of the reviewers (TFM) and later
160 checked for accuracy by another reviewer (RSA). Disagreements between reviewers
161 were resolved by consulting a third reviewer (EMSD). The following data were
162 extracted: first author; geographic location where the study was performed; study
163 period; statistical model; index test(s); antigen(s); type(s) of antigen(s); strain(s) used in
164 antigen(s); cut-off point(s); classification of clinical signs; use of animals to assess
165 cross-reactivity; use of animals vaccinated with leishmaniasis; negative and positive
166 reference standard (gold standard) (for studies that used the frequentist approach);
167 number of true positives (TP), true negatives (TN), false positives (FP) and false
168 negatives (FN); and DSe and DSp values. The determination of whether asymptomatic
169 animals were used was based on the description of the clinical characteristics of the
170 population under study.

171 *2.7 Meta-analysis*

172 To estimate DSe and DSp in the meta-analysis, the TP, TN, FP and FN values
173 were considered for each index test with the antigen used (Dohoo et al., 2012). When
174 evaluating the introduction of bias in the estimation of DSe and DSp, symptomatology
175 and cross-reactivity were considered, respectively, to avoid overestimation of these
176 values. Thus, when estimating DSe, the tests were divided as follows: if they did not
177 report the clinical signs of the animals (NI), presence of symptomatic animals (Symp),
178 presence of asymptomatic animals and symptomatic animals (Asymp + Symp) or
179 presence of asymptomatic animals (Asymp) (Supplementary Table S8). To estimate
180 DSp, the tests were divided into negative animals without cross-reaction (Neg), animals
181 with cross-reaction plus negative animals without cross-reaction (CR+ Neg) or animals
182 with cross-reaction (CR) (Supplementary Table S9).

183 The generalized linear mixing statistical model (binomial with logit link) was
184 used to estimate DSe and DSp in the meta-analysis, where DSe is calculated as
185 TP/(TP+FN), DSp is calculated as TN/(TN+FP) and precision is calculated as
186 (DSe+DSp)/2 (Chu and Cole, 2006; Dohoo et al., 2012). The linear predictor depended

on the fixed effects for the study design (presence/absence of clinical signs and presence/absence of cross-reacting animals) and the random effects for the article and index tests. All adjustments were made using the Ime4 library, and confidence intervals (CIs) were calculated using the emmeans library in the statistical software R version 4.0 (Team, 2021)

3. Results

3.1 Selected studies

The literature search yielded a total of 14,034 articles. A total of 187 duplicates were identified. After screening the titles, 12,878 articles were excluded. Among the 969 articles that underwent abstract screening, 298 articles were excluded. Thus, 671 articles remained for full-text screening. Twenty articles could not be retrieved, and 422 were excluded. Thus, 229 full-text articles remained for eligibility assessment. The quality of these 229 articles was evaluated using the QUADAS-2 tool, leading to a total of 67 articles being included in the qualitative analysis (systematic review) and 63 articles being included in the quantitative synthesis (meta-analysis) (Figure 1). Four articles were excluded from the meta-analysis. It was not possible to extract the data from two articles that used frequentist statistics, written by Dye et al. (1993) and Maurelli et al. (2020) Additionally, two articles that used Bayesian statistics, written by Adel et al. (2015) and Adel et al. (2010) were excluded because they did not determine the mean DSe and DSp of the groups. The main reasons for excluding the 162 articles in the quality assessment are detailed in Supplementary Table S5 and Supplementary Figure S1.

PRISMA 2020 Flow Diagram

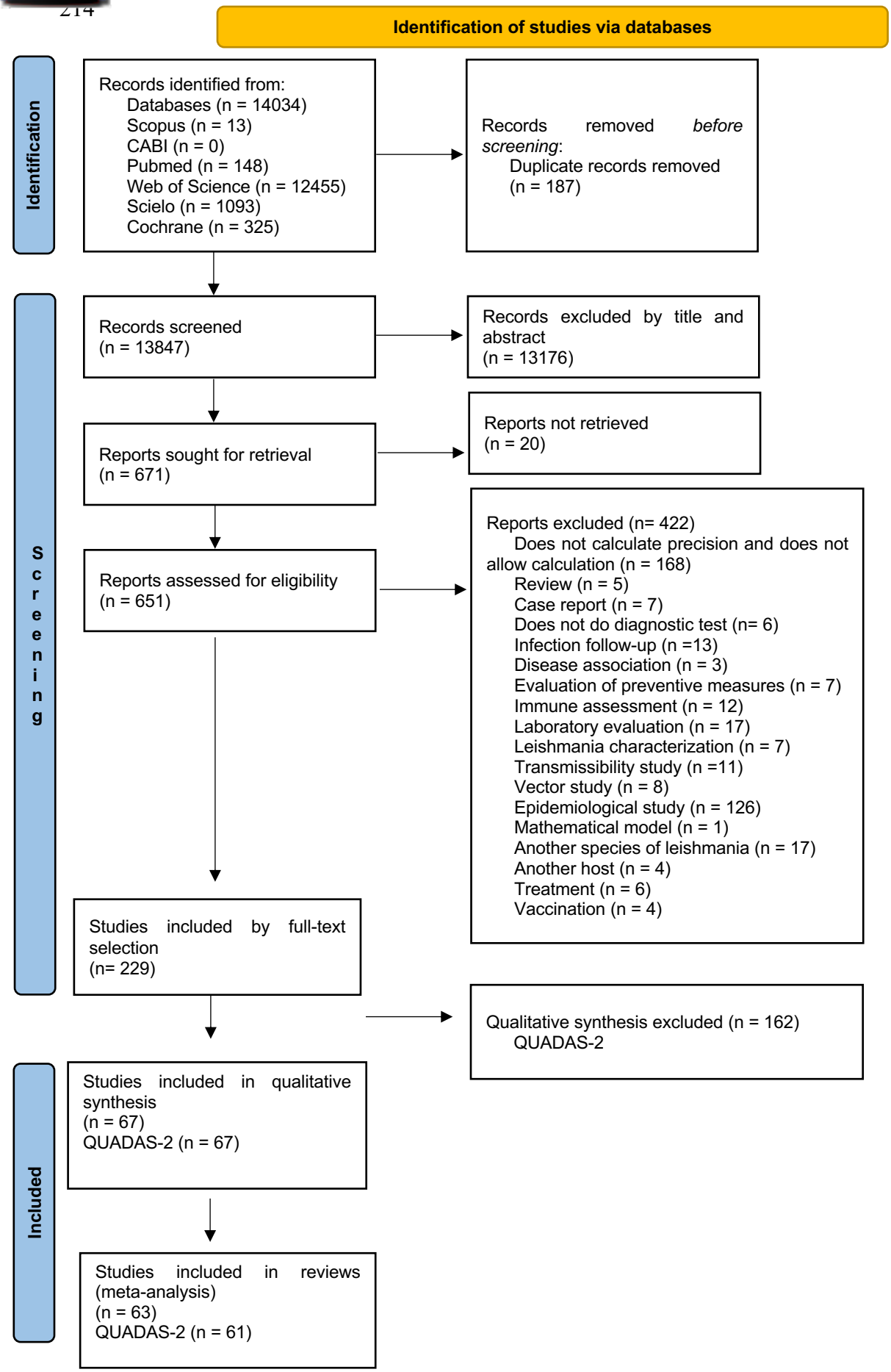
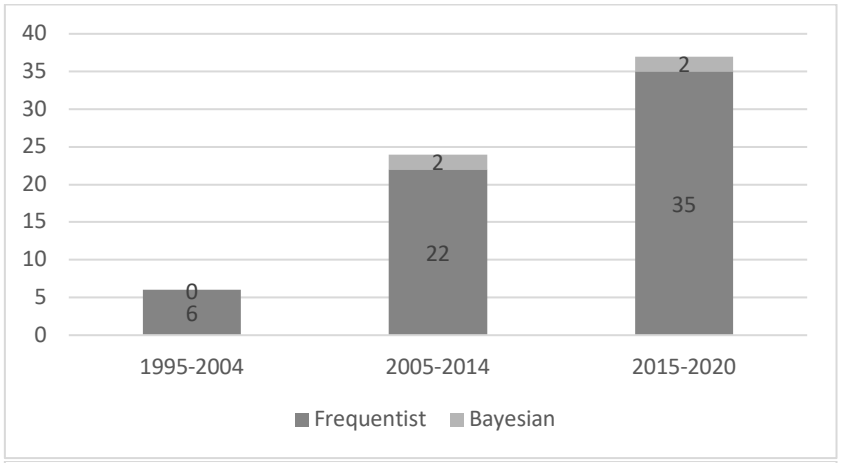


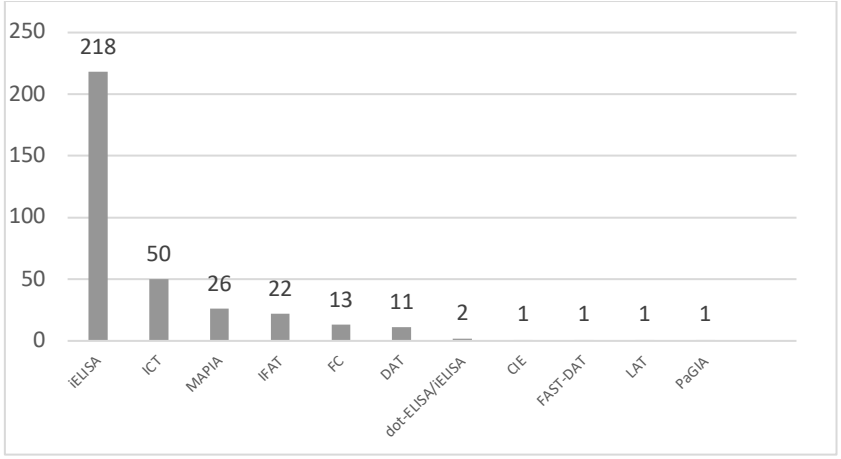
Figure 1: PRISMA flowchart used in the selection of the studies for this systematic review and meta-analysis

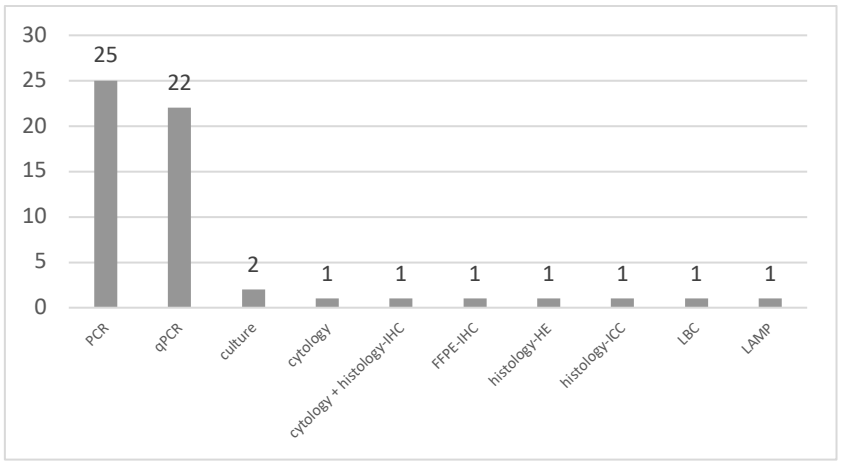
Several diagnostic tests can be evaluated in one study. Therefore, each article was designated as a study, and each diagnostic test (referred to as an index test) was designated as a trial. A total of 67 studies and 402 assays were evaluated. The 67 articles selected for systematic review were published between 1995 and 2020, with 8.96% (6/67) of the articles published between 1995 and 2004 (6 frequentists), 35.82% (24/67) of the articles published between 2005 and 2014 (22 frequentists and 2 Bayesians) and 55.22% (37/67) of the articles published between 2015 and 2020 (35 frequentists and 2 Bayesians) (Figure 2A). Data from the main articles, such as the index tests, the reference standards adopted, the country where the study was performed, the year when it was carried out and the number of evaluated tests, are shown in Supplementary Table S6.

A)



B)





C)

Figure 2. Characteristics of the 67 studies and 402 trials included in the systematic review. A) Distribution of selected studies according to the statistical methodology used and decade of publication. B) Distribution of 346 trials among the 11 different indirect tests for the diagnosis of canine visceral leishmaniasis included in the systematic review. C) Distribution of 56 trials among the 10 different direct tests for the diagnosis of canine visceral leishmaniasis included in the systematic review.

3.2 Evaluated tests and antigens

Of the 402 assays evaluated, 86.07% (346/402) were indirect diagnostic tests, and 13.93% (56/402) were direct diagnostic tests. Among the indirect tests, 63.01% (218/346) were iELISA (indirect enzyme-linked immunosorbent assay), 14.45% (50/346) were immunochromatographic tests (ICTs), 7.51% (26/356) were multiantigen print immunoassay (MAPIA), 6.36% (22/346) were indirect immunofluorescence assay (IFAT), 3.76% (13/346) were flow cytometry (FC), 3.18% (11/343) were direct agglutination tests (DATs), 0.58% (2/346) were dot enzyme-linked immunosorbent assay (dot-ELISA/iELISA), 0.29% (1/346) were counterimmunoelectrophoresis (CIE), 0.29% (1/346) were fast agglutination screening tests (FAST-DATs), 0.29% (1/346) were latex agglutination tests (LATs) and 0.29% (1/346) were particle gel immunoassay (PaGIA). All indirect tests were serological (Figure 2B).

Regarding direct tests, 85.71% (48/56) were molecular techniques, and 14.29% (8/56) were parasitological techniques. Among the molecular techniques, 44.64% (25/56) were conventional polymerase chain reaction (PCR), 39.29% (22/56) were real-time polymerase chain reaction (qPCR), 3.57% (2/56) were culture, 1.49% (1/56) were cytology, 1.49% (1/56) were cytology plus cell block histology combined with immunocytochemistry, 1.49% (1/56) were fixed in formalin and embedded in paraffin wax with immunohistochemistry (FFPE-IHC), 1.49% (1/56) were cell block histology

306 with hematoxylin and eosin (histology-HE), 1.49% (1/56) were histology of cell block
307 combined with immunocytochemistry (histology-ICC), 1.49% (1/56) were liquid-based
308 cytology (LBC) and 1.49% (1/56) were loop-mediated isothermal amplification
309 (LAMP) (Figure 2C).

310 Among the biological samples used in the indirect tests, 97.40% (337/346) were
311 serum, 2.02% (7/346) were blood, 0.29% (1/346) were plasma and 0.29% (1/346) were
312 serum or plasma. Among direct assays, 23.21% (13/56) used popliteal lymph nodes,
313 17.86% (10/56) used bone marrow, 14.29% (8/56) used conjunctival swab, 10.71%
314 (6/56) used skin, 8.93% (5/56) used blood, 8.93% (5/56) used lymph node of any
315 region, 5.36% (3/53) used spleen, 5.36% (3/53) used tissue (lymph-node, bone marrow
316 or spleen necropsy fragments), 3.57% (2/56) used hair, and 1.79% (1/56) used cerumen
317 (Supplementary Table 6).

318 The absolute and relative frequencies of each antigen used in the indirect tests
319 were included and are summarized in Supplementary Table S7. The articles by Maia et
320 al. (2009) and Boelaert et al. (1999) did not mention which antigens they used; Maia et
321 al. (2009) used an IFAT and CIE technique; and Boelaert et al. (1999) used iELISA and
322 IFAT. Molecular techniques promote the amplification of the parasite's DNA, and in this
323 study, we identified which regions were amplified. The 14 PCRs sought to detect
324 *Leishmania* spp. using HSP70/1300 [28.57% (4/14)], HSP70/234 [28.57% (4/14),
325 kDNA/120 [28.57% (4/14)], kDNA 13A 13B [7.14% (1/14)] and DNA [7.14% (1/14)].
326 Six PCRs sought to detect *L. infantum* through kDNA/145 [66.67% (4/6)], DNA
327 [16.67% (1/6)] and kDNA [16.67% (1/6)]. Four PCRs sought to detect *L. donovani*
328 through ITS1/130 [100.00% (4/4)] and *L. infantum* or *L. braziliensis* using [100.00%
329 (1/1)] LINF1B. Of the 7 assays that involved qPCR, they sought to detect *L. infantum*
330 DNA through amplification of kDNA [85.71% (6/7)] and LinJ31 [14.49% (1/7)]; they
331 sought to detect *Leishmania* spp. through amplification kDNA [100.00% (7/7)]; and
332 they sought to detect *L. infantum* or *L. braziliensis* through LinJ31 [100.00% (1/1)]. One
333 trial used the LAMP technique, which involved DNA [100% (1/1)] from *Leishmania*
334 spp.

335

336

337 *3.3 Experimental design*

338 The average number of assays tested per study was 6.0 ± 7.45, ranging from 1 to
339 44 tests across a total of 402 assays. There were some studies that calculated DSe and
340 DSp for different groups considering all animals, only symptomatic animals and/or only
341 asymptomatic animals, and the same occurred considering cross-reaction. Of the 346
342 indirect assays, 49.42% (171/346) considered both the asymptomatic and symptomatic
343 population in the DSe and DSp calculation, 30.63% (104/346) considered only the
344 symptomatic population, 8.95% (31/346) considered only the asymptomatic population
345 and 10.98% (38/346) did not report the clinical condition. Among the 56 direct assays,
346 39.29% (22/56) considered both asymptomatic and symptomatic animals, 19.64%
347 (11/56) considered only symptomatic animals, and 41.07% (23/56) did not report the
348 clinical condition for the calculation of accuracy. Of the 346 indirect assays, 73.70%
349 (255/346) did not use cross-reactive animals, 19.65% (68/346) used negative animals
350 plus cross-reacting animals, and 6.65% (23/346) used only cross-reacting animals. Of
351 the 56 direct assays, 98.21% (55/56) did not use cross-reacting animals, and 1.79%
352 (1/56) used only cross-reacting animals. These data are summarized in Supplemental
353 Table S6.

354 In total, 63 studies had positive and negative quality reference standards
355 according to the QUADAS-2 tool, including a total of 382 assays (335 indirect assays
356 and 47 direct assays). Of the indirect tests, 41.67% (100/244) were positive indirect and
357 direct tests, 41.25% (99/244) were indirect and direct tests, 11.67% (28/244) were direct
358 tests, 5.00% (12/244) were indirect tests, 1.25% (3/244) were positive indirect or direct
359 tests and 0.83% (2/244) were indirect tests or direct tests. As a negative reference
360 standard, the indirect tests used 61.07% (149/244) indirect and direct tests, 24.59%
361 (60/244) direct tests and 14.34% (35/244) indirect tests. When considering direct assays
362 with the appropriate positive reference standard, 77.27% (34/44) used indirect and
363 direct tests, 9.09% (4/44) used indirect tests, 6.82% (3/44) used direct tests, 4.55%
364 (2/44) used direct tests and 4.55% (2/44) used indirect tests or direct tests. For the
365 negative reference standard, 82.22% (37/44) indirect and direct tests, 8.89% (4/44)
366 indirect tests and 8.89% (4/44) direct tests were used. The other 4 included studies used
367 Bayesian methodology.

368

3.3 Meta-analysis

For the meta-analysis of diagnostic tests for CVL, the estimations of DSe and DSp were obtained from 46 studies and 143 assays. Twelve studies and 56 assays from direct tests and 17 studies and 203 assays from indirect tests were excluded because there were not sufficient combinations of technique and antigen/biological sample/amplification of equal regions of the same species of *Leishmania* spp. Thus, a meta-analysis of 18 tests was carried out.

Of these 143 tests, five different indirect techniques were used: 54.55% (78/143) of the tests were iELISA, 28.67% (41/143) of the tests were ICT, 8.39% (12/143) of the tests were IFAT, 6.29% (9/143) of the tests were DAT, and 2.10% (3/143) of the tests were FC. For meta-analysis, the same diagnostic technique with the same antigen was grouped; therefore, there were 18 serological techniques with their respective antigens. The proportions of assays were as follows: 2.10% (3/143) FC tests with *L. infantum* crude antigen, 2.80% (4/143) DATs with *L. donovani* crude antigen, 3.50% (5/143) DATs with crude antigen *L. infantum*, 2.10% (3/143) IFATs with L. major crude antigen, 6.29% (9/143) IFATs with *L. infantum* crude antigen, 1.40% (2/143) ICTs with *L. infantum* crude antigen, 4.89% (7/143) ICTs with rK39 recombinant antigen, 22.38% (32/143) ICTs with rK28 recombinant antigen, 2.10% (3/143) iELISAs with PQ10 recombinant antigen, 2.10% (3/143) iELISAs with recombinant antigen PQ20, 2.10% (3/143) iELISAs with recombinant antigen rK26, 2.80% (4/143) iELISAs with recombinant antigen rK39, 3.50% (5/143) iELISAs with L. major crude antigen, 3.50% (5/143) iELISAs with rC9 recombinant antigen, 5.59% (8/143) iELISAs with rA2 recombinant antigen, 8.39% (12/143) iELISAs with SLA crude antigen, 11.19% (16/143) iELISAs with recombinant antigen rK28 and 13.29% (19/143) iELISAs with *L. infantum* crude antigen.

The DSe varied depending on the category used; when the clinical condition was not reported, the highest DSe was 0.981 (95% CI: 0.956-0.992). In this meta-analysis, we chose to include studies that did not report the clinical condition. The second highest DSe was observed among studies that used symptomatic animals (0.908; 95% CI: 0.837-0.950), followed by studies that used asymptomatic animals plus symptomatic

399 animals (0.891; 95% CI: 0.811-0.939) and studies that used asymptomatic animals
400 (0.790; 95% CI: 0.660-0.879). Similar findings were observed for DSp. The highest
401 DSp was observed among studies that used negative animals (0.954; 95% CI: 0.839-
402 0.643), followed by studies that used cross-reactive and negative animals (0.913; 95%
403 CI: 0.685-0.417) and studies that used cross-reactive animals (0.976; 95% CI: 0.926-
404 0.820). The data are shown in Table 1.

