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ELISA PLASMÔNICA NA DETECÇÃO DE ANTICORPOS IgG anti-*Leishmania* sp.

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Título: *ELISA PLASMÔNICA NA DETECÇÃO DE ANTICORPOS IgG anti-Leishmania sp.*

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"Consagre ao Senhor tudo o que você faz, e os seus planos serão bem-sucedidos."

(Provérbios 16:3)

MACIEL, M.O.S. et al. **ELISA plasmônica na detecção de anticorpos IgG anti-*Leishmania* sp.** 2019. 109 f. Dissertação (Mestrado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2019.

RESUMO

O cão tem sido alvo do controle da Leishmaniose Visceral (LV), pois são reservatórios potenciais de *Leishmania infantum* e desempenham um papel fundamental na cadeia epidemiológica da doença no homem. Portanto, o diagnóstico da leishmaniose canina (Lcan) no Brasil tem sido um desafio para os órgãos de controle de endemias, uma vez que apresentam limitações quanto à sensibilidade e especificidade em áreas endêmicas. Nesta perspectiva a presente pesquisa objetivou desenvolver e validar um ELISA plasmônica indireto rK28 (pELISA) para o diagnóstica da Lcan. Para o desenvolvimento do pELISA, foram realizados diferentes ensaios de otimização, determinação das concentrações ideais de peróxido de hidrogênio, íons ouro, anticorpo IgG anti-dog biotinilado e também do soro. Para a validação do ensaio, 170 amostras de soro de cães de área endêmica para Lcan e 26 amostras de cães saudáveis de área não endêmica para a doença foram testadas pelo pELISA e comparadas com ELISA indireto rk28 e com o teste imunocromatográfico (Dual Path Platform, TR_DPP®) usando como teste padrão-ouro o qPCR em amostras de sangue e/ou swab de subconjuntival. O ensaio foi padronizado com as concentrações de 250 µM de peróxido de hidrogênio, 0,30 mM de íons ouro e a melhor diluição do conjugado de estreptavidina-catalase foi de 1/50. O TR_DPP®, ELISA indireto rK28 e pELISA apresentaram sensibilidade de 79,0%, 89,5% e 94,7% e especificidade de 90,1%, 91,4% e 100,0%, respectivamente. Os maiores valores preditivos positivos (100,0%), negativos (99,3%) e de precisão (99,4%) foram observados no pELISA. O coeficiente Kappa entre o pELISA e a qPCR mostrou excelente concordância (0,970), diferentemente do ELISA indireto rK28 e do TR_DPP®, que mostraram um boa concordância (0,645 e 0,551, respectivamente). O resultados revelaram que o pELISA melhorou a sensibilidade e apresentou alta especificidade em relação ao método oficial recomendado pelo Ministério da Saúde no Brasil e pode aumentar a praticidade do diagnóstico em países com recursos limitados, pois não requer instrumentos sofisticados para leitura, sugerindo que este método pode ser usado como uma ferramenta adicional para o diagnóstico de Lcan nessas áreas.

Palavras-chave: Cães. Diagnóstico. Leishmaniose Visceral. Nanopartículas metálicas.

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ABSTRACT

Dogs have been the target of control of Visceral Leishmaniasis (VL) in humans, as they are potential reservoirs of *Leishmania infantum* and play a key role in the epidemiological chain of the disease. Therefore, the diagnosis of Canine Leishmaniasis (CanL) in Brazil has been a challenge for endemic control organs, since they have limitations on sensitivity and specificity in endemic areas. In this perspective the present research aimed to develop and validate an indirect plasmonic ELISA rK28 (pELISA) for the diagnosis of CanL. For the development of pELISA, different concentrations of hydrogen peroxide, gold ions, biotinylated anti-dog IgG antibody and serum were tested in order to establish ideal values to each parameter. For the validation of the assay, 170 dog serum samples from endemic area to CanL and 26 healthy dog samples from an area nonendemic to the disease were tested by pELISA and compared with indirect ELISA rk28 and the imunocromatografic test (Dual Path Platform, TR_DPP®) using as gold standard assay the real-time PCR in blood samples and/or subconjunctival swab. The assay was standardized with the concentrations of 250 µM hydrogen peroxide, 0.30 mM gold ions, and dilution of the streptavidin-catalase conjugate of 1/50. The TR_DPP®, indirect ELISA rK28 and pELISA presented sensitivity of 79.0%, 89.5% and 94.7% and specificity of 90.1%, 91.4% and 100%, respectively. The highest predictive positive (100%), negative (99.3%) and accuracy (99.4%) values were observed in pELISA. Kappa coefficient between pELISA with real-time PCR showed excellent agreement (0.970), differently of indirect ELISA rK28 and TR_DPP®, which showed good agreement (0.645 and 0.551 respectively). The results revealed that the pELISA improved sensitivity and presented higher specificity compared to official method recommended by the Ministry of Health in Brazil and may increasing the practicality of diagnosis in resource-constrained countries, because it does not require sophisticated instruments to read, suggesting that this method can be used as an additional tool for the diagnosis of CanL in these areas.

Keywords: Dogs. Diagnosis. Leishmaniasis Visceral. Metal nanoparticles.

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 Leishmaniose Visceral

A Leishmaniose Visceral (LV) é uma antropozoonose causada por protozoários do gênero *Leishmania* (Kinetoplastida, Trypanosomatidae), sendo *Leishmania infantum* (previamente *Leishmania chagasi*) o agente etiológico mais comum no Brasil. A forma promastigota do parasita desenvolve-se no tubo digestivo dos vetores, nos hospedeiros mamíferos apresenta-se sob a forma amastigota, arredondadas e sem flagelo livre, multiplicando-se em macrófagos (READY, 2014).

A transmissão entre hospedeiros vertebrados no Novo Mundo ocorre por meio da picada de vetores da Família Phlebotominae, durante o repasto da fêmea, cuja espécie de maior importância no Brasil *é Lutzomyia longipalpis* (READY, 2014), embora *L. cruzi* já tenha sido descrita como espécie vetor (DESJEUX, 2004). Outros artrópodes como carrapatos (COUTINHO et al., 2005; DANTAS-TORRES et al., 2010) e pulgas (COUTINHO; LINARDI, 2007; FERREIRA et al., 2009) podem apresentar formas viáveis de *Leishmania*, no entanto, o papel desses vetores alternativos na transmissão ainda não é conhecido. A transmissão via transfusão sanguínea (OWENS et al., 2001), transmissão venérea (SILVA et al., 2009) e a transmissão vertical (BOGGIATTO et al., 2011) também já foram reportadas.

O ciclo da doença inicia a partir do repasto de fêmeas infectadas com o protozoário, as formas promastigotas são inoculadas na pele de um novo hospedeiro vertebrado, onde dentro dos macrófagos diferenciam nas formas amastigotas. Rapidamente, ocorre então a disseminação linfática para outros tecidos ricos em células do sistema mononuclear fagocitário, tais como, linfonodos, baço e medula óssea dentro das primeiras horas (READY, 2014). Outros órgãos como, bexiga, sistema digestivo, pele, pulmões e órgãos reprodutores também podem ser parasitados (MOLYNEUX; ASHFORD, 1983).

Os sintomas da LV variam em decorrência dos mecanismos imunológicos ativados pelo hospedeiro bem como dos órgãos acometidos. As manifestações clínicas são similares no homem e no cão, e incluem alterações inespecíficas tais como apatia, emagrecimento progressivo, hiporexia ou anorexia, linfoadenomegalia, hepatoesplenomegalia dentre outras (SOLANO-GALLEGO et al., 2011).

A LV é considerada uma das doenças parasitarias de maior impacto sobre a Saúde Pública mundial, é uma doença sistêmica grave, fatal se não diagnosticada e tratada. Nas Américas, a LV é endêmica em 12 países, com 59.769 novos casos registrados entre 2001 e 2017, com uma média de 3.516 casos por ano. Aproximadamente 96% dos casos foram relatados pelo Brasil, no entanto, países da América do Sul como Argentina, Colômbia, Paraguai e Venezuela estão entre aqueles com os maiores registros de casos (WHO, 2019)

Mudanças ambientais como desmatamento e a migração de pessoas das zonas rurais para áreas urbanas pode ter sido determinante para que ocorresse uma rápida urbanização da LV no Brasil, aumentando o risco de infecção em humanos pelo aumento da exposição ao vetor flebotomíneo (DESJEUX, 2001). Além disso, as formas graves e letais da LV podem surgir quando associada à desnutrição ou em indivíduos imunossuprimidos (WHO, 2019).

A expansão da LV no Brasil ocorreu principalmente no Nordeste (Dantas-Torres, 2006) e Sudeste (Santiago et al., 2013). De acordo com dados do Ministério da Saúde (MS), em média, cerca de 3.500 casos de LV são registrados anualmente no Brasil, representando um coeficiente de incidência de 2,0 casos/100,000 habitantes. Nos últimos anos, a letalidade vem aumentando gradativamente, passando de 3,1% em 2000 para 7,1% em 2012 (BRASIL, 2014). Os primeiros casos autóctones de LV no estado de São Paulo foram notificados na cidade de Araçatuba em 1999 (FEITOSA; LUVIZOTTO, 2000). Isto ocorreu após a detecção de *L. longipalpis* em área urbana em 1997, seguido da detecção em cães, em 1998 (CAMARGO-NEVES et al., 2001). Desde então, a LV se expandiu para diversos municípios do estado de São Paulo, sendo que as regiões de Araçatuba, Presidente Prudente e Marília apresentam maiores taxas de incidência e mortalidade humana por LV (CARDIM et al., 2013).

A ocorrência da LV em uma determinada área depende basicamente da presença do vetor e de hospedeiros suscetíveis sendo que, em meio urbano, o cão é o principal reservatório (READY, 2014). No ambiente silvestre, já foram descritos reservatórios como as raposas (*Dusicyon vetulus* e *Cerdocyon thous*) e os marsupiais (*Didelphis albiventris*) e outros canídeos selvagens (LIMA et al., 2009). Em algumas regiões endêmicas, estudos de análises espaço-temporal urbanos concluem que a ocorrência de casos humanos da doença tende a se concentrar em áreas com maior incidência de Leishmaniose canina (Lcan) (CAMARGO-NEVES et al., 2001;

TEIXEIRA-NETO et al., 2014), o qual estudos mostram uma associação entre a prevalência canina e incidência da doença em humanos (PALATNIK-DE-SOUSA et al., 2001; ARAÚJO et al., 2013).