405 **Table 1:** Diagnosis sensitivity (DSe) and diagnosis specificity (DSp) meta-analysis
406 estimates for 18 serological techniques used for the diagnosis of canine leishmaniasis
407 visceral, according to symptomatology or cross-reactivity.

		DSe[a]	95% CI[b]	
			Lower	Upper
Clinical condition	[d]NI	0.981	0.956	0.992
	[e]Symp	0.908	0.837	0.950
	[f]Asymp + Symp	0.891	0.811	0.939
	[g]Asymp	0.790	0.660	0.879
		DSp[c]	**95% CI**	
			Lower	**Upper**
Cross-reactivity	[h]Neg	0.954	0.913	0.976
	[i]CR + Neg	0.839	0.685	0.926
	[j]CR	0.643	0.417	0.820

408

409 [a] DSe: Diagnosis sensitivity; [b] 95% CI: confidence intervals of 95%; [c] DSp: Diagnosis
410 specificity; [d] NI: Uninformed; [e]Symp: Symptomatic; [f]Asymp + Symp: asymptomatic
411 plus symptomatic; [g] Asymp: Asymptomatic; [h] Neg: Negative; [i] CR+Neg: Cross-
412 reactivity plus Negative; [j] CR: Cross-reactivity

413

414 For both DSe and DSp, the order of tests did not vary regardless of the category
415 used. The best DSe was obtained using the DAT with *L. donovani* crude antigen. When
416 the clinical condition was not reported, the DSe was 0.9942 (95% CI: 0.9892-0.9969);
417 when the study included symptomatic animals, the DSe was 0.9703 (95% CI: 0.9456-
418 0.9840); when the study included asymptomatic animals plus symptomatic animals, the
419 DSe was 0.9643 (95% CI: 0.9348-0.9807); and when the study included asymptomatic
420 animals, the DSe was 0.9258 (95% CI: 0.8690-0.9591). The second-best DSe values
421 were obtained using the iELISA with rA2 recombinant antigen. Among studies that did

not report the clinical condition, the DSe was 0.9926 (95% CI: 0.9863-0.9961; among studies using symptomatic animals, the DSe was 0.9624 (95% CI: 0.9316-0.9796); among studies using asymptomatic animals plus symptomatic animals, the DSe was 0.9548 (95% CI: 0.9183-0.9754); and among studies using asymptomatic animals, the DSe was 0.9071 (95% CI: 0.8387-0.9482). The third-best DSe results were obtained using the iELISA with recombinant antigen PQ20. When the clinical condition was not reported, the DSe was 0.9926 (95% CI: 0.9867-0.9960); among studies using symptomatic animals, the DSe was 0.9624 (95% CI: 0.9334-0.9790); among studies using asymptomatic animals plus symptomatic animals, the DSe was 0.9548 (95% CI: 0.9205-0.9747); and among studies using asymptomatic animals, the DSe was 0.9071 (95% CI: 0.8426-0.9469). The test that had the lowest DSe was the ICT with rK39 recombinant antigen. Among studies that did not report the clinical condition, the DSe was 0.9509 (95% CI: 0.9231-0.9690); among studies that included symptomatic animals, the DSe was 0.7859 (95% CI: 0.6947-0.8555); among studies that included asymptomatic animals plus symptomatic animals, the DSe was 0.7519 (95% CI: 0.6527-0.8302); and among studies that included asymptomatic animals, the DSe was 0.5837 (95% CI: 0.4650-0.6934). These results are shown in Table 2.

439 **Table 2:** Diagnosis sensitivity (DSe) meta-analysis estimates for 18 serological techniques used for the diagnosis of canine leishmaniasis
440 visceral, according to clinical condition.

Tests plus antigen	Clinical condition											
	NI[a]			Symp[b]			Asymp + Symp[c]			Asymp[d]		
	DSe[f]	95% CI[e]		DSe	95% CI		DSe	95% CI		DSe	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
[g]DAT_*L. donovani*	0.9942	0.9892	0.9969	0.9703	0.9456	0.9840	0.9643	0.9348	0.9807	0.9258	0.8690	0.9591
[h]iELISA_rA2	0.9926	0.9863	0.9961	0.9624	0.9316	0.9796	0.9548	0.9183	0.9754	0.9071	0.8387	0.9482
iELISA_PQ20	0.9926	0.9867	0.9960	0.9624	0.9334	0.9790	0.9548	0.9205	0.9747	0.9071	0.8426	0.9469
iELISA_PQ10	0.9915	0.9846	0.9953	0.9565	0.9237	0.9755	0.9478	0.9091	0.9705	0.8936	0.8222	0.9384
iELISA_rC9	0.9883	0.9774	0.9940	0.9413	0.8911	0.9691	0.9297	0.8710	0.9629	0.8596	0.9230	0.9297
[i]FC_*L.infantum*	0.9866	0.9527	0.9963	0.9330	0.7924	0.9807	0.9200	0.7591	0.9767	0.8417	0.9509	0.9200
iELISA_rK39	0.9841	0.9711	0.9913	0.9214	0.8644	0.9557	0.9064	0.8403	0.9469	0.8175	0.7088	0.8918
iELISA_rK26	0.9835	0.9554	0.9940	0.9189	0.8023	0.9693	0.9034	0.7702	0.9631	0.8122	0.6079	0.9235
[j]IFAT_*L. infantum*	0.9812	0.9691	0.9886	0.9080	0.8560	0.9425	0.8907	0.8308	0.9312	0.7903	0.6942	0.8622
[k]ICT_*L. infantum*	0.9723	0.9401	0.9874	0.8694	0.7483	0.9371	0.8461	0.7105	0.9248	0.7176	0.5317	0.8505
iELISA_*L. major*	0.9722	0.9536	0.9834	0.8688	0.7957	0.9184	0.8454	0.7628	0.9029	0.7166	0.5980	0.8113
ICT_rK28	0.9714	0.9590	0.9801	0.8654	0.8159	0.9031	0.8414	0.7854	0.8850	0.7105	0.6285	0.7807
iELISA_*L. infantum*	0.9673	0.9516	0.9781	0.8488	0.7883	0.8943	0.8225	0.7546	0.8747	0.6818	0.5871	0.7636
DAT_*L. infantum*	0.9656	0.9345	0.9822	0.8417	0.7299	0.9128	0.8145	0.6905	0.8963	0.6700	0.5079	0.7998
iELISA_SLA	0.9647	0.9387	0.9799	0.8383	0.7438	0.9025	0.8106	0.7056	0.8843	0.6644	0.5257	0.7795
iELISA_rK28	0.9610	0.9422	0.9738	0.8236	0.7554	0.8758	0.7940	0.7184	0.8535	0.6406	0.5412	0.7292
IFAT_*L.major*	0.9607	0.9301	0.9782	0.8226	0.7161	0.8950	0.7929	0.6757	0.8756	0.6391	0.4907	0.7649
ICT_rK39	0.9509	0.9231	0.9690	0.7859	0.6947	0.8555	0.7519	0.6527	0.8302	0.5837	0.4650	0.6934

441 [a] NI: Uninformed; [b] Symp: Symptomatic; [c] Asymp + Symp: asymptomatic plus symptomatic; [d] Asymp: Asymptomatic; [e] 95% CI: confidence
442 intervals of 95%; [f] DSe: Diagnosis sensitivity; [g] DAT: direct agglutination test; [h] iELISA: indirect enzyme-linked immunosorbent assay; [i] FC:
443 flow cytometry; [j] IFAT: indirect immunofluoresence assa; [k] ICT: immunochromatographic test.

The test that yielded the best DSp was the DAT with *L. donovani* crude antigen. Among studies that included negative animals, the DSp was 0.9796 (95% CI: 0.9492-0.9920); among studies with more negative cross-reactive animals, the DSp was 0.9243 (95% CI: 0.8260-0.9691); and among studies using cross-reactive animals, the DSp was 0.8080 (95% CI: 0.6208-0.9154). The second-best DSp was obtained using the ICT with rK28 recombinant antigen. Among studies using negative animals, the DSp was 0.9762 (95% CI: 0.9635-0.9845); among studies using cross-reactive and negative animals, the DSp was 0.9124 (95% CI: 0.8704-0.9417); and among studies using cross-reactive animals, the DSp was 0.7822 (95% CI: 0.6984-0.8478). The third-best DSp results were obtained using the DAT with crude antigen *L. infantum*. Among studies using negative animals, the DSp was 0.9740 (95% CI: 0.9368-0.9896); among studies using more negative cross-reactive animals, the DSp was 0.9051 (95% CI: 0.7902-0.9603); and among studies using negative animals, the DSp was 0.7669 (95% CI: 0.5650 0.8928). The test that yielded the worst DSp was the IFAT with L. major crude antigen. Among studies using negative animals, the DSp was 0.7927 (95% CI: 0.7121-0.8553); among studies using more negative cross-reactive animals, the DSp was 0.4929 (95% CI: 0.3860-0.6004); and among studies using cross-reactive animals, the DSp was 0.2510 (95% CI: 0.1781 0.3412). These DSp values are shown in Table 3.

Table 3: Diagnosis specificity (DSp) meta-analysis estimates for 18 serological techniques used for the diagnosis of canine leishmaniasis visceral, according to cross reaction.

Tests plus antigen	Cross-reaction								
	Neg[a]			CR + Neg[b]			CR[c]		
	DSp[e]	95% CI[d]		DSp	95% CI		DSp	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
[f]DAT_*L. donovani*	0.9796	0.9492	0.9920	0.9243	0.8260	0.9691	0.8080	0.6208	0.9154
[g]ICT_rK28	0.9762	0.9635	0.9845	0.9124	0.8704	0.9417	0.7822	0.6984	0.8478
DAT_*L. infantum*	0.9740	0.9368	0.9896	0.9051	0.7902	0.9603	0.7669	0.5650	0.8928
[h]iELISA_rC9	0.9683	0.9363	0.9845	0.8859	0.7890	0.9417	0.7281	0.5631	0.8476
ICT_*L. infantum*	0.9677	0.8978	0.9903	0.8838	0.6907	0.9629	0.7240	0.4349	0.8994
[i]FC_*L. infantum*	0.9611	0.8751	0.9886	0.8626	0.6404	0.9568	0.6840	0.3804	0.8841
ICT-rK39	0.9600	0.9052	0.9837	0.8591	0.7082	0.9387	0.6776	0.4556	0.8408
iELISA_PQ20	0.9594	0.9157	0.9810	0.8574	0.7341	0.9290	0.6746	0.4877	0.8186
iELISA_rA2	0.9570	0.9194	0.9775	0.8499	0.7435	0.9171	0.6612	0.4998	0.7922
iELISA_PQ10	0.9524	0.9022	0.9775	0.8358	0.7011	0.9169	0.6369	0.4470	0.7919
iELISA_*L. infantum*	0.9505	0.9208	0.9695	0.8301	0.7472	0.8898	0.6275	0.5047	0.7357
iELISA_rK39	0.9452	0.8885	0.9739	0.8144	0.6695	0.9048	0.6020	0.4112	0.7661
iELISA_*L. major*	0.9449	0.9173	0.9637	0.8135	0.7381	0.8709	0.6005	0.4928	0.6993
iELISA_rK26	0.9444	0.8528	0.9803	0.8120	0.5956	0.9269	0.5983	0.3367	0.8137
iELISA_rK28	0.9403	0.9118	0.9600	0.8002	0.7242	0.8593	0.5799	0.4752	0.6779
iELISA_SLA	0.9299	0.8697	0.9635	0.7713	0.6291	0.8702	0.5376	0.3690	0.6981
[j]IFAT_*L. infantum*	0.8785	0.8109	0.9241	0.6475	0.5216	0.7559	0.3878	0.2732	0.5163
IFAT_*L.major*	0.7927	0.7121	0.8553	0.4929	0.3860	0.6004	0.2510	0.1781	0.3412

[a] Neg: Negative; [b] CR + Neg: Cross-reaction plus Negative; [c] CR: Cross-reaction; [d] 95% CI: confidence intervals of 95%; [e] DSp: Diagnosis sensitivity; [f] DAT: direct agglutination test; [g] ICT: immunochromatographic test; [h] iELISA: indirect enzyme-linked immunosorbent assay, [i] FC: flow cytometry; [j] IFAT: indirect immunofluoresence assay.

4. Discussion

This systematic review and meta-analysis examined the accuracy of serological, parasitological and molecular tests for diagnosing CVL by compiling the results of several studies (Sargeant and O'Connor, 2020). CVL is a public health problem, and the accurate diagnosis of the disease is difficult due to the absence of an adequate reference standard. To identify a reference standard, it is necessary to reach a consensus on how to determine whether a dog is infected (Teixeira et al., 2019). Therefore, in our research,

162 studies out of 229 were excluded due to lack of an adequate reference standard according to the adopted criteria. In addition, dogs without clinical signs represent a challenge in the diagnosis of CVL because, according to Pessoa et al. (2019), concordance in diagnostic tools is not good and further declines with the absence of clinical signs in dogs. Faced with this challenge, our study aimed to systematically review the literature and recalculate the accuracy (DSe and DSp) of CVL diagnostic tests. Our results show that 30.63% of the indirect tests and 19.64% of the direct tests considered only symptomatic animals in their experiments, thus increasing the sensitivity of these tests. We confirmed these findings by performing meta-analysis of the indirect tests, as we noticed that DSe decreases with the use of asymptomatic animals – among studies using only asymptomatic animals, the DSe was lower than 79.00%. For cross-reactive animals, 73.70% of indirect tests and 98.21% of direct tests did not use cross-reactive animals; among studies that used only cross-reactive animals, the DSp was lower than 64.30%. Thus, it is necessary for studies to include asymptomatic and cross-reactive animals in order to obtain reliable sensitivity and specificity results, respectively. The use of asymptomatic animals to predict DSe and the use of cross-reactive animals to predict DSp are important to avoid overestimation of these values since serological techniques are limited by cross-reactivity with other disease parasites; furthermore, in cases of asymptomatic animals, there is a low parasite load, making it more difficult to detect this patient (Gomes et al., 2008).

Despite the importance that CVL has in human health, it is considered a neglected disease according to the WHO, and there are relatively few studies on the diagnostic tests for this disease. In the literature, we identified a high-quality systematic review and meta-analysis of serological tests for CVL in Brazil; however, the study focused only on serological tests and only included studies carried out in Brazil (Peixoto et al., 2015). Our systematic review and meta-analysis had a different objective: to recalculate the accuracy of all diagnostic tests for CVL worldwide. However, by establishing a quality standard for the methodology applied to samples from positive and negative dogs used, we did not identify many studies that carried out adequate molecular and parasitological tests because meta-analysis requires the use of identical techniques, the use of identical biological samples and, in the case of molecular tests, the amplification of equal regions of the same species of *Leishmania* spp. Thus, the systematic review covered all diagnostic tests for CVL, including serological,

parasitological, and molecular tests, but the meta-analysis used serological tests applied worldwide.

The lack of a perfect gold standard test for CVL leads to difficulty in evaluating diagnostic tests. In this case, when the studies were analyzed using the QUADAS-2 tool, it was observed that 70.74% (162/229) were excluded because they did not have an adequate reference standard with what was proposed (Supplementary Table S4). Of the 67 studies with an adequate reference standard, 4 were Brazilian and used latent class analysis (LCA). Of these, 2 were excluded from the meta-analysis because they did not determine the mean DSe and DSp of the groups. The other two articles, which were written by Solcà Mda et al. (2014) and Fraga et al. (2016), were included in the meta-analysis. They used a gold standard that was considered to adequate according to the results of the quality assessment. In this way, it was possible to calculate the meta-analysis together with the frequentist studies. LCA is an alternative in the absence of an adequate gold standard, as it analyses test results for the same disease through a common latent variable that accurately reflects the disease (Fraga et al., 2016).

The systematic review showed that most of the diagnostic tests were serological (256/305). This is explained by the fact that they are less invasive tests, viable in the fields and have a relatively low cost when compared to molecular techniques (WHO, 2006). In addition, it was possible to carry out a meta-analysis of only the serological tests because there were more studies and because they used the appropriate reference standards according to our criteria, which were established by specialists in the subject, to classify the samples with less risk of bias positive and negative. Currently, serological tests are considered a main tool for epidemiological surveys to control CVL, but there is a possibility of incorrect results. Dogs that are seronegative mistakenly remain a reservoir for a longer duration without any intervention; thus, diagnostic methods that can accurately diagnose them, including asymptomatic animals, are essential. Searching for studies that did not overestimate these sensitivity and specificity values enables us to identify a successful path to controlling this disease (Teixeira et al., 2019).

In many countries, the treatment of these infected dogs and the slaughter of dogs can be abandoned; therefore, vigilant monitoring of infection rates is essential. Furthermore, current serological tests do not detect most asymptomatic infected animals (Dantas-Torres et al., 2019). To overcome this challenge, molecular diagnosis has been

used since it can be used to provide a diagnosis at different stages of the disease; however, this is a more laborious technique, and in epidemiological surveillance programs, the costs of these tests make their application difficult (Fraga et al., 2016; Teixeira et al., 2019). VL surveillance programs aim to control and prevent this zoonosis; thus, tests with high sensitivity are essential to detect asymptomatic dogs and avoid the unnecessary slaughter of dogs, consequently increasing the adherence of dog owners, since the slaughter of seropositive dogs and apparently normal dogs has a negative impact and does not lead to a change in the rate of CVL seropositivity (Dantas-Torres et al., 2019; Rocha et al., 2020). In addition, the high specificity of the tests is necessary for prevention, as it allows only truly negative animals to remain in the environment (Fraga et al., 2016; Duthie et al., 2018). Thus, serological tests with high DSe and DSp and practical, fast and low cost are highly efficient. In this meta-analysis, we used real samples with true negative and cross-reactive animals, asymptomatic and symptomatic animals, which his like the real-world situation. We found that the DAT with crude *L. donovani* antigen yielded the highest DSe (99.42% to 92.58% depending on the use of symptomatic and asymptomatic animals) and DSp (97.96% to 80.80% depending on the use or not of cross-reacting animals) values. This test can easily be adopted in practice.

Detection by PCR or qPCR in blood or tissues is considered the most sensitive technique because it is possible to diagnose the infection early before seroconversion, but its application in surveillance programs is questionable due to the costs (Maia et al., 2009; Maurelli et al., 2020). Generally, tests that detect the immune response are chosen because they are less expensive, practical, and durable; however, they are better at detecting dogs that show clinical signs (Pessoa et al., 2019). In 2011, the Program for Control and Prevention of Visceral Leishmaniasis (PCVLV) of Brazil changed the diagnostic protocol for LVC from ELISA followed by DPP to DPP followed by ELISA due to the high number of false-positive dogs, but there was no significant change in the number of false-positive dogs. Thus, test standardization is adequate for its purpose because the quality, reliability, sensitivity, and specificity of the diagnostic techniques guide the epidemiological strategy of canine screening adopted (Fraga et al., 2016; Duthie et al., 2018; Rocha et al., 2020). When stating that a test has a high DSe and DSp, it is essential that quality-related criteria are adopted for this calculation, such as the use of an adequate reference standard or the LCA methodology. Although molecular

tests are techniques that were, until recently, considered more sensitive, we found numerous studies that aimed to minimize bias. Therefore, it was not possible to perform a meta-analysis due to the low number of studies carried out with adequate quality standards in molecular techniques.

Veterinary clinics aim to perform highly sensitive tests to treat truly positive animals and highly specific tests to provide preventive vaccination or for blood donor dogs (Duthie et al., 2018). For CVL screening in healthy animals, isolated serology, or combined serology with a molecular technique, such as PCR, is recommended. In seropositive asymptomatic dogs with low titers, cytological or histopathological evaluation is recommended; if the presence of amastigotes is verified, this patient is considered positive. However, if serology is low without the presence of amastigotes in tissues, a molecular technique must be performed, and if it is negative, that animal is considered a true negative (LeishVet, 2018). The molecular technique in the clinical routine is more commonly applied, but due to the need for more invasive samples such as bone marrow puncture, it is often not well accepted by tutors, so a serological technique with high reliability can replace the use of more invasive techniques. When performing the meta-analysis of the serological tests, we noticed that as we added asymptomatic animals, the DSe value decreased. A similar phenomenon occurred when we added cross-reacting animals because, consequently, we decreased the DSp value. This finding shows that we must be careful when recommending a test without evaluating how the DSe and DSp calculation was performed because in a real population when we test the animals, we usually find both asymptomatic animals and animals with other diseases that lead to a cross-reaction.

A limitation found in this study was the bias found in the DSe and DSp calculations, which was due to the use of samples with a quality standard considered inappropriate or not using the LCA methodology. When using a biased serum bank, there may be samples with false results, which leads to the overestimation of DSe and DSp values. In addition, for the cross-reaction serum bank, it is also important that these samples have been tested for CVL, as there may be coinfection between the diseases. All these issues need to be considered because estimating the value of DSe and DSp with samples with inaccurate results can promote the adherence of diagnostic tests for CVL with low performance and, consequently, the maintenance of the protozoan in the

environment. It was noted that molecular and parasitological tests are less studied than serological tests and that standardization of these tests is essential so that in the future, we can determine which technique has superior performance. This systematic review and meta-analysis highlight the most used diagnostic tests and their limitations, and it estimates their accuracy for better application in epidemiological surveillance programs and in veterinary clinical routine.