1.2 Leishmaniose Canina (Lcan)

Os cães tem sido alvo do controle da LV em área endêmica, uma vez que são importantes para a manutenção do ciclo epidemiológico da doença, dentre os fatores que justificam essa importância é possível citar; I) LV é mais prevalente na população canina que na humana; II) a infecção no homem normalmente é precedida por casos caninos; III) cães apresentam maior quantidade de parasitos na pele do que o homem, fato que favorece a infecção dos vetores (SANTA ROSA; OLIVEIRA, 1997).

Nos cães, a doença apresenta um período de incubação variável de 1 mês a 4 anos e tem caráter multissistêmico, sendo o parasito pode ser encontrado em diferentes tecidos e órgãos, provocando reações imunológicas e alterações tissulares características de Lcan. O desenvolvimento da doença vai depender especificamente do tipo de resposta imune estabelecido pelo hospedeiro e da proliferação do parasita. Em alguns animais o desenvolvimento da LV pode ser latente, evoluindo inclusive para a cura espontânea (ALVAR et al., 2004).

O estado clínico do cão está diretamente relacionado ao tipo de resposta imunológica que será desencadeada (SOLANO-GALLEGO et al., 2011). Embora o termo "cão assintomático" ainda seja utilizado na literatura, ele está caindo em desuso (DANTAS-TORRES et al., 2014; DANTAS-TORRES; OTRANTO, 2014) e os termos cães infectados “saudáveis” ou “doentes” com base não apenas em exames clínicos físicos, como também nas anormalidades patológicas, como por exemplo; hemograma, perfil bioquímico e análise de urina, têm sido utilizado para o estadiamento da doença e classificação dos animais (SOLANO-GALLEGO et al., 2011).

A presença de resposta imunológica celular tem sido observada em animais resistentes à doença, especialmente do grupo “clinicamente saudável”, podendo evoluir para cura; enquanto a resposta humoral é observada em animais suscetíveis à doença com manifestação da forma sintomática (MORENO; ALVAR, 2002).

A resistência à infecção está associada à ativação e diferenciação das células efetoras T helper (TH) CD4+ do tipo Th1, com um padrão de secreção de citocinas específicas, tais como, IL 2, IL12 (IL-12), IFN-γ e TNF-α que aumentam a eficiência das células fagocíticas e dos linfócitos citotóxicos (SADICK et al., 1991). A supressão da imunidade celular é o aspecto mais importante na patogênese e progressão da doença canina. Em cães, a ausência de resposta das células T aos antígenos de *Leishmania* sp. foi observada *in vivo*, com um teste de Montenegro negativo (DOS-SANTOS et al., 2008). Foi verificado também que cães infectados com *Leishmania infantum*, apresentam uma redução do número de linfócitos T em sangue periférico (BOURDOISEAU et al., 1997; LIMA et al., 2012) e desorganização da polpa branca do baço (SANTANA et al., 2008).

Em contraste, a suscetibilidade à infecção está associada a um perfil do tipo Th2, com predomínio de citocinas anti-inflamatórias (IL-4, IL-5, IL-10, IL- 13) e TGF-β, as quais desencadearão uma resposta imune humoral com a proliferação de linfócitos B com subsequente superprodução de imunoglobulinas específicas e inespecíficas. Esses anticorpos não são efetivos contra parasitos intracelulares e podem agravar ainda mais a inflamação dos tecidos via ativação do sistema complemento, que tem um papel direto na imunopatologia de diversos órgãos e tecidos. Portanto, a maioria dos cães desenvolvem uma resposta imune humoral, com altos títulos de IgG anti-*Leishmania* sp. e uma grande variedade de sinais clínicos da leishmaniose (DE ALMEIDA LEAL et al., 2014).

A soroprevalência da Lcan em áreas endêmicas pode atingir de 67% da população canina (SOLANO-GALLEGO et al., 2001), essa alta prevalência pode ser justificada devido ao fato de que os cães apresentam uma maior quantidade de parasitas na pele (COURTENAY et al., 2014), o que favorece a infecção dos vetores (DESJEUX, 2001). Há ainda evidências de que a taxa de prevalência da infecção em áreas endêmicas pode ser mais alta do que as obtidas por estudos sorológicos (LOPES et al., 2017), uma vez que nessas áreas, a doença pode permanecer clinicamente inaparente por longos períodos, possibilitando a ocorrência de cães infectados, porém “clinicamente saudáveis” e com baixos títulos de anticorpos (SOLANO-GALLEGO et al., 2001).

O papel dos cão como fonte de infecção em áreas endêmicas para LV de acordo com o seu estado clínico ainda é controverso, enquanto alguns autores mostraram que os cães infectados com *L. infantum* mas “clinicalmente saudáveis” não

são eficazes na transmissão do vetor em comparação aos cães infectados “doentes” (COURTENAY et al., 2014); outros autores sugerem que esse grupo de cães possuem capacidade de infectar os vetores em uma proporção semelhante aos cães “doentes” (LAURENTI et al., 2013). A avaliação de cães infectados por *L. infantum* em áreas endêmicas, mostraram que cães classificados como “clinicamente saudáveis” demonstraram alta grau de parasitismo na pele (SOLANO-GALLEGO et al., 2004) e no sangue (DE ASSIS et al., 2010), o que alerta para a chance de transmissão da doença. Este fato reforça a importância da utilização de novas técnicas altamente sensíveis capazes de identificar cães com LV, tanto em áreas endêmicas como em áreas silenciosas (SOLANO-GALLEGO et al., 2011).

1.3 Diagnóstico laboratorial da Lcan

O diagnóstico da Lcan no Brasil tem sido um desafio para os órgãos de controle de endemias. O diagnóstico clínico pode ser confirmado por métodos laboratoriais, dentre eles, exames parasitológicos, imunológicos e moleculares (WHO, 2010). A detecção do parasita, por meio do exame parasitológico direto com a visualização da forma amastigota em aspirados de baço, fígado, medula óssea ou linfonodos, e apesar das discordâncias entre alguns autores, é considerado, ainda, “padrão ouro” para o diagnóstico da doença (SOLANO-GALLEGO et al., 2009). A especificidade é de 100% e a sensibilidade é variável, pois depende do grau de parasitismo, do tipo de material biológico coletado, do seu processamento e coloração, além da prática do observador.

A análise histopatológica de fragmentos teciduais também é importante no diagnóstico da Lcan, pois permite identificar as formas parasitárias e ao mesmo tempo contribuir para o diagnóstico diferencial com outras doenças (LIMA et al., 2004). Outro método que também pode detectar antígenos parasitários nos tecidos é a imunohistoquímica (TAFURI et al., 2004) . No entanto, biópsias e as punções esplênicas e de medula óssea são consideradas procedimentos invasivos e exigem ambientes apropriados para a coleta, não sendo procedimentos adequado para estudos epidemiológicos em larga escala (GONTIJO; MELO, 2004).

A detecção do DNA parasitário em diversas amostras biológicas, tais como sangue periférico, biópsia de pele, mucosa subconjuntival e aspirados de medula óssea, fígado e baço por meio da reação em cadeia da polimerase em tempo real

(qPCR) vem sendo descrito na literatura. De modo geral, há grande variação na sensibilidade do método de qPCR, particularmente no que se refere à escolha dos oligonucleotídeos iniciadores utilizados (LACHAUD et al., 2002; IKONOMOPOULOS et al., 2003) e das amostras clínicas utilizadas (REALE et al., 1999; MANNA et al., 2004; NUNES et al., 2007; QUARESMA et al., 2009).

Vários sistemas baseados em qPCR têm sido desenvolvidos para *Leishmania* sp. No que se refere ao tipo de amostra ideal, alguns relatam que as amostras de medula óssea, baço, linfonodo e pele demonstraram ser mais adequadas para a detecção de *Leishmania sp.* por qPCR (REALE et al., 1999; QUARESMA et al., 2009; COURTENAY et al., 2014; LOPES et al., 2017). No entanto, métodos não-invasivos, tais como sangue periférico (LACHAUD et al., 2002; IKONOMOPOULOS et al., 2003; MANNA et al., 2004) e mucosa subconjuntival são preferidos porque permitem uma fácil amostragem em pesquisas de campo, especialmente para triagem em massa e têm uma melhor aceitação do proprietário (STRAUSS-AYALI et al., 2004; FERREIRA et al., 2008; LEITE et al., 2010; LOMBARDO et al., 2012; LOPES et al., 2017).

O qPCR em sangue periférico tem se tornado um bom instrumento para o diagnóstico da Lcan, pois apresenta uma boa sensibilidade e especificidade (LACHAUD et al., 2002; IKONOMOPOULOS et al., 2003; MANNA et al., 2004). No entanto, opiniões divergentes, quanto ao uso do sangue periférico foram descritos por outros autores (REALE et al., 1999; DE ALMEIDA LEAL et al., 2014). Para Nunes et al., (2007) a baixa sensibilidade (55%) e especificidade (66,3%) observada no diagnóstico da Lcan em amostras de sangue periférico por qPCR pode estar relacionada a qualidade das amostras clínicas, uma vez que se as coletas de sangue forem realizadas por agentes de saúde não capacitados e não forem adequadamente homogeneizadas podem resultar em amostras com presença de coágulos que dificultarão a extração do DNA alvo (NUNES et al., 2007).

A quantificação do DNA parasitário de *L. infantum* em amostras de subconjuntivais tem se destacado na literatura. Ferreira et al. (2008) destacam que a carga parasitária em amostras subconjuntivais foi equivalente à medula óssea em cães. Este resultado destaca o potencial de uma amostra obtida de forma não invasiva como um recurso no diagnóstico molecular Lcan (FERREIRA et al., 2008).

Embora o qPCR aparentemente tenha melhorado a sensibilidade do diagnóstico da LV, é preciso considerar as dificuldades intrínsecas de incluir testes

moleculares nos serviços de saúde pública. As limitações do qPCR e outras técnicas moleculares, incluem necessidade de instalações e equipamentos que pode implicar em um alto custo, tornando-os difíceis de ser incluídos em programas de controle de financiamento público, especialmente nos países com restrição de recursos (GONTIJO; MELO, 2004). Além disso, recomenda-se ainda, evitar o rastreio de cães infectados mas “clinicalmente saudáveis” usando apenas testes moleculares, pois um resultado positivo confirma infecção por *Leishmania* sp., mas não a doença no cão (SOLANO-GALLEGO et al., 2011).

Os métodos imunológicos baseados na detecção de anticorpos IgG anti-*Leishmania* sp. circulantes no soro se destacam, principalmente para triagem de casos em massa, tais como em inquéritos epidemiológicos (SOLANO-GALLEGO et al., 2011). Diferentes técnicas sorológicas têm sido utilizadas no diagnóstico da Lcan, sendo métodos recomendados pelo Ministério da Saúde para os órgãos de saúde pública no Brasil, o teste rápido imunocromatográfico TR-DPP® (Dual Path Plattaform) como triagem e o teste imunoenzimático [enzyme-linked immunosorbent assay (ELISA)] como confirmatório (BRASIL, 2014).