The diagnosis of CVL must be considered very seriously because when there are false-positive results, dogs can be unnecessarily euthanized or treated unnecessarily, contributing to drug resistance. Furthermore, false-negative results lead to infected dogs remaining in the environment as reservoirs of this protozoan. To define DSp and DSe, it is essential to use cross-reactive animals and asymptomatic animals, respectively, because when these animals are omitted, the values are overestimated. Serological tests are more commonly used than parasitological and molecular tests, and it is essential that new studies use an adequate reference standard or the LCA methodology so that we can estimate the DSe and DSp values of these direct tests.

5. Conclusion

Among the methods for diagnosing CVL, the most used are serological tests. Even for research purposes, these tests are more standardized, thus enabling comparison. Among these tests, the DAT with the *L. donovani* crude antigen yielded the highest DSe and DSp values.

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Declaration of conflicts of interest

The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; the collection, analyses, or interpretation of the data; the writing of the manuscript; or the decision to publish the results.

Authors' contributions

TFM, EMSD, RTF and APP conceived and designed the systematic review and meta-analysis. TFM, JAMP, RSA, MMO and EMSD performed the systematic review analyses. TFM, EMSD, JSSBF and RSA performed the meta-analysis. TFM, JAMP, RSA, GATG, JMPB and EMSD prepared the draft manuscript. All authors critically revised the manuscript and approved the final version.

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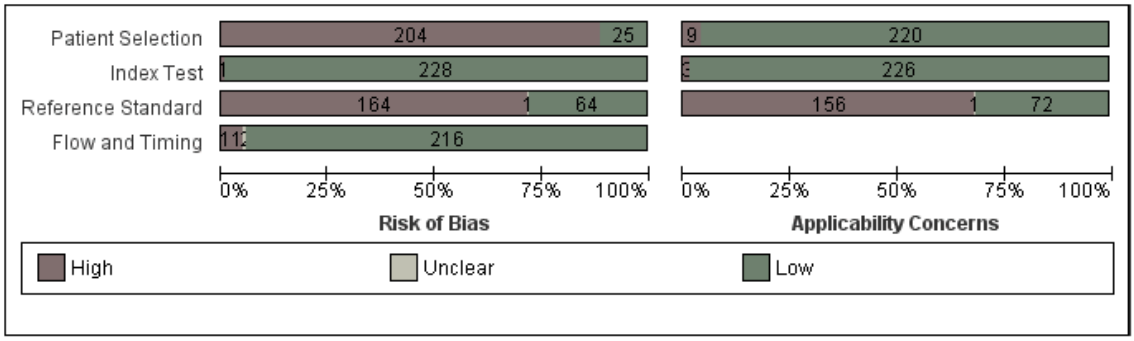
Supplementary figures

Figure S1. Risk of bias and applicability concerns graph: review authors' judgements about each domain presented as percentages across included studies – QUADAS-2



Supplementary tables

Supplementary Table S1 – Guidelines of PRISMA statement

Section and Topic	Item #	Checklist item	Paragraph where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	§1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	§1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	§1, 2, 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	§4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	§4 Tab[1]. S2
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	§1 Tab. S3
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	§4
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	§1,2,3

Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	§2
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	§5-6 Tab. S2
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	§6-7
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	§6
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	§7
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	§7
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	§7
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	§8
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	§8 Tab. S4
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	§8,9
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	§8,9
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	§9

Section and Topic	Item #	Checklist item	Location where item is reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	§9-10
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	§1 Fig[2].1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	§1 Tab. S5
Study characteristics	17	Cite each included study and present its characteristics.	§2 Tab. S6
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	§1 Tab. S4
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	§4-13
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	§6-7
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	§9-12
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	§9
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	§11
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	-

Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	§9-13
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	§1
	23b	Discuss any limitations of the evidence included in the review.	§4-5
	23c	Discuss any limitations of the review processes used.	§4-5
	23d	Discuss implications of the results for practice, policy, and future research.	§11
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	-
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	-
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	-
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	§1
Competing interests	26	Declare any competing interests of review authors.	§2
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	-

[1]Tab.: Table;[2]Fig.: Figure

Supplementary Table S2 – Search terms used in CABI, Cochrane, Pubmed, Scielo, Scopus and Web of Science databases, based on the PICOTS terms.

PICOTS	Search terms
Population	canine OR dog* OR pupp* OR canis AND familiaris OR CanL
Intervertion	diagnostic* OR method* OR test OR serolog* OR parasit* OR molec* AND leishman*
Comparison	DPP OR immunochromatographic dual-path platform OR KDDR PLUS OR recombinant kinesin-derived degenerate repeat OR ELISA OR enzyme linked immunosorbent OR cELISA OR immuno-enzymatic tests OR IFAT OR indirect immunofluorescence OR DAT OR direct agglutination test OR HAI OR indirect hemagglutination OR direct search method OR PCR OR polymerase chain reaction OR RT-PCR OR RTPCR OR real-time polymerase chain reaction OR qPCR OR NESTED PCR OR NPCR OR serologic test OR serologic methods OR indirect test OR indirect methods OR immunologic techniques OR diagnosis
Outcomes	sensitiv* OR specific* OR diagnostic* OR validation OR optimization OR assess* OR evaluation
Time	-
Setting	Systematic review and meta-analysis

Supplementary Table S3 – Inclusion and exclusion criteria for selection of articles in this systematic review.

Inclusion criteria	**Exclusion criteria**
All countries	Written in languages other than English, Spanish, French or Portuguese
Visceral Leishmaniasis	Full text not available
Canine	Not a research article
Diagnostic tests	Evaluation of the response to vaccination
Serological test	Did not perform the accuracy assessment
Parasitological test	It was not a diagnostic test
Molecular test	Another animal species
Assessment of diagnostic sensitivity and specificity	Another microorganism
	Tests accuracy for a group of species

Supplementary Table S4: Criteria for assessment of risk of bias and applicability concerns according to QUADAS-2 used to classify articles in this systematic review.

Domain 1: participant selection		
A. Risk of bias	Was a consecutive or random sample of patients enrolled?	Yes: if a consecutive or random sample of animals was selected. No: if sampling was non-consecutive or non-random, convenience sampling. Unclear: if sufficient information is not available to make a judgment on the spectrum or the sampling method.
	Was a case-control design avoided?	Yes: groups with and without leishmaniosis were recruited from the same population. No: groups with and without leishmaniosis were recruited separately. Unclear: if there is not enough information available to make a judgment about the spectrum or the sampling method.
	Did the study avoid inappropriate exclusions?	Yes: if there were no participants excluded from the analysis, or if the exclusions were adequately described. No: if there is an unexplained exclusion of participants. Unclear: if insufficient information was provided to assess whether any participants were excluded from the analysis.
	Could the selection of patients have introduced bias?	Low risk of bias: if 'yes' classification for all of the above 3 items. High risk of bias: if 'no' classification for any of the above 3 items. Unclear risk of bias: if 'unclear' classification for any of the above 3 items without a 'no' classification.
B. Concerns regarding applicability	As there concerns that the included patients and setting do not match the review question?	Low concerns: if animals are the unit of investigation, and if the population characteristics are representative for those who would receive the test in practice. High concerns: if the conditions stated above are not met. Unclear concern: if insufficient information was provided.
Domain 2: index test		
A. Risk of Bias	Were the index test results interpreted without knowledge of the results of the reference standard?	Yes: if the results of the index test were interpreted blind to the results of reference standard. No: if it is clear that the results of the index test were interpreted with knowledge of the reference standard. Unclear: if it is unclear whether blinded assessments were performed.
	If a threshold was used, was it pre-specified?	Yes: if the threshold values were prespecified before start of the study. No: if the threshold values were selected based on the collected data. Unclear: if there is insufficient information to make a judgement.
	Could the conduct or interpretation of the index test have introduced bias?	Low risk of bias: if all or at least one is “yes”. High risk of bias: if 'no' classification for all of the above 2 items. Unclear risk of bias: if 'unclear' classification for any of the above 2 items without a 'no' classification.

B. Concerns regarding applicability	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Low concerns: if the conduct or interpretation of the index test and comparison tests differed from how they were likely to be used in clinical practice. High concerns: if the conduct or interpretation of the index test or comparison tests differed from how they were likely to be used. Unclear concern: if insufficient information was provided.
Domain 3: reference standard		
A. Risk of Bias	Is the reference standards likely to correctly classify the target condition?	Yes: at least two different serological tests or a direct test (parasitological or molecular) were used to define the disease. For definition of non-diseased: at least two negative results in different serological tests or one negative serological test and one direct negative test or two negative direct tests. No: if the reference standard was other than the described above. Unclear: if it is unclear what reference standard was used.
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes: if the reference standard was interpreted without the knowledge of the results of the index test. No: if the reference standard was interpreted with the knowledge of the results of the index test. Unclear: it was not clear if the reference standard was interpreted without the knowledge of the results of the index test
	Could the reference standard, its conduct, or its interpretation have introduced bias?	Low risk of bias: if 'yes' classification for all of the above 2 items. High risk of bias: if 'no' classification for any of the above 2 items. Unclear risk of bias: if 'unclear' classification for any of the above 2 items without a 'no' classification.
B. Concerns regarding applicability	Are there concerns that the target condition as defined by the reference standard does not match the question?	Low concerns: if the classification of the target condition was defined as in the above criteria. High concerns: if the classification of the target condition has not been defined or was partially defined as in the above criteria. Unclear concern: if insufficient information was provided on the classification of the target condition.
Domain 4: flow and timing		
A. Risk of Bias	Was there an appropriate interval between index test and reference standard?	Yes: if there is no interval between the index test and the reference standard. No: if there is interval between the index test and the reference standard. Unclear: if information on timing between tests was not provided.
	Did all patients receive the same reference standard?	Yes: if it is clear that all animals who received the index test also received the reference standard. No: if not all animals who received the index test also received the reference standard. Unclear: if this information is not reported.

Were all patients included in the analysis?	Yes: if the number of participants in the two-by-two table matched the number of participants recruited into the study or if sufficient explanation was provided for any discrepancy. No: number of participants in the two-by-two table did not match the number of participants recruited into the study and insufficient explanation was provided for any discrepancy. Unclear: if insufficient information was given to permit judgement.
Could the patient flow have introduced bias?	Low risk of bias: if the “yes” classification is for all the above items or “no” for at most one of the criteria. High risk of bias: if the rating is 'no' more than one item above. Unclear risk of bias: if 'unclear' classification for any of the above 3 items without a 'no' classification.

Supplementary Table S5. Characteristics and reason of the 162 studies excluded from the systematic review based on quality assessment

First Author (Year)	Title	Model	Main reason
Abrantes (2016)	Identification of canine visceral leishmaniasis in a previously unaffected area by conventional diagnostic techniques and cell-block fixation	Frequentist	Standard inappropriate reference based on QUADAS-2
Aisa (1998)	Diagnostic potential of western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern	Frequentist	Standard inappropriate reference based on QUADAS-2
Akhoundi (2013)	Rapid detection of human and canine visceral leishmaniasis: Assessment of a latex agglutination test based on the A2 antigen from amastigote forms of Leishmania infantum	Frequentist	Standard inappropriate reference based on QUADAS-2
Andreadou (2014)	A novel non-amplification assay for the detection of Leishmania spp. in clinical samples using gold nanoparticles	Frequentist	Standard inappropriate reference based on QUADAS-2
Andreadou (2012)	Evaluation of the performance of selected in-house and commercially available PCR and real-time PCR assays for the detection of Leishmania DNA in canine clinical samples	Frequentist	Standard inappropriate reference based on QUADAS-2
Anfossi (2018)	A versatile and sensitive lateral flow immunoassay for the rapid diagnosis of visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Athanasiou (2014)	Comparison of two commercial rapid in-clinic serological tests for detection of antibodies against Leishmania spp. in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Babakhan (2009)	Rapid detection of Leishmania infantum infection in dogs: a comparative study using fast agglutination screening test (FAST) and direct agglutination test (DAT) in Iran	Frequentist	Standard inappropriate reference based on QUADAS-2
Barbosa (2012)	Detection of amastigote forms in a parasitological exam of smears made from conjuntival swab in dogs with visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Barbosa-de-Deus (2002)	Leishmania major-like antigen for specific and sensitive serodiagnosis of human and canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Barrouin-Melo (2004)	Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of Leishmania chagasi infection in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Bernadina (1997)	An immunodiffusion assay for the detection of canine leishmaniasis due to infection with Leishmania infantum	Frequentist	Standard inappropriate reference based on QUADAS-2
Boarino (2008)	Application of a recombinant protein for the serological diagnosis of canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Boarino (2005)	Development of recombinant chimeric antigen expressing immunodominant B Epitopes of Leishmania infantum for serodiagnosis of visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Boelart (1999)	The potential of Latent Class Analysis in diagnostic test validation for canine Leishmania infantum infection	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason
Braga (2014)	Evaluation of canine and feline leishmaniasis by the association of blood culture, immunofluorescent antibody test and polymerase chain reaction	Frequentist	Standard inappropriate reference based on QUADAS-2
Camargo (2010)	Performance of IFAT, ELISA, direct parasitological examination and PCR on lymph node aspirates for canine visceral leishmaniasis diagnosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Campos (2017)	Accuracy of quantitative polymerase chain reaction in samples of frozen and paraffin-embedded healthy skin for the diagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Candido (2008)	Comparative evaluation of enzyme-linked immunosorbent assay based on crude and purified antigen in the diagnosis of canine visceral leishmaniasis in symptomatic and oligosymptomatic dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Cantos-Barreda (2017)	Quantification of anti-Leishmania antibodies in saliva of dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Cantos-Barreda (2017)	New wide dynamic range assays for quantification of anti-Leishmania IgG2 and IgA antibodies in canine serum	Frequentist	Standard inappropriate reference based on QUADAS-2
Cardoso (1998)	Use of a Leishmania skin test in the detection of canine Leishmania-specific cellular immunity	Frequentist	Standard inappropriate reference based on QUADAS-2
Carson (2009)	Selection of appropriate serological tests to measure the incidence of natural Leishmania infantum infection during DNA/MVA prime/boost canine vaccine assays	Frequentist	Standard inappropriate reference based on QUADAS-2
Carson (2010)	Comparison of Leishmania OligoC-TesT PCR with Conventional and Real-Time PCR for Diagnosis of Canine Leishmania Infection	Frequentist	Standard inappropriate reference based on QUADAS-2
Carvalho Neta (2006)	Citometria de fluxo no diagnóstico da leishmaniose visceral canina	Frequentist	Standard inappropriate reference based on QUADAS-2
Carvalho (2017)	An ELISA immunoassay employing a conserved Leishmania hypothetical protein for the serodiagnosis of visceral and tegumentary leishmaniasis in dogs and humans	Frequentist	Standard inappropriate reference based on QUADAS-2
Carvalho (2009)	An enzyme-linked immunosorbent assay (ELISA) for the detection of IgM antibodies against Leishmania chagasi in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Carvalho (2002)	Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant Leishmania donovani A2 antigen	Frequentist	Standard inappropriate reference based on QUADAS-2
Castellanos-Gonzalez (2015)	A Novel Molecular Test to Diagnose Canine Visceral Leishmaniasis at the Point of Care	Frequentist	Standard inappropriate reference based on QUADAS-2
Cavalcanti (2009)	The development of a real-time PCR assay for the quantification of Leishmania infantum DNA in canine blood	Frequentist	Standard inappropriate reference based on QUADAS-2
Ceccarelli (2014)	Application of qPCR in conjunctival swab samples for the evaluation of canine leishmaniasis in borderline cases or disease relapse and correlation with clinical parameters	Frequentist	Standard inappropriate reference based on QUADAS-2
First Author (Year)	**Title**	**Model**	**Main reason**

Chaouch (2013)	Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of Leishmania infantum in canine leishmaniasis based on cysteine protease B genes	Frequentist	Standard inappropriate reference based on QUADAS-2
Chargui (2009)	Use of PCR, IFAT and in vitro culture in the detection of Leishmania infantum infection in dogs and evaluation of the prevalence of canine leishmaniasis in a low endemic area in Tunisia	Frequentist	Standard inappropriate reference based on QUADAS-2
Costa (2017)	Antigenicity of phage clones and their synthetic peptides for the serodiagnosis of canine and human visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Costa (2015)	Evaluation of PCR in the diagnosis of canine leishmaniasis in two different epidemiological regions: Campinas (SP) and Teresina (PI), Brazil	Frequentist	Standard inappropriate reference based on QUADAS-2
Costa (2012)	Improved Canine and Human Visceral Leishmaniasis Immunodiagnosis Using Combinations of Synthetic Peptides in Enzyme-Linked Immunosorbent Assay	Frequentist	Standard inappropriate reference based on QUADAS-2
Courtenay (2002)	Infectiousness in a cohort of Brazilian dogs: Why culling fails to control visceral leishmaniasis in areas of high transmission	Frequentist	Standard inappropriate reference based on QUADAS-2
da Costa (2003)	Standardization of a rapid immunochromatographic test with the recombinant antigens K39 and K26 for the diagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
da Silva (2013)	Assessment of serological tests for the diagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Dantas-Torres (2018)	Level of agreement between two commercially available rapid serological tests and the official screening test used to detect Leishmania seropositive dogs in Brazil	Frequentist	Standard inappropriate reference based on QUADAS-2
Dapra (2008)	Validation of a recombinant based antibody ELISA for diagnosis of human and canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
de Andrade (2006)	Use of PCR-RFLP to identify Leishmania species in naturally-infected dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
de Carvalho (2013)	A simple immune complex dissociation ELISA for leishmaniasis: Standardization of the assay in experimental models and preliminary results in canine and human samples	Frequentist	Standard inappropriate reference based on QUADAS-2
de Lima (2010)	Comparison between ELISA using total antigen and immunochromatography with antigen rK39 in the diagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
de Mendonca (2017)	The performance of serological tests for Leishmania infantum infection screening in dogs depends on the prevalence of the disease	Frequentist	Standard inappropriate reference based on QUADAS-2
de Mendonca (2017)	Serological tests fail to discriminate dogs with visceral leishmaniasis that transmit Leishmania infantum to the vector Lutzomyia longipalpis	Frequentist	Standard inappropriate reference based on QUADAS-2
de Sa (2019)	Molecular Methodology for the Detection of the Leishmania Genus in Different Biological Samples Extracted from Dogs	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason

de Souza (2013)	Recombinant Leishmania (Leishmania) infantum Ecto-Nucleoside Triphosphate Diphosphohydrolase NTPDase-2 as a new antigen in canine visceral leishmaniasis diagnosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Dhomi-Lemos (2019)	Leishmania infantum recombinant kinesin degenerated derived repeat (rKDDR): A novel potential antigen for serodiagnosis of visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Di Muccio (2012)	Diagnostic Value of Conjunctival Swab Sampling Associated with Nested PCR for Different Categories of Dogs Naturally Exposed to Leishmania infantum Infection	Frequentist	Standard inappropriate reference based on QUADAS-2
Dias (2019)	Cytological and molecular detection of Leishmania spp. in different biological tissues of dogs in areas endemic for visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Dias (2018)	Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved Leishmania hypothetical protein	Frequentist	Standard inappropriate reference based on QUADAS-2
Dietze (1995)	DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS WITH A DOT-ENZYME-LINKED IMMUNOSORBENT-ASSAY	Frequentist	Standard inappropriate reference based on QUADAS-2
Diotallevi (2020)	Real-time PCR to differentiate among Leishmania (Viannia) subgenus, Leishmania (Leishmania) infantum and Leishmania (Leishmania) amazonensis: Application on Brazilian clinical samples	Frequentist	Standard inappropriate reference based on QUADAS-2
Duarte (2017)	Performance of Leishmania braziliensis enolase protein for the serodiagnosis of canine and human visceral leishmaniosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Fallah (2011)	Serological survey and comparison of two polymerase chain reaction (PCR) assays with enzyme-linked immunosorbent assay (ELISA) for the diagnosis of canine visceral leishmaniasis in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Farahmand (2018)	Assessment of Recombinant A2-Latex Agglutination Test (RA2-LAT) and RA2-ELISA for Detection of Canine Visceral Leishmaniasis: A Comparative Field Study with Direct Agglutination Test in Northwestern Iran	Frequentist	Standard inappropriate reference based on QUADAS-2
Farahmand (2015)	Comparison of recombinant A2-ELISA with rKE16 dipstick and direct agglutination tests for diagnosis of visceral leishmaniasis in dogs in Northwestern Iran	Frequentist	Standard inappropriate reference based on QUADAS-2
Farash (2020)	The rK39 Antigen from an Iranian Strain of Leishmania infantum: Detection of Anti-Leishmania Antibodies in Humans and Dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Ferreira (2012)	Canine Skin and Conjunctival Swab Samples for the Detection and Quantification of Leishmania infantum DNA in an Endemic Urban Area in Brazil	Frequentist	Standard inappropriate reference based on QUADAS-2
Ferroglio (2007)	Evaluation of an ELISA rapid device for the serological diagnosis of Leishmania infantum infection in dog as compared with immunofluorescence assay and Western blot	Frequentist	Standard inappropriate reference based on QUADAS-2
Ferroglio (2013)	Evaluation of a Rapid Device for Serological Diagnosis of Leishmania infantum Infection in Dogs as an Alternative to Immunofluorescence Assay and Western Blotting	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	**Title**	**Model**	**Main reason**