A sensibilidade e especificidade dos métodos sorológicos dependem do tipo de antígeno empregado. Além do antígeno total, atualmente diferentes antígenos recombinantes sozinhos ou combinados têm sido descritos na literatura, sendo os principais o rK39, rK26, rK9 e o rK28. O antígeno recombinante rK39 possui uma seqüência idêntica em sete espécies de *Leishmania* sp. sem demonstrar reatividade com outros tripanossomatídeos (BURNS et al., 2006). Apresenta alta densidade de epítopos de célula B imunodominantes da proteína relacionada à kinesina de 230 kDa de *L. infantum* (anteriormente *L. chagasi)* (BURNS et al., 2006). Em *Leishmania* sp., o antígeno K39 é expresso principalmente no estágio amastigota e induz uma forte resposta imune em cães infectados, assintomáticos ou doentes (ROSATI et al., 2003). A massa molecular esperada para o antígeno recombinante rK9 é de 35 kDa e para o rK26 é de 46 kDa (ROSATI et al., 2003). Ambos representam uma região central de repetição de 14 aminoácidos de proteínas hidrofílicas que é específica de *L. chagasi* e *L. donovani,* alem disso, foi demonstrado que os pacientes com LV desenvolvem respostas de anticorpos fortes e específicas contra o K26, o que poderia complementar o rK39 em um diagnóstico mais preciso da LV humana (BHATIA et al., 1999). A reatividade específica e anticorpos independentes para cada um desses três antígenos (rK9, rK26 e rK39) também já foi estudada e utilizada no diagnóstico

sorológico de Lcan (ROSATI et al., 2003). No que se refere ao antígeno recombinante rK28, ele foi gerado a partir de um gene sintético, fundindo-se múltiplas sequências genicas repetidas dos genes haspb1 e k39 kinesina de *L. donovani*, aumentando desse modo a densidade do epitopo antigênico, enquanto proporcionava epitopos complementares na proteína recombinante resultante (PATTABHI et al., 2010).

O teste imunocromatográfico usado no diagnóstico oficial da Lcan, TR-DPP®, pode ser caracterizado como um teste qualitativo para detecção de anticorpos anti-*Leishmania* sp. que emprega de um lado, a combinação de proteína A conjugada a partículas de ouro coloidal, e de outro, antígenos recombinantes k28 (fusão do rk39, rk26 e rk9) específicos de *Leishmania* sp. ligados a uma membrana. As vantangens são de um teste rápido, simples e de fácil uso, podendo ser executado em campo e apresenta sensibilidade de 98,3% (167/171) e especificidade de 97,4% (175/186) (BIO-MANGUINHOS, 2011).

O ELISA é um tipo particular de imunoensaio em que um sinal é gerado principalmente pela conversão biocatalítica de uma molécula por uma enzima (CLARK; LISTER; BAR-JOSEPH, 1986). O ELISA indireto utilizando antígeno total de *Leishmania* sp. tem apresentado uma sensibilidade variando de 88% a 90% e especificidade variando de 93% a 100% no diagnóstico da Lcan (DO ROSÁRIO et al., 2005; PORROZZI et al., 2007; CÂNDIDO et al., 2008). No entanto, estudos comparativos entre o antígeno bruto e recombinantes mostraram que antígenos recombinantes tem sido mais precisos na detecção da Lcan (DO ROSÁRIO et al., 2005; PORROZZI et al., 2007). Em levantamentos sorológicos humanos, o ELISA indireto com o antígeno recombinante rK28 mostraram alta sensibilidade e especificidade (100%) no Sudão (PATTABHI et al., 2010) e na Índia (VAISH et al., 2012). Na Lcan, o ELISA com antígeno recombinante rK28 apresentou um sensibilidade variando de 91% a 100% e especificidade de 100% (VENTURIN et al., 2015; LAURICELLA et al., 2016).

Diversas limitações do ELISA indireto para o diagnóstico da Lcan têm sido descrito na literatura, tais como baixos valores de sensibilidade, acarretando taxas de infecções subestimadas e consequentemente permitindo a manutenção de animais infectados em áreas endêmicas. Um recente estudo, revelou que um a cada cinco cães soronegativos estão infectados com *Leishmania* sp. Os autores avaliaram 975 cães de uma área endêmica para LV utilizando os testes TR-DPP®, ELISA indireto com antígeno recombinante rK39 e qPCR em amostras de sangue periférico,

aspirados de linfonodos e mucosa subconjuntival. Verificou-se que o diagnóstico oficial da Lcan nas áreas endêmicas brasileiras não conseguiu acusar um número expressivo de animais infectados quando comparados com os testes moleculares. Na primeira amostragem, cerca de dois em dez animais TR-DPP® negativos estavam infectados e na segunda amostragem, essa proporção aumenta para cinco em dez, ou seja, metade dos animais soronegativos no ELISA indireto estariam infectados (LOPES et al., 2017). Essas limitações podem estar relacionadas com o limite de detecção do ELISA indireto, que normalmente é de aproximadamente 1 ng/mL de anticorpos (WU et al., 2007; OGISO et al., 2013), sendo, portanto, pouco preciso na detecção de cães infectados no período pré-patente e antes da soroconversão, cães que nunca farão soroconversão (“clinicalmente saudáveis”) ou mesmo àqueles que apresentam um baixo título de anticorpos, mas ainda permanecem infectados (SOLANO-GALLEGO et al., 2011). Uma outra limitação do ELISA indireto principalmente com antígeno total é a possibilidade de reações cruzadas com outros tripanosomatídeos que podem infectar cães (ROSYPAL et al., 2007).