Figueiredo (2010)	Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples	Frequentist	Standard inappropriate reference based on QUADAS-2
Fisa (1997)	Serologic diagnosis of canine leishmaniasis by dot-ELISA	Frequentist	Standard inappropriate reference based on QUADAS-2
Fraga (2014)	A multicentric evaluation of the recombinant Leishmania infantum antigen-based immunochromatographic assay for the serodiagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Francino (2006)	Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Gouvêa (2016)	Predictive factors for Leishmania infantum infection in dogs examined at a veterinary teaching hospital in Teresina, State of Piauí, Brazil	Frequentist	Standard inappropriate reference based on QUADAS-2
Gualda (2015)	NEW PRIMERS FOR DETECTION OF Leishmania infantum USING POLYMERASE CHAIN REACTION	Frequentist	Standard inappropriate reference based on QUADAS-2
Guerbouj (2014)	Evaluation of a gp63-PCR Based Assay as a Molecular Diagnosis Tool in Canine Leishmaniasis in Tunisia	Frequentist	Standard inappropriate reference based on QUADAS-2
Habibzadeh (2007)	Rk39 dipstick test as a practical solution for canine leishmaniasis diagnosis among nomadic populations	Frequentist	Standard inappropriate reference based on QUADAS-2
Larson (2017)	Semi-quantitative measurement of asymptomatic L-infantum infection and symptomatic visceral leishmaniasis in dogs using Dual-Path PlatformA (R) CVL	Frequentist	Standard inappropriate reference based on QUADAS-2
Laurenti (2014)	Comparative evaluation of the DPP (R) CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Lauricella (2016)	An rK28-Based Immunoenzymatic Assay for the Diagnosis of Canine Visceral Leishmaniasis in Latin America	Frequentist	Standard inappropriate reference based on QUADAS-2
Lima (2017)	The use of Escherichia coli total antigens as a complementary approach to address seropositivity to Leishmania antigens in canine leishmaniosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Lima (2019)	Diagnostic performance of a qPCR for Leishmania on stained cytological specimens and on filter paper impressions obtained from cutaneous lesions suggestive of canine leishmaniosis	Frequentist	Standard inappropriate reference based on QUADAS-2
Lira (2006)	Canine visceral leishmaniosis: A comparative analysis of the EIE-leishmaniose-visceral-canina-Bio-Manguinhos and the IFI-leishmaniose-visceral-canina-Bio-Manguinhos kits	Frequentist	Standard inappropriate reference based on QUADAS-2
Lopez-Cespedes (2012)	Leishmania spp. Epidemiology of Canine Leishmaniasis in the Yucatan Peninsula	Frequentist	Standard inappropriate reference based on QUADAS-2
Machado (2020)	A Leishmania infantum hypothetical protein evaluated as a recombinant protein and specific B-cell epitope for the serodiagnosis and prognosis of visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
First Author (Year)	**Title**	**Model**	**Main reason**

Machado (2020)	A new Leishmania hypothetical protein can be used for accurate serodiagnosis of canine and human visceral leishmaniasis and as a potential prognostic marker for human disease	Frequentist	Standard inappropriate reference based on QUADAS-2
Maciel (2020)	Plasmonic rK28 ELISA improves the diagnosis of canine Leishmania infection	Frequentist	Standard inappropriate reference based on QUADAS-2
Magalhaes (2017)	Evaluation of a new set of recombinant antigens for the serological diagnosis of human and canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Macianti (1996)	Evaluation of dot enzyme-linked immunosorbent assay (dot-ELISA) for the serodiagnosis of canine leishmaniosis as compared with indirect immunofluorescence assay	Frequentist	Standard inappropriate reference based on QUADAS-2
Mathis (1995)	PCR and in-vitro cultivation for detection of *Leishmania* spp in diagnostic samples from humans and dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Mendes (2017)	Epitope mapping of recombinant Leishmania donovani virulence factor A2 (recLdVFA2) and canine leishmaniasis diagnosis using a derived synthetic bi-epitope	Frequentist	Standard inappropriate reference based on QUADAS-2
Mendonça (2017)	The performance of serological tests for Leishmania infantum infection screening in dogs depends on the prevalence of the disease	Frequentist	Standard inappropriate reference based on QUADAS-2
Mendonça (2017)	Serological tests fail to discriminate dogs with visceral leishmaniasis that transmit Leishmania infantum to the vector Lutzomyia longipalpis	Frequentist	Standard inappropriate reference based on QUADAS-2
Menezes (2013)	Sensitivity and Specificity of In Situ Hybridization for Diagnosis of Cutaneous Infection by Leishmania infantum in Dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Menezes-Souza (2015)	Linear B-cell epitope mapping of MAPK3 and MAPK4 from Leishmania braziliensis: implications for the serodiagnosis of human and canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Menezes-Souza (2014)	Epitope mapping of the HSP83.1 protein of Leishmania braziliensis discloses novel targets for immunodiagnosis of tegumentary and visceral clinical forms of leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Menezes-Souza (2014)	Mapping B-cell epitopes for the peroxidoxin of Leishmania (Viannia) braziliensis and its potential for the clinical diagnosis of tegumentary and visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Menezes-Souza (2015)	Improving Serodiagnosis of Human and Canine Leishmaniasis with Recombinant Leishmania braziliensis Cathepsin L-like Protein and a Synthetic Peptide Containing Its Linear B-cell Epitope	Frequentist	Standard inappropriate reference based on QUADAS-2
Mettler (2005)	Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic Leishmania infections in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason
Mohammadi-Ghalehbin (2011)	A Leishmania infantum FML-ELISA for the Detection of Symptomatic and Asymptomatic Canine Visceral Leishmaniasis in an Endemic Area of Iran	Frequentist	Standard inappropriate reference based on QUADAS-2
Mohammadiha (2013)	Comparison of real-time PCR and conventional PCR with two DNA targets for detection of Leishmania (Leishmania) infantum infection in human and dog blood samples	Frequentist	Standard inappropriate reference based on QUADAS-2
Mohebali (2015)	Modification on Direct Agglutination Antigen Preparation for Simplified Sero-Diagnosis of Human and Canine Visceral Leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Mohebali (2004)	Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick rk39 test and direct agglutination	Frequentist	Standard inappropriate reference based on QUADAS-2
Morales (2012)	Epidemiological implications of the use of various methods for the diagnosis of canine leishmaniasis in dogs with different characteristics and in differing prevalence scenarios	Frequentist	Standard inappropriate reference based on QUADAS-2
Moreira (2007)	Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs	Frequentist	Standard inappropriate reference based on QUADAS-2
Nasereddin (2006)	Serological survey with PCR validation for canine visceral leishmaniasis in northern Palestine	Frequentist	Standard inappropriate reference based on QUADAS-2
Nunes (2015)	Serological, parasitological and molecular tests for canine visceral leishmaniosis diagnosis in a longitudinal study	Frequentist	Standard inappropriate reference based on QUADAS-2
Nunes (2007)	Polymerase chain reaction evaluation for canine visceral leishmaniasis diagnosis in dog blood samples	Frequentist	Standard inappropriate reference based on QUADAS-2
Oliva (2006)	Incidence and time course of Leishmania infantum infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons	Frequentist	Standard inappropriate reference based on QUADAS-2
Oliveira dos Santos Maciel (2020)	Plasmonic rK28 ELISA improves the diagnosis of canine Leishmania infection	Frequentist	Standard inappropriate reference based on QUADAS-2
Oliveira (2011)	Characterization of Novel Leishmania infantum Recombinant Proteins Encoded by Genes from Five Families with Distinct Capacities for Serodiagnosis of Canine and Human Visceral Leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Oliveira (2008)	A study of cross-reactivity in serum samples from dogs positive for Leishmania sp., Babesia canis and Ehrlichia canis in enzyme-linked immunosorbent assay and indirect fluorescent antibody test	Frequentist	Standard inappropriate reference based on QUADAS-2
Oliveira-da-Silva (2020)	Evaluation of Leishmania infantum pyridoxal kinase protein for the diagnosis of human and canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Otranto (2009)	Toward diagnosing Leishmania infantum infection in asymptomatic dogs in an area where leishmaniasis is endemic	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason
Otranto (2005)	Recombinant K39 dipstick immunochromatographic test: a new tool for the serodiagnosis of canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Otranto (2004)	Rapid immunochromatographic test for serodiagnosis of canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Otranto (2010)	Diagnosis of Canine Vector-Borne Diseases in Young Dogs: a Longitudinal Study	Frequentist	Standard inappropriate reference based on QUADAS-2
Pinheiro (2009)	A recombinant cysteine proteinase from Leishmania (Leishmania) chagasi as an antigen for delayed-type hypersensitivity assays and serodiagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Porrozzi (2007)	Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic Leishmania infantum visceral infections in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Proverbio (2013)	Comparison of a clinic-based ELISA test kit with the immunofluorescence antibody test for assaying Leishmania infantum antibodies in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Proverbio (2016)	Comparison of a rapid immunochromatographic assay with an immunofluorescent antibody test for detection of Leishmania infantum antibodies in dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Quinnell (2013)	Evaluation of rK39 Rapid Diagnostic Tests for Canine Visceral Leishmaniasis: Longitudinal Study and Meta-Analysis	Frequentist	Standard inappropriate reference based on QUADAS-2
Quinnell (2001)	Detection of Leishmania infantum by PCR, serology and cellular immune response in a cohort study of Brazilian dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Rajasekariah (2008)	A novel exo-antigen-based ELISA for the detection of canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Ramírez (2020)	Improving the serodiagnosis of canine Leishmania infantum infection in geographical areas of Brazil with different disease prevalence	Frequentist	Standard inappropriate reference based on QUADAS-2
Rampazzo (2017)	A ready-to-use duplex qPCR to detect Leishmania infantum DNA in naturally infected dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Reale (1999)	Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood	Frequentist	Standard inappropriate reference based on QUADAS-2
Regina-Silva (2014)	Evaluation of parasitological examination, kDNA polymerase chain reaction and rK39-based immunochromatography for the diagnosis of visceral leishmaniasis in seropositive dogs from the screening-culling program in Brazil	Frequentist	Standard inappropriate reference based on QUADAS-2
Reis (2013)	Molecular diagnosis of canine visceral leishmaniasis: A. comparative study of three methods using skin and spleen from dogs with natural Leishmania infantum infection	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason
Reithinger (2002)	Rapid detection of Leishmania infantum infection in dogs: Comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR	Frequentist	Standard inappropriate reference based on QUADAS-2
Rodrigues (2019)	Immunodiagnosis of human and canine visceral leishmaniasis using recombinant Leishmania infantum Prohibitin protein and a synthetic peptide containing its conformational B-cell epitope	Frequentist	Standard inappropriate reference based on QUADAS-2
Romero Peñuela (2010)	DIAGNÓSTICO DE LA LEISHMANIASIS VISCERAL CANINA MEDIANTE LAS TÉCNICAS WESTERN BLOT E INMUNOFLUORESCENCIA INDIRECTA	Frequentist	Standard inappropriate reference based on QUADAS-2
Rosa (2013)	Molecular diagnosis of canine visceral leishmaniasis through the technique of probe gold nanoparticles (AuNPprobes)	Frequentist	Standard inappropriate reference based on QUADAS-2
Rosário (2005)	Evaluation of enzyme-linked immunosorbent assay using crude Leishmania and recombinant antigens as a diagnostic marker for canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Rosati (2003)	Prokaryotic expression and antigenic characterization of three recombinant Leishmania antigens for serological diagnosis of canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Rosypal (2005)	Utility of diagnostic tests used in diagnosis of infection in dogs experimentally inoculated with a North American isolate of Leishmania infantum	Frequentist	Standard inappropriate reference based on QUADAS-2
Ryan (2002)	Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM) and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Santarem (2010)	Application of an Improved Enzyme-Linked Immunosorbent Assay Method for Serological Diagnosis of Canine Leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Santarem (2020)	Challenges in the serological evaluation of dogs clinically suspect for canine leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Santos (2019)	Recombinant Leishmania eukaryotic elongation factor-1 beta protein: A potential diagnostic antigen to detect tegumentary and visceral leishmaniasis in dogs and humans	Frequentist	Standard inappropriate reference based on QUADAS-2
Santos (2020)	Gene design, optimization of protein expression and preliminary evaluation of a new chimeric protein for the serological diagnosis of both human and canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Saridomichelakis (2005)	Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (Leishmania infantum) in symptomatic and asymptomatic	Frequentist	Standard inappropriate reference based on QUADAS-2
Sassaki (2011)	Comparison of different diagnostic tests in dogs uninfected and naturally infected with visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Schallig (2004)	Development of a dipstick assay for detection of Leishmania-specific canine antibodies	Frequentist	Standard inappropriate reference based on QUADAS-2
First Author (Year)	**Title**	**Model**	**Main reason**
Schallig (2002)	Development of a fast agglutination screening test	Frequentist	Standard inappropriate

	(FAST) for the detection of anti-Leishmania antibodies in dogs		reference based on QUADAS-2
Seimiao-Santos (2014)	Performance of an indigenous beta-mercaptoethanol-modified antigen in comparison with a commercial reference in direct agglutination test for detection of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Syyedtabaei (2017)	Detection of Potentially Diagnostic Leishmania Antigens with Western Blot Analysis of Sera from Patients with Cutaneous and Visceral Leishmaniases	Frequentist	Standard inappropriate reference based on QUADAS-2
Silva (2001)	Short report: Detection of Leishmania DNA by polymerase chain reaction on blood samples from dogs with visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Silva (2016)	Epidemiological aspects of canine visceral leishmaniasis in the semi-arid region of Paraiba and analysis of diagnostic techniques	Frequentist	Standard inappropriate reference based on QUADAS-2
Solca (2012)	Qualitative and quantitative polymerase chain reaction (PCR) for detection of Leishmania in spleen samples from naturally infected dogs	Frequentist	Standard inappropriate reference based on QUADAS-2
Soto (1998)	Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Sousa (2013)	Development of a Fluorescent Based Immunosensor for the Serodiagnosis of Canine Leishmaniasis Combining Immunomagnetic Separation and Flow Cytometry	Frequentist	Standard inappropriate reference based on QUADAS-2
Sousa (2011)	Seroepidemiological survey of Leishmania infantum infection in dogs from northeastern Portugal	Frequentist	Standard inappropriate reference based on QUADAS-2
Souza (2019)	Evaluation of DPP® and SNAP® Rapid Tests for diagnosis of Leishmania infantum canine infections	Frequentist	Standard inappropriate reference based on QUADAS-2
Staniek (2019)	eNose analysis of volatile chemicals from dogs naturally infected with Leishmania infantum in Brazil	Fraquentist	Standard inappropriate reference based on QUADAS-2
Taran (2007)	Diagnosis of canine visceral leishmaniasis by ELISA using K39sub recombinant antigen	Frequentist	Standard inappropriate reference based on QUADAS-2
Teran-Angel (2007)	The direct agglutination test as an alternative method for the diagnosis of canine and human visceral leishmaniasis	Frequentist	Standard inappropriate reference based on QUADAS-2
Travi (2001)	Canine visceral leishmaniasis in Colombia: Relationship between clinical and parasitologic status and infectivity for sand flies	Frequentist	Standard inappropriate reference based on QUADAS-2
Vargas-Duarte (2009)	Evaluacion por Western Blot, Inmunofluorescencia Indirecta y ELISA de Perros Infectados con Leishmania (Leishmania) infantum	Frequentist	Standard inappropriate reference based on QUADAS-2
Vasconselos (2016)	Reliability evaluation between two evaluators in the cytopathologic and immunocytochemical exams from bone marrow aspirate in the canine visceral leishmaniasis diagnosis	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author (Year)	Title	Model	Main reason

Velez (2019)	Seroprevalence of canine Leishmania infantum infection in the Mediterranean region and identification of risk factors: The example of North-Eastern and Check for Pyrenean areas of Spain	Frequentist	Standard inappropriate reference based on QUADAS-2
Vercammen (1997)	Development of a slide ELISA for canine leishmaniasis and comparison with four serological tests	Frequentist	Standard inappropriate reference based on QUADAS-2
Wolf (2014)	Serological diagnosis of canine leishmaniosis: comparison of three commercially available tests	Frequentist	Standard inappropriate reference based on QUADAS-2

Supplementary Table S6. Main characteristics of the 67 studies included in the systematic review.

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
Abad (2017)	Brazil	NI	Frequentist	negative for ICT and ELISA	100	positive for ICT and ELISA	100	200	No	iELISA	3
Adel (2015)	Algeria	2008-2009	Bayesiana	NA	NA	NA	NA	NA	No	IFAT and DAT	3
Adel (2010)	Algeria	2004-2005	Bayesiana	NA	NA	NA	NA	NA	No	IFAT and DAT	3
Alves (2012)	Brazil	NI	Frequentist	negative for ELISA and IFAT and culture and Nested-PCR	39	positive for culture isolation and isoenzyme technique	69	78	No	iELISA, IFAT and ICT	5
Arruda (2016)	Brazil	NI	Frequentist	negative for parasitological examination and culture	1327	positive for parasitologicalexamination or culture	98	1425	No	iELISA and IFAT	1
Ashford (1995)	Brazil	NI	Frequentist	negative for culture and hamster inoculation and FAST-ELISA and PCR	35	positive for culture and hamster inoculation and FAST-ELISA and PCR	25	60	No	dELISA and *c*PCR	2
Barral-Veloso (2013)	Portugal	NI	Frequentist	negative for DAT and parasitological examination	37	positive for parasitological examination	31	68	No	ELISA	2
Belinchón-Lorenzo (2013)	Spain	NI	Frequentist	negative for lymph node PCR and ELISA-SLA and blood PCR and IFAT	15	positive for lymph node PCR and ELISA-SLA and/or blood PCR and/or IFAT	13	28	Yes	iELISA, IFAT and *q*PCR	5
Belinchón-Lorenzo (2016)	Spain	NI	Frequentist	negative for ELISA-SLA and IFAT	5	positive for ELISA-SLA and IFAT	26/ 32/ 33	32/ 37/ 38	Yes	*q*PCR	4
Bivona (2019)	Argentina	NI	Frequentist	negative for IFAT, ICT-rK39 (Kalazar Detect rapid test) and ICT-Snap Leishmania (IDEXX)	26	positive for parasitological examination and IFAT and ICT-rK39 (Kalazar Detect rapid test) and ICT-Snap Leishmania (IDEXX)	15	41	Yes	iELISA	4

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
Borja (2018)	Brazil	NI	Frequentist	negative for culture and qPCR	51/ 178	positive for culture or qPCR	73/ 183/ 24/ 60/ 49/ 123	124/ 361/ 75/ 238/ 100/ 301	Yes and No	iELISA and ICT	30
Caldas (2020)	Brazil	2015	Frequentist	negative for ICT, ELISA, parasitological examination and culture	10	positive for ICT, ELISA and/or parasitological examination and/or culture or immunohistochemistry	60	70	Yes	*q*PCR	3
Chávez-Fumagalli (2013)	Brazil	NI	Frequentist	negative for ELISA, IFAT and PCR	40	positive for PCR-RFLP, ELISA and IFAT or positive for PCR	25/20	65/60	Yes and No	iELISA	8
Coelho (2016)	Brazil	NI	Frequentist	negative for serological tests and parasitological tests	60	positive for IFAT, ELISA and PCR	29	89	Yes	iELISA	5
Coelho (2009)	Brazil and Spain	NI	Frequentist	negative for serological tests and parasitological tests	47	positive for parasitological examination	44	91	Yes	iELISA	2
Colombo (2015)	Brazil	2011	Frequentist	negative for PCR (three different sets of primers), DPP, ELISA and IFAT	97	positive for PCR (three different sets of primers) and at least two of the serological tests (DPP and/or ELISA and/or IFAT)	101	198	No	*q*PCR	1
Costa (2014)	Brazil	NI	Frequentist	negative for ELISA, IFAT and PCR	61	positive for ELISA, IFAT and PCR-RFLP	31	92	Yes	iELISA	19
Da Silva (2006)	Brazil	NI	Frequentist	negative for microscopy, culture and PCR	67	positive for microscopy, culture and PCR	36	103	No	IFAT and DAT	2
De Arruda (2013)	Brazil	2008-2010	Frequentist	negative for microscopy, immunohistochemical	1327	positive for microscopy and/or immunohistochemical	98	1425	Yes	iELISA	2

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
				and culture		and/or culture					
De Arruda (2016)	Brazil	2008-2010	Frequentist	negative for microscopy, immunohistochemical and culture	1327	positive for microscopy and/or immunohistochemical and/or culture	98	1425	Yes	iELISA and IFAT	2
De Oliveira (2015)	Brazil	NI	Frequentist	negative for serology, culture, *q*PCR of splenic aspirate	40	positive for culture	57	97	Yes	MAPIA	13
De Souza (2019)	Brazil	NI	Frequentist	negative for IFAT and ELISA	22	positive for isolation and PCR	164/72	186/94	No	iELISA and ICT	2
De Souza (2012)	Brazil	2006-2008	Frequentist	negative for serological test, parasitological test and molecular test	119	positive for serological test, parasitological test and molecular test	138	257	No	iELISA and ICT	2
De Souza (2016)	Brazil	NI	Frequentist	negative for ELISA and IFAT	40	positive for ELISA, IFAT, histopathology, culture and direct exam	38/41	78/81	Yes	ICT	2
Dias (2017)	Brazil	NI	Frequentist	negative for ELISA, IFAT and PCR	30	positive for ELISA, IFAT and PCR	23	53	Yes	iELISA	3
Do Rosário (2005)	Brazil		Frequentist	negative for IFAT and parasitological examination	25	positive for IFAT and parasitological examination	106	131	Yes	iELISA	8
Dos Santos (2019)	Brazil	NI	Frequentist	negative for ICT, DPP, ELISA and microscopy or culture or PCR	76	positive for ICT, DPP, ELISA and microscopy or culture or PCR	109	185	Yes	iELISA	6
Dye (1999)	France	1989 - 1991	Frequentist	negative for culture on more than one occacision	NI	positive for culture on at least one occasion	NI	NI	No	IFAT	1
Faria (2011)	Brazil	NI	Frequentist	negative for parasitological test and serological test	20	positive for parasitological examination	62	82	Yes	iELISA	12

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
Faria (2015)	Brazil	NI	Frequentist	negative for IFAT and ELISA and parasitological	131	positive for IFAT and ELISA and parasitological	231	362	Yes	iELISA	2
Ferreira (2007)	Brazil	NI	Frequentist	negative for parasitological examination, ELISA, DAT and IFAT	20	positive for parasitological examination	112	132	Yes	iELISA	3
Figueiredo (2018)	Brazil	NI	Frequentist	negative for culture, immunohistochemistry and histopatology	128	positive for culture, immunohistochemistry or histopatology	1318/ 448/ 721/ 149	1446/ 576/ 643/ 140	Yes and No	ICT	4
Fonseca (2014)	Brazil	NI	Frequentist	negative for culture, immunohistochemistry and histopatology	10	positive for IFAT and parasitological examination	31	41	Yes	iELISA and ICT	6
Fonseca (2019)	Brazil	NI	Frequentist	negative for IFAT and ELISA	30	positive for IFAT and ELISA	100	130	No	iELISA	3
Fraga (2016)	Brazil	2011-2012	Bayesiana	NA	NA	NA	NA	670	Yes	iELISA, IFAT, ICT, *q*PCR and culture	5
Garcia (2017)	Argentina	NI	Frequentist	negative for DPP and microscopy	20	positive for DPP and microscopy	150	170	No	LAT	1
Gonçalves (2016)	Brazil	NI	Frequentist	negative for ELISA, culture and PCR	8	positive for PCR spleen	15	23	No	*c*PCR	1
Goncalves-de-Albuquerque (2015)	Brazil	NI	Frequentist	negative for PCR and qPCR	42	positive for PCR and qPCR	53	95	No	*c*PCR	1
Grimaldi (2012)	Brazil	2003-2004	Frequentist	negative for ELISA-rk39, ELISA-rK26 and ELISA-rA2	59	positive for microscopy	120	179	Yes	ICT	1

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
Guerra (2019)	Brazil	2015-2016	Frequentist	negative for ELISA and PCR	10	positive for ELISA and PCR	50	60	No	cytology, LBC, histology-HE, histology-ICC, FFPE-IHC and cytology + histologyIHC	6
Jusi (2015)	Brazil	NI	Frequentist	negative for IFAT and ELISA-SLA	170	positive for IFAT and ELISA-SLA	482	652	No	iELISA	1
Ker (2013)	Brazil	NI	Frequentist	negative for IFAT, ELISA, parasitological examination and PCR-RFLP	70	positive for parasitological examination	36	106	Yes	FC	1
Ker (2019)	Brazil	NI	Frequentist	negative for DPP, ELISA and PCR-RFPL	30	positive for DPP, ELISA and PCR-RFLP	60	90	Yes	FC	3
Lage (2016)	Brazil	NI	Frequentist	negative for IFAT, ELISA and PCR	124	positive for IFAT, ELISA and PCR	53	177	Yes	iELISA	6
Ledesma (2017)	Spain and United Kingdom	NI	Frequentist	negative for IFAT and qPCR	25	positive for two ELISA and qPCR	45	70	No	iELISA	1
Lemos (2008)	Brazil	NI	Frequentist	negative for ELISA and parasitological examination	33	positive for parasitological examination	76/ 16/ 17/ 43	109/ 49/ 50/ 106	Yes and No	iELISA and ICT	8
Llera (2002)	Spain	NI	Frequentist	negative for IFAT *L. infantum*, IFAT *E. canis* and IFAT *E. canis* and *L. infantum*	40	positive for IFAT *L. infantum*, IFAT *E. canis* and IFAT *E. canis* and *L. infantum*	120	160	No	IFAT	2
Maia (2009)	Portugal	NI	Frequentist	negative for culture in all organs (liver, bone marrow and lymph	87/ 69	positive for culture in any of the organs (liver, bone marrow and lymph nodes)	35/ 26	122/ 95	Yes	CIE, IFAT and *c*PCR	4

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
				nodes)							
Marcelino (2020)	Brazil	NI	Frequentist	negative for serological tests and parasitological tests	10	positive for DPP, ELISA and microscopy, culture or immunohistochemistry	65	75	No	*c*PCR	20
Marcondes (2011)	Brazil	NI	Frequentist	negative for serological tests and parasitological tests	117	positive for IFAT, ELISA and microscopy	283	400	No	iELISA	1
Maurielli (2020)	Italy	2018-2019	Frequentist	negative for all the techniques serological and molecular	NI	positive for two or more techniques serological and molecular	NI	60	Yes	iELISA, ICT, IFAT, *q*PCR and LAMP	5
Nogueira (2018)	Brazil	NI	Frequentist	negative for IFAT, ELISA and parasitological examination	50	positive for IFAT, ELISA and parasitological examination	74	124	Yes	iELISA	1
Oliveira (2016)	Brazil	2013	Frequentist	negative for IFAT and ELISA	66	positive for IFAT, ELISA and culture	34	100	No	DAT	1
Portela (2019)	Brazil	NI	Frequentist	negative for parasitological examination and PCR	68/ 98	positive for parasitological examination and PCR	97	165/ 195	Yes	iELISA and ICT	4
Ribeiro (2015)	Brazil	2010-2013	Frequentist	negative for ELISA and IFAT and/or parasitological examination and molecular test	76	positive for parasitological examination and PCR	40	116	Yes	iELISA, ICT IFAT, FAST-DAT and DAT	5
Ribeiro (2019)	Brazil	NI	Frequentist	negative for ICT, DAT, IFAT and ELISA	47/ 45/ 46	positive for isolation and PCR	142/ 139/ 140	189/ 184/ 189	No	iELISA and IFAT	6
Rodríguez-Cortés (2013)	Spain	NI	Frequentist	negative for ELISA and PCR	19	positive for ELISA and PCR	41	60	No	ELISA, ICT and IFAT	7
Salles (2019)	Brazil	NI	Frequentist	negative for IFAT and ELISA	145	positive for IFAT, ELISA and PCR	55	200	Yes	iELISA	3

First Author (Year)	Country	Years	Model	Reference standard (negative)	N Neg	Reference standard (positive)	N Pos	Total animals	Asymptomatic animals	Index tests	N index test
Scalone (2002)	Italy and USA	NI	Frequentist	negative for IFAT and parasitological tests	81	positive for parasitological test	209	290	No	iELISA	1
Schubach (2014)	Brazil	2009	Frequentist	negative for histopatology, culture and immunohistochemical	281/ 308/ 301	positive for histopatology or culture or immunohistochemical	24/ 25/ 11/ 4	305/ 333/ 312/ 305/ 310/ 325	Yes and No	ICT	7
Silvestre (2008)	NI	NI	Frequentist	negative IFAT and ELISA	44	positive for microscopy or culture	56	100	Yes and No	FC	6
Solano-Gallego (2014)	Italy, United Kingdom, Cyprus and Spain	2011-2012	Frequentist	negative for two ELISA and qPCR	32	positive for two ELISA and qPCR	36	68	No	ELISA, IFAT and ICT	5
Solca (2014)	Brazil	2010	Bayesiana	NA	NA	NA	NA	NA	No	ICT, culture, *q*PCR	3
Teixeira (2019)	Brazil	2015-2017	Frequentist	negative for microscopy, culture, PCR and qPCR	54	positive for microscopy, culture, PCR or qPCR	69	123	Yes	iELISA, ICT and DAT	4
Toledo-Machado (2015)	Brazil	NI	Frequentist	negative for IFAT and microscopy	38	positive for IFAT, ELISA and microscopy	38	76	No	iELISA	6
Toz (2004)	Turkey	1996-2000	Frequentist	negative for IFAT and microscopy	10	positive for IFAT and microscopy	11	21	No	ICT	1
Vercammen (1998)	Belgium	NI	Frequentist	negative for IFAT, slide-ELISA and/or DAT	38	positive for IFAT, slide-ELISA and DAT	40	78	No	dot-ELISA	1

Supplementary Table S7. Most commonly used antigens in indirect diagnostic techniques.

Indirect Test	Antigen	Absolute Frequency (n)	Relative Frequency (%)
iELISA	*L. infantum*	20	9.17
	rK28	16	7.34
	SLA	12	5.50
	rA2	8	3.67
	L. major	5	2.29
	NI	5	2.29
	rK39	4	1.83
	Lci1	3	1.38
	Lci12	3	1.38
	Lci2	3	1.38
	Lci4	3	1.38
	Lci5	3	1.38
	Lci8	3	1.38
	PepL1	3	1.38
	PepL1 plus PepLi2	3	1.38
	PepL15	3	1.38
	PepL2	3	1.38
	PepL3	3	1.38
	PepL6	3	1.38
	PepL7	3	1.38
	PepLi1 plus PepLi2 plus PepLi7	3	1.38
	PepLi1 plus PepLi7	3	1.38
	PepLi2 plus PepLi7	3	1.38
	peptide - *L. infantum*	3	1.38
	PQ10	3	1.38
	PQ20	3	1.38
	rC9	3	1.38
	rFc	3	1.38
	rK26	3	1.38
	rLci5	3	1.38
	C9	2	0.92
	L. amazonensis	2	0.92
	L. donovani	2	0.92
	LPG	2	0.92
	MIX 10	2	0.92
	PSLc10	2	0.92
	PSLc6	2	0.92
	PSLc8	2	0.92
	5	1	0.46
	6	1	0.46
	11	1	0.46
	A1	1	0.46

Indirect Test	Antigen	Absolute Frequency (n)	Relative Frequency (%)
iELISA	A8	1	0.46
	A9	1	0.46
	Ag	1	0.46
	C1	1	0.46
	C18 (mix C1 + C8)	1	0.46
	C7	1	0.46
	C8	1	0.46
	C-CPB	1	0.46
	D9	1	0.46
	E5	1	0.46
	E7	1	0.46
	E8	1	0.46
	F1	1	0.46
	F10	1	0.46
	F12	1	0.46
	F45	1	0.46
	F-CPB	1	0.46
	G7	1	0.46
	G8	1	0.46
	H1	1	0.46
	H5	1	0.46
	H7	1	0.46
	H9	1	0.46
	Leishmania spp.	1	0.46
	LRPs	1	0.46
	N-CPB	1	0.46
	PepL10	1	0.46
	PepL11	1	0.46
	PepL12	1	0.46
	PepL13	1	0.46
	PepL14	1	0.46
	PepL16	1	0.46
	PepL17	1	0.46
	PepL4	1	0.46
	PepL5	1	0.46
	PepL8	1	0.46
	PepL9	1	0.46
	Pep-Mix (5/6/11)	1	0.46
	PRF1	1	0.46
	PSLc1	1	0.46
	PSLc2	1	0.46
	PSLc3	1	0.46
	PSLc4	1	0.46

	PSLc5	1	0.46
Indirect Test	**Antigen**	**Absolute Frequency (n)**	**Relative Frequency (%)**
	PSLc7	1	0.46
	PSLc9	1	0.46
	rCcOx	1	0.46
	rCcOx plus rHRF	1	0.46
	rHRF	1	0.46
	rKLO8	1	0.46
	rKLO8+ rK26	1	0.46
	rLc36	1	0.46
	rLci1A	1	0.46
	rLci2B	1	0.46
	rLiHyD	1	0.46
	RP	1	0.46
	rSGT	1	0.46
	rSMP-3	1	0.46
	TLA	1	0.46
	Total	218	100.00
ICT	rK28	32	64.00
	rK39	7	14.00
	L. infantum	4	8.00
	NI	2	4.00
	PQ10	1	2.00
	PQ20	1	2.00
	rA2	1	2.00
	rC9	1	2.00
	rFc	1	2.00
	Total	50	100.00
MAPIA	rLci10	2	7.69
	rLci11	2	7.69
	rLci12	2	7.69
	rLci13	2	7.69
	rLci1A	2	7.69
	rLci1A + rLci2B + rLci3 + rLci4 + rLci4 + rLci5 + rLci6 + rLci7 + rLci8 + rLci10 + rLci11 + rLci12 + rLci13	2	7.69
	rLci2B	2	7.69
	rLci3	2	7.69
	rLci4	2	7.69
	rLci5	2	7.69
	rLci6	2	7.69
	rLci7	2	7.69
	rLci8	2	7.69
	Total	26	100.00
IFAT	*L. infantum*	13	59.09

	NI	5	22.73
Indirect Test	**Antigen**	**Absolute Frequency (n)**	**Relative Frequency (%)**
IFAT	*L. major*	3	13.64
	E. canis and L. infantum	1	4.55
	Total	22	100.00
FC	L. infantum	7	53.85
	A4–rLci1A	2	15.38
	A4–rLci1A/E4–rLci2B	2	15.38
	E4–rLci2B	2	15.38
	Total	13	100.00
DAT	*L. infantum*	7	63.64
	L. donovani	4	36.36
	Total	11	100.00
dot-ELISA/iELISA	*L. infantum*	2	100.00
	Total	2	100.00
FAST-DAT	*L. donovani*	1	100.00
	Total	1	100.00
PaGIA	rK39	1	100.00
	Total	1	100.00
LAT	LeQuiDi	1	100.00
	Total	1	100.00
CIE	NI	1	100.00
	Total	1	100.00

NI: uninforme

Supplementary Table S8. Grouped studies for sensitivity calculation

Category	Subcategory	N studies	N assays	References
ICT_rK28	Asymp	4	4	Borja_2018; Figueiredo_2018; Grimaldi_2012; Schubach_2014;
ICT_rK28	Symp	8	12	Borja_2018; De Souza_2016; Figueiredo_2018; Fonseca_2014; Grimaldi_2012; Ribeiro_2015; Schubach_2014; Solca_2014
ICT_rK28	Asymp+Symp	10	14	Borja_2018; De Souza_2016; Figueiredo_2018; Fonseca_2014; Fraga_2016; Grimaldi_2012; Portela_2019; Ribeiro_2015; Teixeira_2019; Schubach_2014
ICT_rK39	Symp	3	5	Lemos_2008; Ribeiro_2019; Toz_2004
iELISA_SLA	Asymp	2	2	Chávez-Fumagalli_2013; Lemos_2008
iELISA_SLA	Symp	2	4	Chávez-Fumagalli_2013; Lemos_2008
iELISA_SLA	Asymp+Symp	6	6	Coelho_2009; Coelho_2016; Dias_2017; Lage_2016; Lemos_2008; Salles_2019
iELISA_rC9	Asymp+Symp	3	3	Costa 2014; Dos Santos_2019; Fonseca_2014
iELISA_*L.infantum*	Symp	5	9	De Souza_2019; Fonseca_2019; Ribeiro_2019; Solano-Gallego_2014; Solca_2014
iELISA_*L.infantum*	Asymp+Symp	2	3	Belinchón-

				Lorenzo_2013; Do Rosário_2005
iELISA_*L.major*	Asymp+Symp	4	4	De Arruda_2013; De Arruda_2016, Fonseca_2015; Ribeiro_2015
iELISA_rA2	Asymp+Symp	6	6	Coelho_2016; Dias_2017; Dos Santos _2019; Jusi_2015; Lage_2016; Salles_2019
iELISA_rK28	Symp	2	3	Borja_2018; Toledo-Machado_2015
iELISA_rK28	Asymp+Symp	5	6	Borja_2018; De Arruda_2013; Faria_2011; Fraga_2016; Teixeira_2019
iELISA_rK39	Asymp+Symp	2	3	Do Rosário_2005; Teixeira_2019
IFAT_*L. infantum*	Symp	2	3	Ribeiro_2019; Solano-Gallego_2014;
IFAT_*L. infantum*	Asymp+ Symp	3	4	Belinchón-Lorenzo_2013; Ferreira_2007; Llera_2002
IFAT_*L. major*	Asymp+Symp	2	2	De Arruda_2016; Ribeiro_2015
DAT_*L. donovani*	Asymp+Symp	2	3	Ferreira_2007; Ribeiro_2015
DAT_*L. infantum*	Symp	2	5	Oliveira_2016; Teixeira_2019
FC_*L. infantum*	Asymp+Symp	2	3	Ker_2013; Silvestre_2008

Supplementary Table S9. Grouped studies for specificity calculation

Category	Subcategory	N studies	N assays	References
ICT_rK28	CR+ Neg	2	2	Alves_2012; Grimaldi_2012
ICT_rK28	Neg	15	30	Alves_2012; Borja_2018; De Souza_2016; De Souza_2019; Fraga_2016; Figueiredo_2018; Fonseca_2014; Fonseca_2019; Grimaldi_2012; Portela_2019; Ribeiro_2015; Ribeiro_2019; Schubach_2014; Solca_2014; Teixeira_2019
ICT_rK39	Neg	3	6	Lemos_2008; Ribeiro_2019; Toz_2004
ICT_*L. infantum*	Neg	2	2	Solano-Gallego_2014; Rodríguez-Cortés_2013
iELISA_*L. infantum*	CR	2	4	Barral-Veloso_2013; Solano-Gallego_2014
iELISA_*L. infantum*	Neg	9	13	Barral-Veloso_2013; Belinchón-Lorenzo_2013; De Souza_2019; Do Rosário_2005; Fonseca_2019; Marcondes_2011; Ribeiro_2019; Solano-Gallego_2014
iELISA_L. infantum	CR + Neg	2	2	Alves_2012; Marcondes_2011
iELISA_L. major	Neg	5	5	De Arruda_2013; De Arruda_2016; Fonseca_2014; Ribeiro_2015; Solca_2014
iELISA_rA2	Neg	2	4	Dos Santos _2019; Jusi_2015
iELISA_rA2	CR + Neg	4	4	Coelho_2016; Dias_2017; Lage_2016, Salles_2019
iELISA_rK28	Neg	7	14	Alves_2012;

				Arruda_2016; Borja_2018; De Arruda_2013; Fraga_2016; Faria_2011; Teixeira_2019
iELISA_rK28	CR + Neg	2	2	Alves_2012; Toledo-Machado_2015
iELISA_SLA	Neg	3	7	Chávez-Fumagalli_2013; Coelho_2009; Lemos_2008
iELISA_SLA	CR + Neg	4	4	Coelho_2016; Dias_2017; Lage_2016; Salles_2019
iELISA_PQ10	Neg	2	3	Faria_2015; Fonseca_2019
iELISA_PQ20	Neg	2	3	Faria_2015; Fonseca_2019
iELISA_rK26	Neg	2	3	Abad_2017; Do Rosário_2005
iELISA_rK39	Neg	3	4	Do Rosário_2005; Scalone_2002; Teixeira_2019
iELISA_rC9	Neg	2	4	Dos Santos_2019; Fonseca_2014
IFAT_*L. infantum*	CR	2	2	Solano-Gallego_2014; Ferreira_2007
IFAT_*L. infantum*	Neg	5	5	Belinchón-Lorenzo_2013; Ferreira_2007; Ribeiro_2019; Rodríguez-Cortés_2013; Solano-Gallego_2014
IFAT_*L. infantum*	CR + Neg	2	2	Alves_2012; Llera_2002
IFAT_*L. major*	Neg	3	3	Arruda_2016; De Arruda_2016; Ribeiro_2015
DAT_*L. donovani*	Neg	3	3	Da Silva_2006; Ferreira_2007; Ribeiro_2015
DAT_*L. infantum*	Neg	2	5	Oliveira_2016; Teixeira_2019
FC_*L.infantum*	Neg	2	3	Ker_2013; Silvestre_2008

ARTIGO 2

This paper is a preliminary version, prepared following the guidelines of the *Journal of Veterinary Science* (JCR 1.8) to which it will be submitted later. The journal's editorial board may suggest changes to suit its style.

ALERT TO HEALTH PROFESSIONALS AND AUTHORITY FOR THE FIRST REPORT OF VISCERAL LEISHMANIASIS IN ABADONADO DOGS IN CENTRAL COLOMBIA

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Conflict of Interest

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ABSTRACT

This study aimed to diagnose the visceral leishmaniasis (VL) in abandoned dogs from the central region of Colombia and the identification of species in circulation of *Leishmania*. For this, Canines from a shelter were sampled by convenience at four different moments for laboratory testing, including, serological, molecular, and sequencing analysis. For serological diagnosis, the immunochromatographic test rKDDR-plus (ICT-rKDDR-plus) and the immunoenzymatic assay rKDDR-plus (ELISA-rKDDR-plus) were used. For molecular diagnosis, a real-time polymerase chain reaction (RT-PCR) was realized using the GAPDH gene, and a conventional polymerase chain reaction (PCR) was conducted using the genes RV1-RV2 for amplified *Leishmania* spp. and finally the multiplex PCR was conducted to amplified five species of *Leishmania* spp. The product of PCR using the gene RV1-RV2 was used for genomic sequencing and bioinformatic analysis. The seropositivity using both tests showed 25.3% and 37.5% in the dogs of the first and second sampling, respectively. These animals also showed relevant clinical and laboratory findings consistent with VL.40 samples were used for PCR with the GAPDH gene, 46.5% were positive. Of these, 19 were selected for RV1-RV2 PCR, of which 10.5% were positive, and 17 were selected for multiplex PCR, of which 11.8% were positive for *L. infantum*. The same positive samples in RV1-RV2 PCR were also positive in multiplex PCR and were sent for genetic sequencing, which showed 99.0% similarity to *L. infantum*. Thus, this study confirmed for the first time the presence of *L. infantum* in the central region of Colombia through serological and molecular methods, this emphasizes the need to develop effective control strategies to prevent this neglected disease.

KEYWORDS: Parasitic diseases, Zoonosis, Serology, Molecular, South America.

INTRODUCTION

Leishmaniasis is a neglected zoonosis caused by obligate intracellular protozoa of the *Leishmania* species. In mammalian hosts, two main forms of the disease are recognized and called, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL), with VL being the most severe systemic form of the disease. In South America, *L. infantum* is the most common species involved in VL and the sandflies of the species *Lutzomyia* are the main vector in rural and urban scenarios (1, 2). Currently, the epidemiology of leishmaniasis is changing, traditionally it was reported worldwide except in Oceania, but nowadays there are new scenarios of emergence. Previous findings showed that tropical developing countries are mainly affected by this disease, however, the new scenarios of dispersion appear according to climate change, movement of human populations and reservoirs, urbanization, and social inequalities that facilitate the epidemiological outbreaks. In these dispersion factors, the movement of infected dogs from tropical and subtropical regions to temperate regions without the vector and infected host contributes to leishmaniasis emergence and the involvement of dogs as the primary reservoir of VL (2, 3). To minimize the transmission of the protozoan and reduce the occurrence of the disease, reservoirs and circulating species of parasites must be identified, essentially to diagnose the early incidence of disease in a territory (4, 5).