1.4 ELISA plasmônica indireto versus ELISA indireto

No ELISA indireto para o diagnóstico da Lcan, antígenos solúveis e purificados de *Leishmania* sp. obtidos a partir de cultura “in vitro” são previamente adsorvidos nas cavidades de microplacas e posteriormente é adicionado o soro do cão. Na presença do anticorpo IgG anti-*Leishmania* sp. ocorre uma ligação antígeno/anticorpo específica, sendo a visualização desta interação possível com o uso de uma antiglobulina de cão marcada com a enzima peroxidase, que na presença de peróxido de nitrogênio (H_2O_2) e cromógeno gera um sinal colorido. O sinal é gerado pela conversão do substrato enzimático em uma molécula colorida e a intensidade da cor da solução pode ser observada pela medição da absorbância com um leitor de placas (espectrofotometria) (LIMA et al., 2003).

Embora a distinção entre solução com presença de cor e sem cor possa ser detectada a olho nu, a detecção adequada só é possível quando há concentrações elevadas do anticorpo. Por exemplo, quando o anticorpo estiver presente em baixas concentrações no soro pode resultar na geração de uma solução levemente corada, o qual seria difícil diferenciar a partir da solução não corada obtida na ausência dele. Além disso, em situações reais, uma solução levemente corada também pode ser

gerada na ausência deste anticorpo por causa de interações inespecíficas entre os elementos do imunoensaio e o substrato (DE LA RICA; STEVENS, 2012). Portanto, a detecção de anticorpos IgG anti-*Leishmania* sp. em baixas concentrações poderia ocasionar um resultado equivocado. Além disso, o limite de detecção de anticorpos específicos é relativamente alto, ou seja, esses métodos podem ser inadequados para atingir o limiar clínico de muitos biomarcadores, principalmente na fase inicial das doenças (RISSIN et al., 2010; DE LA RICA; STEVENS, 2012; NIE et al., 2014).

Com o intuito de suprir esta lacuna surge uma nova variação do ELISA, conhecida como ELISA plasmônica (pELISA), a qual é caracterizada por uma combinação de uma Ressonância Plasmônica de Superfície Localizada (LSPR) de nanopartículas metálicas nobres (por exemplo ouro e cobre) associada à técnica ELISA (DE LA RICA; STEVENS, 2013). O LSPR é um fenômeno óptico especial conferido pela interação da luz com elétrons nas superfícies metálicas das nanopartículas (EUSTIS; EL-SAYED, 2006; GUO; KIM, 2012). A luz, sob a qual ocorre LSPR, é fortemente dependente do tamanho, forma, superfície e estado de agregação das nanopartículas metálicas (OGISO et al., 2013a). Assim, variações no estado de agregação das nanopartículas metálicas acarreta em alterações nas propriedade ópticas, oferecendo uma abordagem potencial para ensaios ultra-sensíveis de moléculas alvo (SATIJA et al., 2016).

Neste novo método, o ciclo biocatalítico do rótulo enzimático está ligado ao crescimento de nanopartículas ouro em diferentes estados de agregação, para obter soluções de cor azul ou vermelha na presença ou ausência do analito (DE LA RICA; STEVENS, 2013). O fato do resultado do pELISA apresentar soluções coradas diferentemente ocorre pela alteração nas condições da superfície das nanopartículas, ou seja, as nanopartículas de ouro possuem elétrons livres e na sua superfície esses elétrons formam uma nuvem eletrônica que se move com a interação de uma onda eletromagnética (a luz). Com uma mudança na morfologia, essa superfície muda e, portanto a maneira com que essa nuvem eletrônica interage com a luz também muda, e consequentemente, a solução coloidal poderá apresentar diferentes cores dependendo do estado de agregação das nanopartículas. Além disso, a LSPR pode ser influenciada por condições que rodeiam a partícula, como o tipo de ligante (moléculas que interagem com nanopartículas) (EUSTIS; EL-SAYED, 2006).

O peróxido de hidrogênio é uma das moléculas capazes de interagir com as nanopartículas ouro, assim, a concentração dele é um fator chave para o estado

de agregação delas. A fim de testar o impacto da concentração de peróxido de hidrogênio, na morfologia e propriedades ópticas de nanopartículas ouro, um estudo analisou diferentes concentrações de peróxido de hidrogênio, que foram adicionados a uma solução contendo íons de ouro (0,1 mM) em tampão (MÊS) (1 mM, pH 6,5). Os autores observaram que a tonalidade da solução muda de vermelho para azul em uma faixa de concentração estreita entre 120 e 100 mM, sugerindo que o crescimento de nanopartículas ouro no estado agregadas ocorre em baixas concentrações de peróxido de hidrogênio. Além disso, também é possível monitorar esse crescimento medindo a absorbância a 550 nm com um leitor de placas convencional (DE LA RICA; STEVENS, 2013).