Leishmaniasis in the Americas is no longer exclusive to wild and rural areas but is now spreading to large urban scenarios (6). In Colombia, some reports demonstrated the increase of urban leishmaniasis in the coexistence with *Lutzomyia* insects and dogs (7-10). This country ranks as second in the incidence of CL, right after Brazil, a neighboring country, in which, in addition to CL, it is endemic for VL and the control of leishmaniasis is challenging (11-13). In this country, military operations cover the entire

length of the country and commonly involve dogs, and there are reports of outbreaks in different regions involving humans and military dogs caused by *L braziliensis* and *L. panamensis* with CL (11, 13).

However, canine Leishmaniasis in Colombia is underdiagnosed, and most of the epidemiologic evidence comes from research, with less routine surveillance by authorities (11). This suggests the existence of *Leishmania* spp. in other country areas without a diagnosis. Recently, a study showed a seroprevalence of 12% for leishmaniasis in dogs with cutaneous and visceral lesions, but it is not known about clinical findings and the species of protozoan involved in the central region (14). Similarly, in dogs species such as *L. amazonensis* traditionally associated with CL could promote clinical signs of VL, indicating an interface between *Leishmania* species (5). These findings indicate the importance of semiological, clinical and molecular analysis of canine leishmaniasis in the new scenarios of presentation. Similarly, the identification of species that could circulate throughout the territory is important to establish control and prevention measures (4, 5, 15). Therefore, the objective of this study is to carry out the serological and molecular diagnosis of VL in dogs under conditions of abandonment in the central region of Colombia and to determine for first time the species that circulate in the territory.

MATERIAL AND METHODS

Declaration of ethics

The research proposal was approved by the Bioethics Committee of the University of Applied and Environmental Sciences (UDCA) nº 001/2019. It also had the verbal consent of the authorities of the Albergue Canine of Girardot-Cundinamarca.

Study location

The study was carried out at the Albergue Canine of Girardot-Cundinamarca located in the municipality of Girardot, in the Department of Cundinamarca, Alto Magdalena Province, Colombia, from January 2020 to March 2023. The city of Girardot is located 134 km from the capital Bogotá, it is considered one of the cities with the largest floating population in the country. The average temperature is 33.3ºC and the relative humidity is 66% (16). The country has a high incidence of CL and has few studies on VL, and it has a territorial extension of 1664.2 km with Brazil, which is endemic for VL (11, 12, 17). Another important factor to consider is that this shelter is close to the region of the military kennel, where an outbreak of CL has already been reported (11, 13).

Sample collection

The samples were collected at four different moments, in which a prospective cohort case study was carried out at the population level, in this way, the serological diagnosis was made at different times and the persistence of antibodies was verified, indicating the presence of the etiological agent in this population. To collect these samples, a non-probabilistic convenience sample was performed.

The collection of samples took place at different times due to the availability of the team. In the first collection, the clinical signs of the dogs were evaluated and blood samples were collected in a tube without anticoagulant for serology. In the second

collection, blood was collected in EDTA tubes for a hemogram and a tube without anticoagulant for serology. In the third collection, blood was collected in a tube without anticoagulant for serology. In the fourth collection, a bone marrow puncture was performed for molecular diagnosis and later, genetic sequencing.

All collections take place in the kennel itself, with a team of Veterinarians and Veterinary Medicine students duly trained and qualified to collect the samples. Animal data, physical examination, and parameters are noted (Appendix 1). For bone marrow collection from dogs, the animals were submitted to general anesthesia with 0.5 mg/kg of intramuscular xylazine hydrochloride and 2 to 5 mg/kg of intramuscular ketamine. Subsequently, for analgesia of these animals, 25 mg/Kg of Dipyrone Sodium intramuscular was applied.

Clinical evaluation

Clinical examination was performed and the values were recorded in a standardized model (Appendix 1). In all collections, data such as animal number, name, date, age, sex, species, breed, weight, coat, and color for each animal were noted, in addition, photographs of each canine were taken. The physical examination of these animals, such as heart rate, respiratory rate, rectal temperature (ºC), dehydration (%), capillary reperfusion time, mucous membranes, state, body score, and the presence of alterations in lymph nodes, dermatological alterations, ocular alterations, and presence of ectoparasites.

Laboratory analysis

Blood samples were processed at the UDCA for laboratory analysis, including a complete blood count and biochemical profile (urea, ALT, AST, GGT, total proteins, and plasma proteins), and serum was also extracted. Bone marrow samples were subjected to DNA extraction for molecular diagnosis.

The blood count was evaluated for hematocrit (%), hemoglobin (g/dL), mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin concentration (MCHC) (g/dL), mean corpuscular hemoglobin (MCH) (pg), platelets (x 106 cells/mL), total leukocytes (cells/mL), lymphocytes (cells/mL) and reticulocytes (%). The biochemical profile evaluated urea (mg/dL), alanine aminotransferase (ALT) (U/L), aspartate aminotransferase (AST) (U/L), alkaline phosphatase (AP) (U/L), gamma-glutamyl transpeptidase (GGT) (U/L), total proteins (g/dL) and plasma proteins (g/dL). The hematological and biochemical profile was made using HUMAN kits and equipment.

Serum samples were sent lyophilized to the Laboratory of Immunology and Control of Parasites (LICP) of the Institute of Biological Sciences of the Universidade Federal de Minas Gerais (UFMG), in Brazil.

Serological diagnosis

During data collection and sampling, the ICT-rKDDR-plus was performed using blood samples, only in the sampled process was possible. In cases where this was not possible, this test was performed in LICP with serum samples lyophilized. This test uses an antigen derived from recombinant *L. infantum* kinesin produced at LICP and currently sold by ECO Diagnóstica (Brazil). The test consists of an initial region, which has a pad for application of a release membrane conjugated with colloidal gold nanoparticles bound to rKDDR-plus mouse antigen or IgG antibody, and a final region, which has an absorbent pad. To read the test, formed lines were observed, in the positive result there is the formation of two lines (control and test) and in the negative result, there is the formation of only the control line. If there is not a formation of the control line, this test is considered invalid. This test has satisfactory results in VL tests in Brazil (18).

The serum sent to Brazil was lyophilized and, upon arrival, it was resuspended to perform the ELISA-rKDDR-plus in the LICP. The ELISA test is recommended by the Brazilian Ministry of Health for confirming animals that are positive in immunochromatographic or indirect immunofluorescence tests. Thus, the ELISA was also performed in duplicate following a protocol of three consecutive days. On the first day, each well of the 96-wells microtiter plate (Costar, Cambridge, MA, USA) was sensitized with 100 ng of rKDDR-Plus antigen and incubated overnight, for at least 12 hours at 4°C. On the second day, the plate was washed 3 times with 200 µl of washing solution (PBS-0.05% Tween 20) and, subsequently, 200 µl of nonspecific site blocking solution (PBS-0.5% + BSA 1 %) was added to the board. After incubation for 60 minutes at room temperature (26°C) the plate waspoured to discard the washing solution. Soon after, 100 µl of cat sera diluted 1:100 in PBS-0.05% Tween 20 wasadded in each well and then incubated overnight, for at least 12 hours at a temperature of 4°C. The controls (2 positive samples, 3 negative samples, and 2 blanks) were placed in the last wells of each plate. On the third and last day, each plate was washed five times with 200 µl of washing solution (PBS-0.05% Tween 20). The Anti-IgG conjugate - Peroxidase produced in rabbit (Bio-rad - HRP AAI26P) was diluted in 1:5000 in PBS-0.05% Tween 20, then 100 µl of diluted conjugate was applied to each well of the plate and incubated for 60 minutes at room temperature. Each plate was washed five times with 200 µl of washing solution (PBS-0.05% Tween 20), and 100 µl of developing solution containing 0.1 M citric acid, 0.2 M Na2PO4, OPD was added to each well 0.05% and H2O2 0.1%. After incubation for 20 minutes at room temperature and in a dark place, the reaction ended with the addition of 50 µl of H2SO4 (4N) (sulfuric acid), finally the reading was performed using a wavelength of 492 nm. For each plate, the cut-off point was determined from the average of the absorbance readings of the six

negative sera, plus twice the standard deviation. The mean absorbance of the tested sera was divided by the cut-off value found in each plate, to determine the reactivity index (RI), thus normalizing the optical density (OD) values. Therefore, seras with RI>1.0 were considered positive and IR<1.0 were considered negative. The reactions were read in an ELISA reader at 492 nm and the results expressed in absorbance values.

Molecular diagnosis

Samples of bone marrow, lymph nodes, and white blood cells had their DNA extracted using the Wizard Genomic DNA Purification kit™ (Promega, Madison, USA) were used for molecular diagnosis and, consequently, confirmation of parasitic circulation in that kennel. Conventional polymerase chain reaction (PCR) and real-time chain reaction (RT-PCR) were performed at UDCA to detect deoxyribonucleic acid (DNA) from *Leishmania* spp.

Immediately after DNA extraction, RT-PCR was performed with an endogenous gene to verify the viability of DNA after extraction. For this, the endogenous GAPDH F (5'-GCCGTGGAATTTGCCGT-3') and GAPDH R (3'-GCCATCAATGACCCCTTCAT-5') described by Leutenegger, Mislin (19) were used, employing the thermocycling amplification involving an initial denaturation cycle at 95 °C for three minutes, and then 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, followed by the final extension step at 72 °C for seven minutes using Light-Cycler 96® (Roche Diagnostics, Switzerland).

Subsequently, we used primers that target the highly reiterated minicircle of kinetoplast DNA (kDNA) (mitochondrial) and amplified *L. donovani* sensu lato (RV1-RV2). This primer performs well because it is extremely sensitive detecting 10−3 parasites per ml of blood and has an expected amplicon of 145 bp for all sequences,

forward 5' CTTTTCTGGTCCCGCGGGTAGG 3' and reverse 5' CCACCTGGCCTATTTTAC 3'. The PCR cycle conditions were adjusted according to the protocol: initial annealing at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, 35 cycles of 59ºC for 30 s, 35 cycles of 68 ºC for 30 s followed by a final elongation at 68 ºC for 5 min (20). All reactions in all PCR assays were used as a no template control, positive control, and negative control. The no template control was without the presence of DNA, the positive control was with a sample known to be positive through prior genetic sequencing, and the negative control was with a negative sample in sequencing.

The multiplex PCR of *Leishmania* sp. was also carried out with primers developed in the LICP under study, this technique allows genotyping, since they are molecular markers. Thus, it is composed of five primers, primers Lam KE specific for *L. amazonesis*, Lbr 19 specific for *L. braziliensis*, Ld 33 specific for *L. donovani* and *L. infantum*, Lguy 12 specific for *L. guyanensis* complex and Lm 13 specific for *L. mexicana*. After the samples have been prepared and placed in a 96-well plate, a thermocycler T100 (Bio-Rad, California, USA) is used with a protocol consisting of initial annealing at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30s, 35 cycles of 59ºC for 30 s, 35 cycles of 68 ºC for 30 s followed by a final elongation at 68 ºC for 5 min.

Sequencing analysis

Genomic sequencing was performed to characterize the species of *Leishmania* spp. present in the city of Girardot, Colombia. With the bone marrow samples obtained previously, sequencing by the Sanger method of the RV1-RV2 PCR product was performed. The sequences obtained were analyzed and the positions with a Phred score >20 were considered to assemble the consensus sequence using the BioEdit 7.2.5

281 software. Subsequently, these sequences were aligned using the Blast nt tool in
282 GenBank and compared with sequences GenBank® homologs were then downloaded
283 and aligned using the Clustal W tool in BioEdit Sequence Alignment Editor 7.2.5 (21).
284 MEGA X software (22) was used to determine the evolutionary model and build a
285 maximum likelihood tree with 1000 Bootstrap replicates.

286 **Statistical analyzes**

287 All data were computed in an Excel spreadsheet. Association analyses were
288 performed between the serological diagnosis and the clinical and laboratory variables of
289 dogs using the R software version 4.2.0 (23). The tabulation of responses was processed
290 using the Independence Test (with test statistics: Chi-square) per question and per
291 course to observe whether there is an association between two qualitative variables
292 measured in the same sample unit (dog).

293 According to Pimentel (2009), the Independence Test is used for large samples,
294 considered above 20. To verify if there is statistical significance between them, it
295 considered the significance value of 10%. Results above 10% means that there is no
296 association between the variables. The following are the Independence test statistics:

297 $$\chi^2 = \Sigma_{i=1}^{I}\Sigma_{J=1}^{J}\frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

298 on what:

299 O_{ij} is the total number of observations in the cell (columns and rows *i* and *j*);

300 E_{ij} is the expected frequency in column *i* in row *j*;

301 I is the *i*-th row of the table;

302 J is the jth column of the table;

303 χ^2 is the chi-square statistic of the Independence test.

In the present study, the non-parametric test of Independence (Chi-square) was performed using the chisq.test function of the stats package of the statistical program R(23). Graphs were made with the ggstatsplot and ggplot2 packages of the statistical program R.

When applying the χ^2 test to i x j contingency tables, it is necessary to consider the following restrictions;

The minimum expected frequency must not be less than 1;

Only in a few cases is the expected frequency less than 5.

For cases where the assumptions above are not met, Fisher's test was used, which performs the exact test of independence of rows and columns in a contingency table with fixed margins. For this reason, Fisher's test was used, in which it performs the exact test of independence of rows and columns in a contingency table with fixed margins. In the present study, Fisher's test was performed using the Fisher test function of the stats package of the R statistical program (23). Graphs were made with the ggstatsplot and ggplot2 packages of the statistical program R.

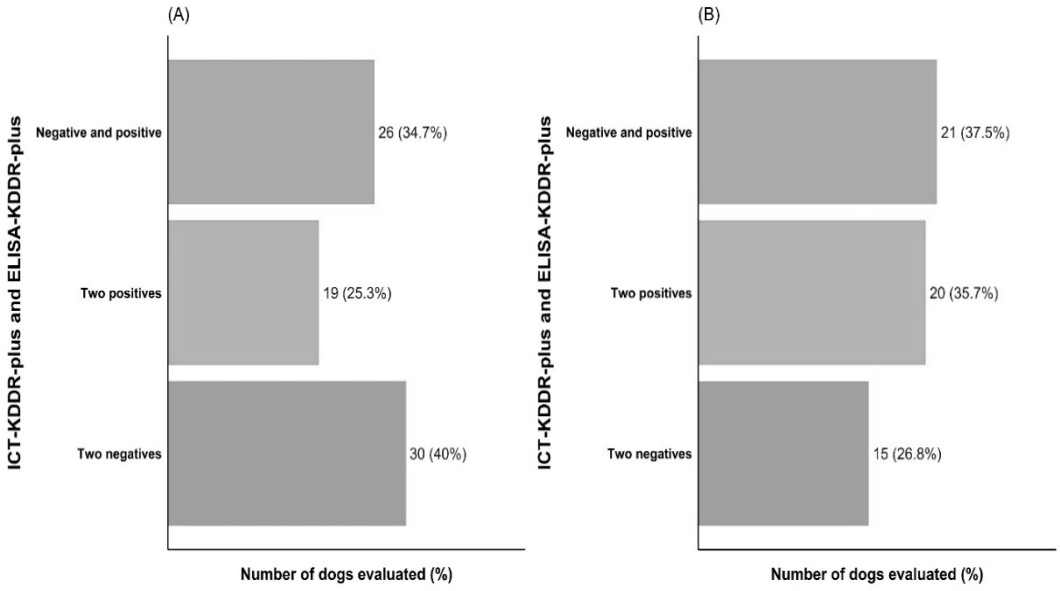
After evaluating the global association through the Chi-square test, Cramer's V was used to measure the degree of association between the ELISA-rKDDR-PLUS test and the variables considered statistically significant in the Independence test. Since, “the values were analyzed in an interval between 0 and 1, in which the value 1 indicates the maximum relationship between the variables and 0 the absence of relationship (24).

RESULTS

Association analysis with clinical findings

Considering that the first two collections of canines were obtained in two stages for clinical and laboratory assessment. In the first stage, a physical examination was performed, and blood samples were obtained from 75 dogs. In the second stage, blood samples were obtained from 56 dogs for laboratory analysis and serology. Serological diagnosis by both tests using dogs sampled for the first time showed seropositivity of 25.3% (19/75), seronegativity of 40% (30/75), and discordant results of 25.3% (19/75) (Graph 1A). Similarly, the second sampling showed seropositivity of 35.7% (20/56), seronegativity of 26.8% (15/56), and discordant results of 35.7% (20/56) (Graph 1B).

Graph 1: Dogs evaluated in the serological diagnosis, ICT-rKDDR-plus, and ELISA-rKDDR-plus, in both samplings.



Subtitle: Absolute and relative total of positive animals in serological tests ICT-rKDDR-plus and ELISA-rKDDR-plus in both samplings. A) First sampling, collection of clinical signs, and serological diagnosis. B) Second sampling, collection of laboratory tests, and serological diagnosis.

342 All positive animals showed at least one clinical associated with positivity of
343 ICT-rKDDR-plus, in a significance level of 10.0% showing association with body
344 condition score condition (CC/5), ticks and the ELISA-rKDDR-plus test, with the *p*-
345 values of 0.0811, 0.0916 and 0.0203, respectively (Table 1).
346

347 Table 1 - Summary of the Independence test for ICT-rKDDR-plus data × clinical signs
348 of leishmaniasis.

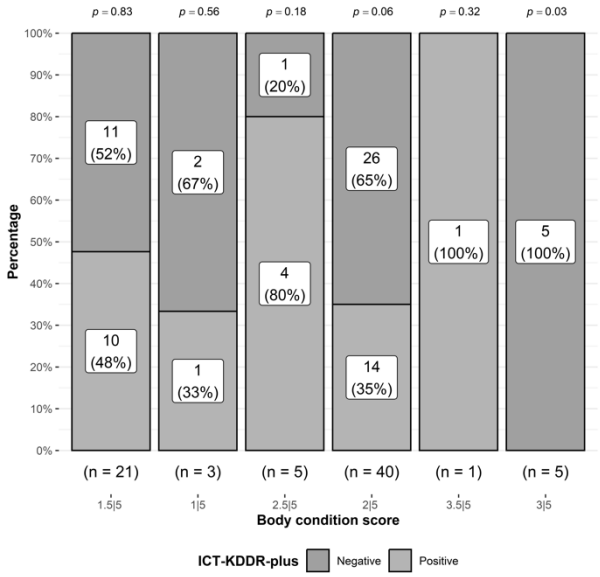
ICT-rKDDR-plus × Clinical signs of CVL	χ^2	**Fisher Exact Test[1]**	**G.L**	**p-value**
Sex	1.7578	-	1	0.1849^{ns}
Age Group	<0.0001	-	1	1.0000^{ns}
Temperature	-		-	1.0000^{ns}
Membranes colours	-		-	0.5146^{ns}
Enlarged lymph nodes	<0.0001	-	1	1.0000^{ns}
CC/5	-		-	**0.0811***
Body condition score	-		-	0.2315^{ns}
Crusts	2.4554	-	1	0.1171^{ns}
Ulcers	1.3951	-	1	0.2375^{ns}
Peeling	0.0217	-	1	0.8829^{ns}
Eythema	0.0028	-	1	0.9581^{ns}
Alopecia	0.2968	-	1	0.5859^{ns}
Dermatological signs	0.0037	-	1	0.9518^{ns}
Fleas	-		-	0.3834^{ns}
Ticks	**2.8454**	-	**1**	**0.0916***
Ectoparasites	2.3572	-	1	0.1247^{ns}
Onychogryphosis	0.4075	-	1	0.5232^{ns}
Conjunctivitis	-		-	1.0000^{ns}
Keratitis	-		-	0.4275^{ns}
Uveitis	-		-	0.5085^{ns}
Eye injuries	0.9160	-	1	0.3385^{ns}
Haemorrhages	-		-	0.7074^{ns}
Presence of other clinical signs	<0.0001	-	1	1.0000^{ns}

ELISA-rKDDR-plus	**5.3824**	**-**	**1**	**0.0203***

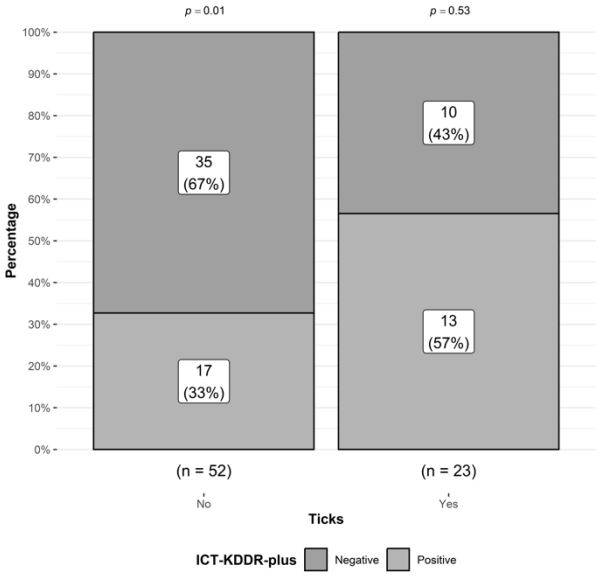
χ^2 is the chi-square statistic of the Independence; G.L: degrees of freedom (number of columns – 1) (number of rows –1); *: significant at 10%; ns: not significant; 1 Fisher's exact test is the calculation of the probability, expressed in the p-value column.

Considering only the seropositivity for the ICT-rKDDR-plus test there was a correlation between body condition score (CC/5) and ticks (Graph 2). The correlation solely between ICT-rKDDR-plus and body condition score (CC/5) exhibited a significant difference in classification score of 2 (p-value = 0.0580), with 35.0% (14/40) testing positive and 65.0% (26/40) testing negatively. The test exhibited a substantial difference in all dogs (5/5) (100.0%) (Graph 2A) when associated with a seronegative body condition score (CC/5) classification of 3 (p-value = 0.0250). In contrast, no significant difference was observed with the other scores. Furthermore, V-Cramer's result indicated an association between ICT-rKDDR-plus and a body condition score of 0.3492 (34.9%), a moderate correlation. Based on the results, there is a correlation between tick infestation and dogs who tested seronegative through the ICT-rKDDR-plus test. Out of the 52 dogs in this study, 35 out of 52 dogs tested negative for the disease showed no infestation, while 17 out of 52 dogs that tested positive for the disease were infested by ticks, indicating a statistically significant difference (p-value = 0.0130) (Graph 2B). Additionally, the result of Cramer's V indicated a 22.4%, a moderate association, between ICT-rKDDR-plus testing and tick infestation.

371 Graph 2. Association between the ICT-rKDDR-plus and clinical signs



372 A)



373 B)

374 Subtitle: Distribution of frequencies when associated with ICT-rKDDR-plus and
375 clinical signs. A) Association between ICT-rKDDR-plus and CC/5. B) Association
376 between ICT-rKDDR-plus and ticks.

377

378

379 The seropositive dogs by ELISA-rKDDR-plus test have been associated with
380 clinical signs at a significance level of 10.0%, showing association with temperature,
381 enlarged lymph nodes and ICT-rKDDR-plus with the following p-values of 0.0130,
382 0.0283 and 0.0203, respectively (Table 2). A more in-depth analysis of these
383 relationships is discussed below.
384
385

386 Table 2 - Summary of the Independence test for the ELISA-rKDDR-plus data × clinical
387 signs.

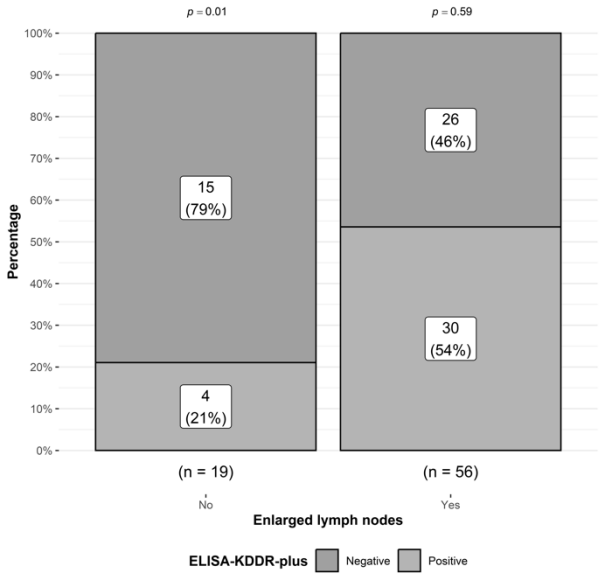
Teste ELISA-rKDDR-plus × Clinical signs CVL	χ^2	**Fisher Exact Test[1]**	**G.L**	**p-value**
Sex	0.1279	-	1	0.7206^{ns}
Age Group	<0.0001	-	1	1.0000^{ns}
Temperature	**-**		**-**	**0.0130***
Mucosal colour	-		-	0.3786^{ns}
Enlarged lymph nodes	**4.8125**	**-**	**1**	**0.0283***
CC/5	-		-	0.8348^{ns}
Enlarged lymph nodes	-		-	0.1188^{ns}
Crusts	0.5178	-	1	0.4718^{ns}
Ulcers	2.4502	-	1	0.1175^{ns}
Peeling	0.01579	-	1	0.9000^{ns}
Erythema	<0.0001	-	1	1.0000^{ns}
Alopecia	<0.0001	-	1	1.0000^{ns}
Dermatological signs	<0.0001	-	1	1.0000^{ns}
Fleas	-		-	0.6535^{ns}
Ticks	0.0013	-	1	0.9706^{ns}
Ectoparasites	0.1209	-	1	0.7281^{ns}
Onychogryphosis	1.7531	-	1	0.1855^{ns}
Conjunctivitis	-		-	0.2022^{ns}
Keratitis	-		-	0.2338^{ns}
Uveitis	-		-	1.0000^{ns}
Eye injuries	0.9871	-	1	0.3204^{ns}
Haemorrhages	-		-	0.6202^{ns}
Presence of other clinical signs	2.6140	-	1	0.1059^{ns}

ICT-rKDDR-plus	**5.3824**	**-**	**1**	**0,0203***

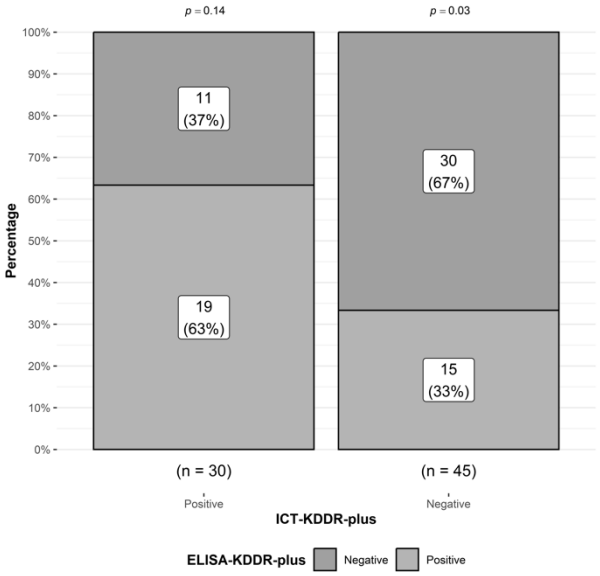
388 χ^2 is the chi-square statistic of the Independence; G.L: degrees of freedom (number of
389 columns – 1) (number of rows –1); *: significant at 10%; ns: not significant; 1 Fisher's
390 exact test is the calculation of the probability, expressed in the p-value column.
391
392

393 An in-depth analysis of seropositivity for the ELISA-rKDDR-plus test has been
394 associated with enlarged lymph nodes and ICT-rKDDR-plus. Regarding enlarged
395 lymph nodes, in dogs that do not have enlarged lymph nodes, 79.0% (15/19) are
396 negative and 21.0% (4/19) are positive, with a significant difference (*p*-value = 0.0120)
397 (Graph 3A). According to the Cramer's V result shown association between ELISA-
398 rKDDR-plus and enlarged lymph nodes is 0.2841 (28.4%), a moderate correlation. The
399 association between the serological tests showed that when negative in the ICT-
400 rKDDR-plus test, 67.0% (30/45) were also negative in the ELISA-rKDDR-plus and
401 only 33.0% (15/45) were positive, indicating that there was a significant difference (*p*-
402 value = 0.025) (Graph 3B). According to Cramer's V result, it showed that the
403 association between ELISA-rKDDR-plus and ICT-rKDDR-plus is 0.2952 (29.5%), a
404 moderate correlation.
405
406

Graph 3. Association between the ELISA-rKDDR-plus and clinical signs and ICT-rKDDR-plus



A)



B)

Subtitle: Distribution of frequencies when associated with ELISA-rKDDR-plus and clinical signs and ICT-rKDRR-plus. A) Association between ELISA-rKDDR-plus and enlarged lymph nodes. B) Association between ELISA-rKDDR-plus and ICT-rKDDR-plus.

Association analysis between laboratory findings

The association between seropositivity in the ICT-rKDDR-plus test and laboratory variables, at a significance level of 10.0%, showed an association with Qualitative Mean Corpuscular Hemoglobin Concentration (MHCH), qualitative Plasma Proteins and ELISA-rKDDR-plus, with the *p*-values of 0.0920, 0.0302 and 0.0060, respectively (Table 3).

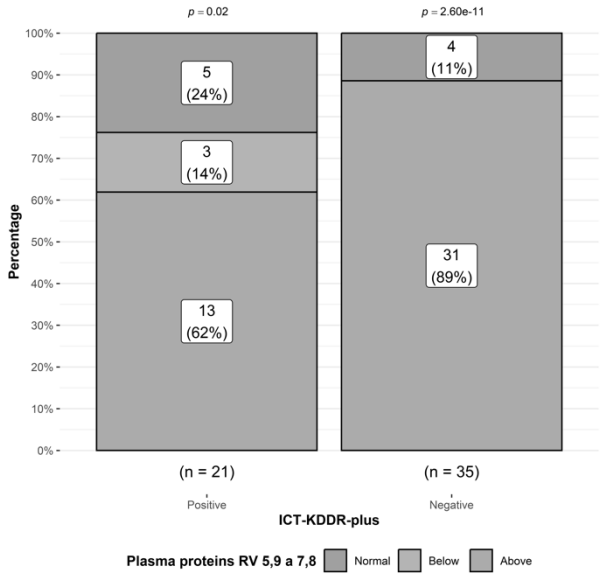
423 Table 3 - Summary of the Independence test for ICT-rKDDR-plus data × laboratory
424 data for leishmaniasis.

ICT-rKDDR-plus × Laboratory data of CVL	χ^2	**Fisher Exact Test[1]**	**G.L**	**p-value**
Qualitative MHCH (RV 32 to 36%)	**-**		**-**	**0.0920***
Qualitative LYM (RV 1000 to 4800/µL)	-		-	0.4014[ns]
Qualitative Hematocrit (RV 37 to 55%)	0.0056	-	1	0.9402[ns]
Plasma Proteins (RV 5.9 to 7.8 g/dL)	**-**		**-**	**0.0302***
Qualitative Reticulocytes (RV 0 to 1.5%)	-		-	1.0000[ns]
Qualitative FA (RV 20 to 156 UI/L)	-		-	0.1722[ns]
Qualitative creatinine (RV 0.5 to 1.5 mg/dL)	-		-	1.0000[ns]
Qualitative AST (RV 23 to 66 UI/L)	-		-	0.2320[ns]
Qualitative ALT (RV 21 to 102 UI/L)	-		-	0.6783[ns]
Qualitative GGT (RV 1,2 to 6,4 UI/L)	-		-	0.8669[ns]
Qualitative Total Protein (RV 5,4 to 7,1 g/dL)	-		-	0.5541[ns]
ELISA-rKDDR-PLUS	**7.5600**	**-**	**1**	**0.0060***

425 χ^2 is the chi-square statistic of the Independence; G.L: degrees of freedom (number of
426 columns – 1) (number of rows –1); *: significant at 10%; [ns]: not significant; [1] Fisher's
427 exact test is the calculation of the probability, expressed in the p-value column; RV:
428 reference value; MHCH: Mean Corpuscular Hemoglobin Concentration; LYM:
429 lymphocytes; FA: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine
430 aminotransferase; GGT: gamma glutamyl transferase.
431

Similarly, there was an association between seropositivity in the ICT-rKDDR-plus test and plasma proteins showing that seropositive dogs with 62.0% (12/21) evidenced plasma proteins above the reference values, and 24.0% (5/21) within the reference values and 14.0% below, with a significant difference (*p*-value = 0.0180). When the dogs were seronegative, 89.0% (31/35) had the values above and of 11.0% (4/35) within normal limits, indicating that there was a significant difference (*p*-value = 2.6×10^{-11}) (Graph 4).

440 Graph 4: Association between ICT-rKDDR-plus and plasma proteins.



441

442 Subtitle: Distribution of frequencies when associated with ICT-rKDRR-plus and plasma

443 proteins.

444

445 Seropositivity in the ELISA-rKDDR-plus test and laboratory values were
446 associated and there was a significant difference with qualitative lymphocytes (LYM)
447 and ICT-rKDDR-plus, with the following *p*-values of 0.0284 and 0.0060, respectively
448 (Table 4), at a significance level of 10.0%.
449

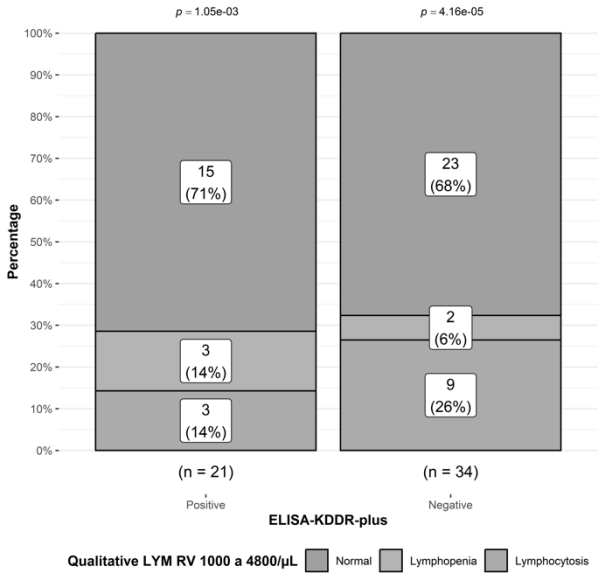
450 Table 4 - Summary of the Independence test for the ELISA-rKDDR-plus data ×
451 laboratory data for leishmaniasis.

ELISA-rKDDR-plus× Laboratory data of CVL	χ^2	**Fisher Exact Test[1]**	**G.L**	**p-value**
Qualitative MHCH (RV 32 to 36%)	-		-	0.2380[ns]
Qualitative LYM (RV 1000 to 4800/µL)	**-**		**-**	**0.0284[*]**
Qualitative Hematocrit (RV 37 to 55%)	-		-	1.0000[ns]
Plasma Protein (RV 5.9 to 7.8 g/dL)	-		-	0.2711[ns]
Qualitative Reticulocytes (RV 0 to 1.5%)	-		-	0.5736[ns]
Qualitative FA (RV 20 to 156 UI/L)	-		-	0.5596[ns]
Qualitative Creatinine (RV 0.5 to 1.5 mg/dL)	-		-	0.4935[ns]
Qualitative AST (RV 23 to 66 UI/L)	-		-	0.8504[ns]
Qualitative (RV 21 to 102 UI/L)	-		-	0.3944[ns]
Qualitative GGT (RV 1.2 to 6.4 UI/L)	-		-	0.3159[ns]
Qualitative Total Protein (RV 5.4 to 7.1 g/dL)	-		-	0.7897[ns]
ICT-rKDDR-plus	**7.5600**	**-**	**1**	**0.0060[*]**

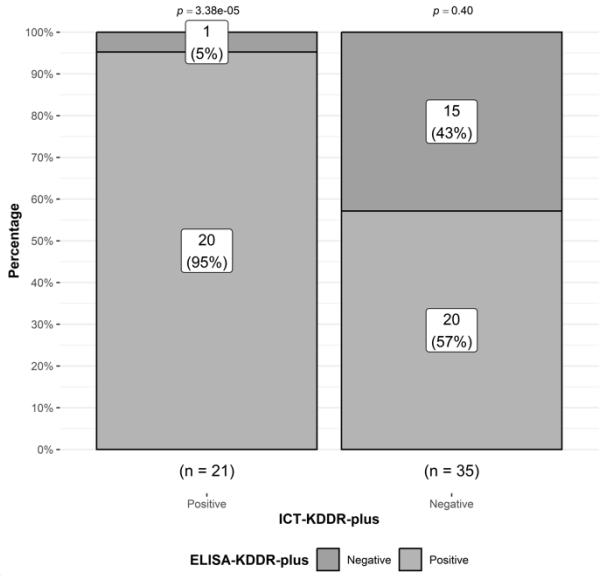
452 χ^2 is the chi-square statistic of the Independence; G.L: degrees of freedom (number of
453 columns – 1) (number of rows –1); [*]: significant at 10.0%; [ns]: not significant; [1] Fisher's
454 exact test is the calculation of the probability, expressed in the p-value column; RV:
455 reference value; MHCH: Mean Corpuscular Hemoglobin Concentration; LYM:
456 lymphocytes; FA: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine
457 aminotransferase; GGT: gamma glutamyl transferase.
458

There was an association between seropositivity in the ELISA-rKDDR-plus test with LYM and ICT-rKDDR-plus. When dogs were seropositive, 71.0% (15/21) had LYM within reference values, 14.0% (3/21) lymphopenia and 14.0% (3/21) lymphocytosis, showing that there was a significant difference (*p*-value = 0.0010). In the case of seronegative dogs, there was also a significant difference (*p*-value = $4.16 \times 10^{-5} = 0.0000416$), with 68.0% (23/34) having LYM values within normal limits, 26.0% (9 /34 lymphocytosis) and 6.0% lymphopenia (Graph 5A). There was also an association between the serological tests in this sample. When dogs are seropositive in the ICT-rKDDR-plus test, 95.0% (21/22) are positive in the ELISA-rKDDR-plus test and 5.0% (1/22) are negative, there was a significant difference (*p*-value = $3.38 \times 10^{-5} = 0.0000338$), (Graph 5B). According to the result of Cramer's V, it showed that the association between these variables was 0.4082 (40.8%), a moderate correlation.

472 Graph 5: Association between the ELISA-rKDDR-plus and LYM and ICT-rKDDR-plus



473 A)



474 B)

475 Subtitle: Distribution of frequencies when associated with ICT-rKDDR-plus and LYM
476 and ICT-rKDDR-plus. A) Association between ICT-rKDDR-plus and LYM. B)
477 Association between ELISA-rKDDR-plus and ICT-rKDDR-plus.

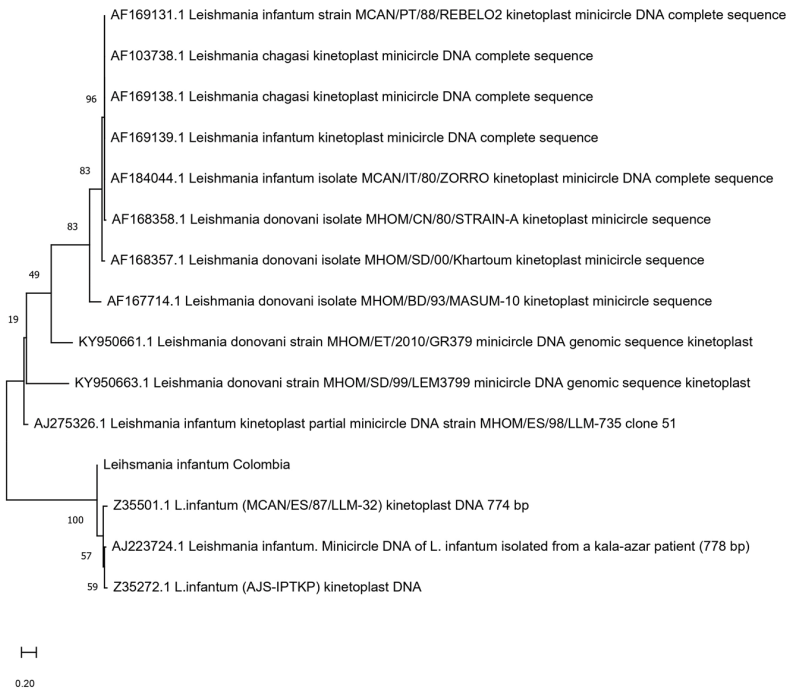
478

479

480 **Molecular and sequencing analysis**

481 In the molecular analysis, we first performed RT-PCR of endogenous genes
482 using 43 dog samples, of which 46.5% (20/43) were positive, showing that these 20
483 samples had good DNA quality. We then proceeded to perform the RV1-RV2 PCR,
484 using 19 samples and 10.5% (2/19) dogs were positive, one sample was from white
485 blood cells and the other from lymph nodes. In the multiplex PCR, we used 17 samples,
486 in which 11.8% (2/17) dogs were positive for *L. infantum*, which were the two positive
487 samples also in the RV1- RV2 PCR. Thus, the PCR RV1-RV2 molecular diagnosis
488 confirmed the presence of *Leishmania* sp. circulating in dogs in Colombia and using
489 multiplex PCR identified the presence of *L. infantum*. The two positive samples in both
490 PCRs were selected for sequencing, however, only one presented an adequate score.
491 This sample, in the similarity analysis (BLAST), presented the genetic sequence
492 obtained from the isolate with 99.0% similarity with *L. infantum* found in Brazil.
493

494 Figure 1. Analysis of the phylogenetic tree based on the partial mitochondrial kDNA
495 sequence in *L. infantum* isolated from Colombian dogs using genetic linkage analysis.



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497

498

DISCUSSION

This work presents for the first time the VL in abandoned dogs from Girardot City including clinical signs, hematology, serology, and molecular findings. These results support the existence of a new scenario for the dispersion of urban Leishmaniasis, which may be related to dog migration and vector endemism. This occurrence may be related to the largest canine floating population that occurred in Girardot City when the dogs may have been taken from other country areas. Similarly, the abandoned dogs of Girardot could enter in contact with military dogs that participate in military operations in other country areas, and previous studies showed the incidence of CL in these dogs (11, 13). Similarly, in this city it was previously reported the presence of *L. longipalpis* in association with ecological factors contributing to the spreading of the *Leishmania* parasites (25). In this sense, the dog migration to places where sandflies can be found is a principal reason to explain the dog participation as a reservoir and important source of infection for other animals and humans (26). This could explain the chain of *Leishmania* sp. transmission and the incidence identified in this study.