Sabendo- se ainda que a enzima catalase pode decompor eficazmente o peróxido de hidrogênio em oxigênio e água (BOUYAHIA et al., 2011), isto poderia ser utilizado para o surgimento desta nova variação de ELISA. Assim no pELISA, é adicionado um anticorpo secundário biotinilado e o conjugado streptavidina/catalase, a enzima catalase consome o peróxido de hidrogênio e isso retarda a cinética do crescimento de nanopartículas de ouro, o que resulta no estado agregado das nanopartículas de ouro, como conseqüência, a solução fica azul. Por outro lado, na ausência da catalase, a redução dos íons ouro com peróxido de hidrogênio ocorre a um ritmo rápido, e sob estas condições, espera-se que haja um crescimento das nanopartículas num estado monodisperso e que a solução coloidal se torne vermelha. A cor azul e vermelha são facilmente distinguíveis, facilitando a detecção a olho nú (DE LA RICA; STEVENS, 2013).

Recentes pesquisas mostram que o pELISA têm sido eficaz na detecção ultrassensível de biomarcadores de doenças e que podem ser visualizadas a olho nu. La-Rica e Stevens (2012) estudaram o impacto de matrizes reais do pELISA na detecção de antígeno de capsídeo do HIV-1 (p24), uma proteína da partícula viral de grande importância clínica, pois pode ser detectado em sangue periférico no estágio inicial, antes da soroconversão. Em conclusão o pELISA detectou em soro integro o antígeno p24 do HIV-1 na concentração ultra-baixa de $1x10^{-18}$ g /ml (DE LA RICA; STEVENS, 2012). Nie et al. (2014) realizaram um estudo analítico do princípio do pELISA para a detecção ultra-sensível de anticorpos de *Treponema pallidum*. O pELISA exibiu uma resposta quase linear para as concentrações de anticorpos logarítmicos de *T. pallidum* na gama de 1pg/mL-10ng/mL com um limite de detecção de 0,98 pg/mL. O limite mínino de detecção foi 1000 vezes mais sensível em relação

ao ELISA convencional (NIE et al., 2014). Satija et al., (2014) por meio de uma revisão de literatura sistemática descrevem que pELISA é eficaz na detecção ultra-sensível de vários biomarcadores de patógenos clinicamente relevantes, e oferece inúmeras vantagens em termos de sensibilidade, confiabilidade, dispensa o uso de instrumentos sofisticados para a leitura do sinal e, portanto, reduz as despesas de detecção, tornando-a uma alternativa viável para regiões com recursos limitados (SATIJA et al., 2016).

Em contrapartida, algumas limitações são citadas na literatura, tais como; a agregação espontânea, também chamada de autoagregação das nanopartículas devido à presença de vários fatores ambientais, dentre elas a existência de impurezas, pH, temperatura extrema, força iônica elevada e outros fatores que podem induzir agregação indesejável de nanopartículas de metal, resultando em falsos positivos; os íons metálicos e as soluções de peróxido de hidrogênio são instáveis, o que pode afetar os resultados do pELISA na ausência de uma rotina rigorosa e reagentes de qualidade para os testes (SATIJA et al., 2016).

2 CAPÍTULO 1 - DEVELOPMENT OF PLASMONIC ELISA FOR THE DETECTION OF anti-*Leishmania* sp. IgG ANTIBODIES

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Journal of Immunological Methods – ANEXO A

2.1 Abstract

Recently, a novel Enzyme-Linked Immunosorbent Assay (ELISA) strategy has emerged, known as “plasmonic ELISA” (pELISA), which enables the detection of disease biomarkers at low concentrations with the naked eyes. For the first time, this research has developed, a signal-generation mechanism for the detection of anti-*Leishmania* sp. IgG antibodies with the naked eye using pELISA. The immunoassay incorporates an indirect ELISA with successive growth of gold nanoparticles to obtain blue or red-colored solutions in the presence or absence of anti-*Leishmania* sp. IgG antibodies in canine serum, respectively. For the development of pELISA, different concentrations of hydrogen peroxide, gold ions, biotinylated anti-dog IgG antibody and serum were tested in order to establish ideal values to each parameter. The assay was standardized with the concentrations of 250 μM hydrogen peroxide, 0.30 mM gold ions, and dilution of the streptavidin-catalase conjugate of 1/50. The technique was successfully tested in canine serum positive and negative for canine leishmaniasis (CanL) and was shown to be an effective method that could be used as an additional tool for CanL diagnosis. It will be particularly useful in resource-constrained countries, because it does not require sophisticated instruments to read the results, increasing the practicality of CanL detection in these areas.

Keywords: Enzyme-Linked Immunosorbent Assay; Metal nanoparticles; Canine Leishmaniasis

2.2 Introduction

Visceral leishmaniasis (VL) is a severe systemic disease that is fatal if not diagnosed and treated. In the Americas, VL is endemic in 12 countries with 59,769 new human cases reported from 2001-2017. Approximately 96% of these cases were reported by Brazil, however, southern countries like Argentina, Colombia, Paraguay and Venezuela are among those with the highest case records (WHO, 2019). In endemic areas for VL, infected dogs are the main reservoir for the zoonotic disease and play an important role in human transmission. Therefore, the diagnosis of canine leishmaniasis (CanL) is an essential measure for disease control (SOLANO-GALLEGO et al., 2009).

Various techniques are available for diagnosing CanL, which are typically classified into parasitological, immunological and molecular methods (SOLANO-GALLEGO et al., 2011). Indirect ELISA is one of the most commonly used immunoassay platforms in official VL control programs in Brazil (BRASIL, 2014). However, recent research has shown the low sensitivity of this method compared with molecular tests, suggesting that one out of five seronegative dogs are infected by *Leishmania infantum* (LOPES et al., 2017).