In this study, we found a satisfactory result for the ICT-rKDDR-plus when compared to the ELISA-rKDDR-PLUS, since there was an agreement between the tests, as 67.0% of the dogs were negative in both tests in the first collection and 95.0% of the dogs were positive in both tests in the second collection. We chose to use the ICT-rKDDR-plus test for the field diagnosis of these animals because it is a fast, practical and inexpensive test, as well as having a lower percentage of cross-reactions, and it was the first time this test was used in epidemiological conditions other than Brazil (18). Another important reason for choosing this test is that we are evaluating a shelter population in which there is a constant change of animals as new ones are added,

adopted, or died. According to Fujisawa, Silcott-Niles (27), stray dogs or dogs living in shelters make up the majority of VL reservoirs in endemic areas and, because they are a volatile population, they are more difficult to monitor, which makes it harder to assess epidemiological conditions. It is therefore essential that this test provides us with reliability and practicality to better detect the spreaders of the disease. In this way, more effective control and prevention measures can be targeted (27). It is work emphasizing that the samples were collected at different times due to the availability of the team and the study was carried out to a population level rather than individually. In this sense, this serological diagnosis in dogs with VL from Girardot City clearly shows the need for an epidemiological investigation of this disease in the central region of Colombia.

For molecular diagnosis, the RT-PCR amplifies endogenous genes and two PCRs, RV1-RV2 PCR which amplifies different species of *Leishmania* sp. and multiplex PCR. The RV1-RV2 PCR amplifies mainly *L. donovani*, in addition, the gene shows good results with Brazilian samples in LICP and this gene allowed us to validate the presence of *Leishmania* sp. before moving on to multiplex PCR and sequencing of the RV1-RV2 PCR product. The endogenous RT-PCR gene was accurate in 46.5% of the samples and of these samples, two were positive in the RV1-RV2 PCR and the multiplex PCR amplified *L. infantum*. The presence of *L. infantum* was also detected by genome sequencing, in which phylogenetic analyses found 99% similarity. In this way, we proved the presence of *L. infantum* in dogs for the first time in this region of the country, corroborating the results found in ICT and ELISA. This result is like that found by Jaimes-Dueñez, Castillo-Castañeda (28), who found serologically and molecularly positive animals in Colombian territory, but close to the Bucaramanga Metropolitan Area.

548 Based on this diagnosis of dogs with VL, it is important to highlight their role as
549 reservoirs. Because canines are considered the main domestic reservoirs and thus
550 maintain the protozoan in the population. In addition, canine cases usually precede
551 human cases, which indicates the need for research and action to understand its
552 distribution in the country (29, 30). Another issue is that we have noticed symptomatic
553 dogs, and having clinical signs increases the potential for transmission, especially when
554 they are cutaneous signs (31). As most of the animals showed some clinical signs, this
555 may indicate that it is a recent urban circulation. In this case, our population presented
556 skin lesions, weight loss and lymphadenopathy, and in subsequent collections, when we
557 compared the dogs present at the first collection with those still present at the fourth
558 collection, there was a worsening of the clinical signs. However, the analysis of clinical
559 signs was carried out on the first collection, and we found that dogs that tested positive
560 for the ELISA-rKDDR-plus had a 54 per cent chance of having enlarged lymph nodes.
561 We also found an association between the presence of ticks and seropositivity in
562 the ICT-rKDDR-plus ($p = 0.0916$), in which 57% of the positive dogs were found to
563 have ticks. In this case, we opted to use the rKDDR-plus recombinant protein for
564 diagnosis to minimize cross-reactions with tick-borne diseases, when compared to the
565 use of other antigens, such as rK39 and rK26 (18). Another issue that confirms that the
566 animals are truly positive is the positivity in molecular diagnosis and sequencing. It is
567 important to remember that the Girardot-Cundinamarca Dog Shelter, despite being on
568 the urban perimeter, is close to the forest, and there is poor management of organic
569 matter, especially animal faces, which attracts and facilitates the proliferation of
570 sandflies. Consequently, the presence of ectoparasites is associated with the
571 seropositivity of dogs with leishmaniasis (32). For strategies to control and prevent this
572 disease, it is also important to observe the characteristics of the dogs' living

environment, since the material with which the shelter was built, its proximity to accumulated organic matter, streams, pipes and coexistence with other animals attract phlebotomine, making it a favourable environment for the *Leishmania* circulation (14).

The animals studied represent a small proportion of the dogs present in Colombian territory, however, studies such as Jaimes-Dueñez, Castillo-Castañeda (28), confirm the presence of *L. infantum* positive dogs and align with this study. Furthermore, we are in an area close to Colombian army kennels that have already diagnosed *Leishmania* sp. It is therefore important to carry out further studies to better elucidate the country's epidemiology. Along with research into the presence of *L. infantum* in other animal species, such as humans. This will make it possible to understand its distribution throughout the country and take effective measures to minimize its spread. By showing positive dogs, we have opened a series of discussions around this disease, such as euthanizing positive dogs. Today, countries in Europe and Brazil allow treatment and are fighting back with preventative measures. In Colombia, however, it is necessary to assess what public measures should be taken to improve control in the territory (15).

The study shows for the first-time canines infected with *L. infantum* in Girardot, however, it is still important to monitor phlebotomine to confirm the species transmit *Leishmania* spp. and the behavior in Colombian territory and to elucidate the chain of transmission. This is the next step in study because by determining positive dogs, the research was extended to felines since there are several studies in Brazil that point to the importance of this secondary reservoir (26). The study indicates the presence of circulating *L. infantum* for the first time in symptomatic dogs in the central region of Colombia, which indicates a potential for zoonotic transmission. Consequently, it is

597 essential to develop effective strategies to control and prevent this neglected disease in
598 the country.
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ABBREVIATION

CL: cutaneous leishmaniasis

CVL: canine visceral leishmaniasis

UDCA: Universidad de Ciencias Aplicadas y Ambientales

UFLA: Universidade Federal de Lavras

UFMG: Universidade Federal de Minas Gerais

VL: leishmaniasis visceral

WHO: World Health Organization

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ARTIGO 3

This paper is a preliminary version, prepared following the guidelines of the journal *Journal Veterinary Science* (JCR 1.8) to which it will be submitted later. The journal's editorial board may suggest changes to suit its style.

FIRST SURVEY OF LEISHMANIASIS IN CATS FROM THE CENTRAL REGION OF COLOMBIA

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Conflict of Interest

The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; the collection, analyses, or interpretation of data; the writing of the manuscript; or the decision to publish the results.

ABSTRACT

This study aimed to diagnose by serological and molecular tests the feline leishmaniasis (FL) from cats abandoned in the central region of Colombia. This study takes place in a shelter located in Giradort, a tourist city with a large floating population. A single sampling of blood and aspirate from popliteal lymph nodes were collected from these cats for serologic diagnosis using the rkDDR-plus immunochromatographic test (ICT-rKDDR-plus) and the rkDDR-plus enzyme-linked immunosorbent assay (ELISA-rKDDR-plus). These samples were diagnosed by real-time polymerase chain reaction (RT-PCR) through GAPDH and HSP-70 genes. As a result, we found 100.00% of cats positive in ELISA-rKDDR-plus and 37.50% of cats positive in ICT-rKDDR-plus. The molecular diagnostics showed 100.00% positivity using the GAPDH gene, indicating a good quality of the samples. These samples diagnosed molecularly by HSP70 showed 66.67% positivity in the first serial, however, the second serial showed 33.33% positivity. The cat samples diagnosed by serological and molecular tests showed complementary positivity for feline leishmaniasis in the population studied suggesting the involvement of this species in the transmission cycle and alerting about the establishment of control and prevention plans for these animals.

KEYWORDS: Zoonosis. Neglected Disease. Dermatological lesions.

INTRODUCTION

Leishmaniasis is a parasitic disease caused by the protozoan species of *Leishmania* spp. commonly transmitted by dipterans of the species *Phlebotomus* and *Lutzomyia* worldwide. It is considered a neglected disease of global importance due to its wide geographical distribution, and for this reason, studies to better understand, control and prevent the disease are essential (1).

In addition to dogs, several non-human hosts can be infected and participate as potential reservoirs in the cycle of transmission in rural and urban scenarios. In urban scenarios, previous findings support the idea that *L. infantum* is not restricted to dogs, and felines such as cats could be involved in this transmission, highlighting the importance of elucidating the participation of these animals in the *Leishmania* spp. circulation (2). The role of cats in the transmission cycle of *Leishmania* spp. in rural and urban areas is not well understood. However, previous studies have shown that this species is the second most common pet diagnosed worldwide (3).

In the areas of enzootic cycle transmission of *L. infantum*, it is common for other host species based on dogs and cats to participate. Reports of visceral leishmaniasis (VL) in cats date back 30 years and have been increasing ever since (4). Additionally, it is well-established that strains of *L. infantum* found in cats often exhibit the same genetic characteristics as those found in humans and dogs (5). In the central region of Colombia, there have been few studies on VL. However, a recent case of visceral leishmaniasis (VL) in cats with co-infection has been reported with a molecular assumption of *Leishmania* spp. participation, suggesting the parasite's circulation in enzootic cycles in this area (6-9).

In this sense, the study of feline leishmaniasis (FL) in Colombia include a sample of cats, providing insight into the role of this species in protozoan transmission and indicating the epidemiology status of infection in a population (4). Considering that this disease is underdiagnosed in many parts of the country, the study of FL could contribute to designing integrated strategies by public health authorities (10). Thus, this study aims to conduct a serological and molecular survey of FL in cats under conditions of abandonment from the central region of Colombia.

MATERIAL AND METHODS

Declaration of Ethics

The research was approved by the Bioethics Committee of the University of Applied and Environmental Sciences (UDCA) nº 001/2019. In addition, the procedures had the verbal consent of the authorities of the Albergue Felino of Girardot-Cundinamarca.

Study location.

The study was carried out at Albergue Felino de Girardot-Cundinamarca located in the city of Girardot, in the department of Cundinamarca, province of Alto Magdalena, Colombia, from January 2020 to March 2023. This is a tourist city that is close to the capital Bogotá. It is important to emphasize that Colombia is the second country in South America with most cases of CL, however, VL is still poorly studied and there are no reports on VL in cats in the country. Brazil, a neighboring country, has a high incidence of both CL and VL and studies are showing the presence of infected cats (9, 11, 12). This study proceeds from a previous study carried out by the same team, in which the serological and molecular diagnosis of dogs with VL was carried out in the same shelter, since positive dogs were found, an attempt was made to identify whether the cats were positive for FeVL.

Sample collection.

A single sampling was carried out with a team of Veterinarians and students who were duly trained and qualified. During this period, the cats were identified and photographed and the rKDDR-plus immunochromatographic test (ICT) was performed. Blood samples were collected in a tube without anticoagulant to perform serological and molecular diagnosis. Also, for molecular diagnosis, popliteal lymph node puncture was performed with the animals anaesthetized with 0.5 mg/Kg of intramuscular Xylazine Hydrochloride and 2 to 5 mg/Kg of intramuscular Ketamine. Subsequently, for analgesia of these animals, 25 mg/Kg of Dipyrone Sodium intramuscular was applied.

Serum samples were sent lyophilized to the Laboratory of Immunology and Control of Parasites (LICP) of the Institute of Biological Sciences of the Federal

University of Minas Gerais (UFMG), in Brazil. To perform the ELISA test with the rKDDR-plus protein in all collected samples.

Serological diagnosis

During data collection and sampling, ICT-rKKDR-plus was performed on blood samples. This test uses an antigen derived from recombinant *L. infantum* kinesin produced in LICP and currently commercialized by ECO Diagnóstica (Brazil). The test consists of an initial region, which has a pad for application of a release membrane conjugated with colloidal gold nanoparticles bound to rKDDR-plus mouse antigen or IgG antibody, and a final region, which has an absorbent pad. To read the test, the lines formed must be observed. A positive result will show the formation of two lines (control and test) and a negative result will only show the control line. If there is no formation of the control line, this test is considered invalid. This test presents satisfactory results in VT tests in Brazil (13).

The serum sent to Brazil was lyophilized and, upon arrival, it was resuspended to perform the ELISA-rKDDR-plus in the LICP. The ELISA test is recommended by the Brazilian Ministry of Health for confirming animals that are positive in immunochromatographic or indirect immunofluorescence tests. Thus, the ELISA was also performed in duplicate following a protocol of three consecutive days. On the first day, each well of the 96-well microtiter plate (Costar, Cambridge, MA, USA) was sensitized with 100 ng of rKDDR-plus antigen and incubated overnight, for at least 12 hours at 4°C. On the second day, the plate was washed 3 times with 200 µl with washing solution (PBS-0.05% Tween 20) and, subsequently, 200 µl of nonspecific site blocking solution (PBS-0.5% + BSA 1 %) was added to the board. After incubation for 60 minutes at room temperature (26°C) the plate was poured to discard the washing solution. Soon after, 100 µl of cat sera diluted 1:100 in PBS-0.05% Tween 20 was applied in each well and then was incubated overnight, for at least 12 hours at a temperature at 4°C. The controls (2 positive samples, 3 negative samples and 2 blanks) were placed in the last wells of each plate. On the third and last day, each plate was washed five times with 200 µl of washing solution (PBS-0.05% Tween 20), and the Anti-IgG conjugate - Peroxidase produced in rabbit (Bio-rad - HRP AAI26P) was diluted in 1:5000 in PBS-0.05% Tween 20, then 100 µl of diluted conjugate was applied to each well of the plate and incubated for 60 minutes at room temperature. Each plate

was washed five times with 200 µl of washing solution (PBS-0.05% Tween 20), and 100 µl of developing solution containing 0.1 M citric acid, 0.2 M Na2PO4, OPD was added to each well 0.05% and H2O2 0.1%. After incubation for 20 minutes at room temperature and in a dark place, the reaction was stopped with the addition of 50 µl of H2SO4 (4N) (sulfuric acid) and the reading was performed using a wavelength of 492 nm. For each plate, the cut-off point was determined from the average absorbance readings of the six negative sera, plus twice the standard deviation. The mean absorbance of the tested sera was divided by the cut-off value found in each plate, to determine the reactivity index (RI), thus normalizing the optical density (OD) values. Therefore, sera with RI>1.0 were considered positive and IR<1.0 were considered negative. The reactions were read in an ELISA reader at 492 nm and the results expressed in absorbance values.

Molecular diagnosis

Samples of lymph nodes and white blood cells had their DNA extracted using the Wizard Genomic DNA Purification™ kit (Promega, Madison, USA) and were used for molecular diagnosis and, consequently, confirmation of parasitic circulation in that kennel. Conventional polymerase chain reaction (PCR) and real-time chain reaction (RT-PCR) were performed at UDCA to detect deoxyribonucleic acid (DNA) of *Leishmania* sp.

After the DNA extraction, RT-PCR was performed with an endogenous gene to verify the viability of DNA after extraction. For this, the endogenous GAPDH F (5'-GCCGTGGAATTTGCCGT-3') and GAPDH R (3'-GCCATCAATGACCCCTTCAT-5') described by Leutenegger, Mislin (14) were used, employing the thermocycling amplification with an initial denaturation cycle at 95 °C for three minutes, and then 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, followed by the final extension step at 72 °C for seven minutes using Light-Cycler 96 (Roche Diagnostics, Switzerland).

Subsequently, primers that amplify the gene fragment of heat shock protein 70 (HSP-70) were used, which was the same used in a Colombian study by Herrera, Castillo (15). This gene performs well because it has a higher resolution power and has an expected amplicon of 337 bp for all sequences. Furthermore, the HSP-70 gene amplifies several species of *Leishmania sp*. mainly *L. braziliensis*, the most frequent in

the territory of Colombian. The forward 5' AGGTGAAGGCGACGAACG 3' and the reverse 5' CGCTTGTCCATCTTTGCGTC 3' for amplification of HSP70 (8, 15, 16).

The RT-PCR cycle conditions were adjusted according to the protocol: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, hybridization at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 minutes (8). After qPCR, the dissociation of the amplicon followed immediately by a fusion step. The thermal profile was consistent of denaturing at 95°C for 10 seconds, annealing at 60°C for 1-minute, high-resolution melting at 95°C for 30 seconds, and a final annealing stage at 60°C for 15 seconds. During this process, the amplicons obtained in PCR were denatured before the development of melting curves at the inflexion point where changes in fluorescence concerning temperature changed (dF / dT) and were recorded with a ramp of 0.1 °C / second. Each DNA sample was analyzed in duplicate. Finally, the profiles of the normalized and derived melting curves were obtained. Analyzes of the melting values obtained were performed using the GraphPad Prism 6 software (GraphPad Software, San Diego California, USA, www.graphpad.com) to determine the mean and standard deviations. Study contamination controls always include two DNA-free reaction mix controls (Non-Template Controls - NTC) (16).

The RT-PCR technique is a more sensitive and specific method than conventional PCR, which is why this technique was chosen. qPCR is also based on the amplification of nucleic acids by the action of the polymerase enzyme, but the product is immediately quantified using a specific reagent called TaqMan, which emits fluorescence during reaction cycles and, consequently, monitors the reaction. Thus, the parasite load of these animals was determined using RT-PCR, since a high parasite load is related to the individual's infectiousness (17).

RESULTS

In this study, there was one moment of collection of feline samples and it was not possible to collect clinical and laboratory signs. In total, samples were collected from eight cats and 37.50% (3/8) were positive in the ICT-rKDDR-plus test and 100.00% (8/8) were positive in the ELISA-rKDDR-plus test.

In the molecular diagnosis, RT-PCR of endogenous genes was initially performed using 3 cat samples that were positive in both serological tests, of which 100.00% (3/3) were positive, indicating good DNA quality. Subsequently, RT- PCR-HSP70 was carried out in which 66.67% (2/3) cats were positive in the first reaction and in the second reaction 33.33% (1/3) were positive, and the positivity was repeated.

DISCUSSION

In this work, we sought to carry out the serological and molecular diagnosis of leishmaniasis in cats that live in an abandoned situation in the central region of Colombia, together with this study, research was carried out on the incidence of positive dogs in that same location, since it is an area with strong tourism and with military kennels with LC positive dogs (8, 9). Aligned with this, felines are the second most frequent pet specie in the world, presenting an intimate relationship with humans and are considered, along with dogs, the most susceptible group to leishmaniasis with subclinical and chronic infections in endemic areas (2, 18). Positive felines were found.

This study found that cats tested positive for leishmaniasis in both serological and molecular diagnoses. In this sense, the serological tests ICT-rKDDR-plus and ELISA-rKDDR-plus are based on an antigen derived from recombinant *L. infantum* kinesin suggesting that cats could be infected by *L. infantum*. In a similar survey, we found dogs positive for *L. infantum* in the same locality, confirmed by genetic sequencing and increases the possibility of vector transmission to cats. The serological and molecular findings aligned with previous findings of low prevalence in cats associated with a high degree of natural resistance showed mild or absence of clinical signs (19, 20). This reinforces the importance of improving diagnostic techniques for hosts other than dogs, especially rapid and low-cost serological techniques to check other hosts such as cats (6).

The findings of this survey suggest that leishmaniasis is well established in this population, reinforced with complementary diagnosis in cats that were tested by serological and molecular tests. In this sense, the animals of this study with serological positivity indicate previous exposure, while those with molecular positivity suggest recent infection. This puts forward, that this population may be exposed in an enzootic cycle through continuous exposure (2). Although the clinical diagnosis of the cats was not able to be done, empirical observation evidence dermatological lesions compatible with FeVL aligned with dermatological sign identified in dogs of the same shelter that were positive for *L. infantum*. However, the fact that the cats showed relative resistance to infection generally evidence of mild clinical signs. It is believed that this is due to the mechanism of feline immune function against *Leishmania*, but this has yet to be

clarified. Thus, the clinical signs of leishmaniasis in cats are not as indicative of the disease as they are in dogs (21).

It was noted that there was a difference in the results between the serological tests, with the ELISA-rKDDR-plus being 100% positive, unlike the ICT-rKDDR-plus, which shows that even using the same antigen there are differences in the sensitivity and specificity of the diagnosis concerning the technique adopted. Immunochromatographic tests have a lower sensitivity than ELISA because the quality of the antigen can be lost over time, so a second test is recommended for confirmation (13, 22). There were also differences in serological and molecular diagnosis, but in this study, we used samples known to be positive in serological diagnosis for molecular diagnosis, as we were looking for samples with a high parasite load. Controversial results may be due to the natural resistance of cats, leading to underreporting, as cats have a lower specific immune response to the parasite than dogs in techniques such as ELISA and IFAT. Thus, cats that are negative in serology may be positive in PCR, as they seem to have a lower humoral and cell-mediated immune response when compared to dogs, which would justify performing PCR on all samples (19, 23). In addition, high seropositivity may be justified by the fact that study, identified higher seropositivity in cats than in dogs, and that, as in our study, these cats have abundant contact with *L. infantum*-positive dogs, considering the presence of sandflies (24).

The identification of the species of *Leishmania* sp. circulating in cats in the central region of Colombia, and study (7) corroborates this result since a positive cat was reported in Ibagué, which is around 70 kilometres from the city. This shows the importance of an epidemiological investigation of the presence of VL in Colombian territory. In our study, there was weak staining in the gel, which suggests a low parasite load in these animals. Furthermore, genetic sequencing was not possible. However, this study (7) identified the presence of *L. infantum* in the cat in the case report and because it is a region close to Girardot and we found *L. infantum* in dogs from that same shelter, it is possible that these cats also have FeVL.

Feline leishmaniasis has increased the fury of being studied and reported worldwide. In conclusion, we found symptomatic cats positive for both serological and molecular tests for leishmaniasis in the central region of Colombia, which shows the

need to include this disease in the clinical routine and at the public health level so that there is epidemiological surveillance.

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ABBREVIATION

CL: cutaneous leishmaniasis

CVL: canine visceral leishmaniasis

FeVL: feline visceral leishmaniasis

UDCA: Universidad de Ciencias Aplicadas y Ambientales

UFLA: Universidade Federal de Lavras

UFMG: Universidade Federal de Minas Gerais

VL: leishmaniasis visceral

WHO: World Health Organization

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