The problem with current indirect ELISA strategies is the high detection limit (1 ng/mL), which means they are inaccurate at the clinical threshold of many biomarkers, especially in the initial phase of the disease (WU et al., 2007; OGISO et al., 2013a). In addition, the need for an expensive instrument to read the results has limited its wide applicability, particularly in resource-constrained countries (SATIJA et al., 2016).

A novel immunoassay pELISA has emerged that enables the detection of a few molecules with the naked eye (DE LA RICA; STEVENS, 2013). This technique is based on the optical properties (Localized Surface Plasmon Resonance, LSPR) of metal nanoparticles, especially gold, and the successive growth of these nanoparticles mediated by a biocatalytic cycle of the enzymes (OGISO et al., 2013a). LSPR may be defined as a special optical phenomenon conferred by the interaction of light with electrons on the metallic nanoparticle surfaces (EUSTIS; EL-SAYED, 2006; GUO; KIM, 2012). Generally, following modulation in the growth (size/morphology/aggregation) of the gold nanoparticles, a prominent color change occurs (e.g. red to blue when there is a growth of gold nanoparticles in their aggregation state) that can be easily differentiated with the naked eye. In addition, minor changes in the state of gold nanoparticles leads to large optical changes in their properties (SATIJA et al., 2016).

Although, pELISA has been used to investigate different diseases diagnoses, including cancer and HIV (de La Rica and Stevens, 2012), fungal (SOJINRIN et al., 2017) and bacterial infections (NIE et al., 2014; CHEN et al., 2015; MOHD BAKHORI et al., 2018), in CanL, this method has not yet been investigated. Thus, for the first time, we developed a signal-generation mechanism to detect anti-*Leishmania* sp. IgG antibodies with the naked eye using pELISA.

2.3 Materials and methods

2.3.1 Reagents and materials

Recombinant antigen rK28 (Infectious Disease Research Institute, Seattle, Washington, USA), streptavidin (Sigma-Aldrich, cat. no. S4762), catalase from bovine liver (Sigma-Aldrich, cat. no. C1345), 24-unit ethylene glycol functionalized with succinimidyl and maleimido ends (Thermo Scientific SM (PEG)24), bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A7030), phosphate-buffered saline (PBS), Tween 20 (Sigma-Aldrich, cat. no. P9416), deionized water (resistivity ~18 MΩ cm), anti-dog IgG biotinylated antibodies and nonbiotinylated antibodies produced in goat (Sigma, St. Louis, MO, USA), MES (Sigma-Aldrich, cat. no. M3671), sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045), gold (III) chloride trihydrate (Sigma-Aldrich, cat. no. 520918), hydrogen peroxide (Sigma-Aldrich, cat. no. H1009), DMSO, carbonate buffer (0.05M and pH 9.6), vacuum filtration systems (0.2 µm), disposable PD-10 desalting columns (GE Healthcare Life Sciences, cat. no. 17-0851-01) and polystyrene microtiter plates, 96 well (Greiner Bio-one, cat. no. 655081 and Corning Inc., Corning, NY, USA, no. 3690) were used.

2.3.2 Samples and dilutions

For assay performance, blood samples of dogs were obtained from the “Luiz Quintiliano de Oliveira” Veterinary Hospital of the School of Veterinary Medicine, São Paulo State University (UNESP), Araçatuba, SP, Brazil. The samples were considered positive and negative by a combination of immunochromatographic test (Dual Path Platform - DPP®) and indirect rK28-ELISA (VENTURIN et al., 2015) followed by confirmation by real-time PCR in peripheral blood (PEROSSO et al., 2014).

In order to determine the optimal concentration of canine serum for use in indirect pELISA, serial dilutions of serum positive and negative for CanL (1/2, 1/50, 1/100, 1/200, 1/300 and 1/400) were analyzed by indirect pELISA. The concentration of the anti-dog IgG biotinylated antibodies was also tested by indirect pELISA in the following dilutions (1/50, 1/100, 1/200, 1/300 and 1/400). Blank wells without samples were included in all assays as control and the samples were analyzed in duplicate. After 15 min, the absorbance values at 550 nm were determined and photographs were taken showing the growth of the gold nanoparticles in different colors and intensities.

2.3.3 Preparation of solutions and conjugation of streptavidin to catalase

The preparation of gold-III and MES stock solution (100 nM), washing and blocking buffer and the procedure for conjugating streptavidin to catalase were performed as previously described (DE LA RICA; STEVENS, 2013). To establish the success of the conjugation reaction and determine a suitable working dilution, microtiter plates (area 1/2) were modified with 50 µl of recombinant antigen and incubated overnight at 4°C. Then, was added 50ul of anti-dog IgG biotinylated and nonbiotinylated antibodies diluted 1/200 in PBS-BSA (PBS and BSA to a 1 mg/mL, pH 7.2) After washing and blocking the plates, the conjugate (streptavidin-catalase) was added at different dilutions (1/10, 1/20, 1/50, 1/100; 1/200, 1/300,1/400, 1/800 and 1/2000) and the plasmonic signal was generated.

2.3.4 Determination of the optimal concentration of hydrogen peroxide and gold ions

To test the impact of the concentration of hydrogen peroxide on morphology and optical properties of the growth of gold nanoparticles, 100 µl of different concentrations of hydrogen peroxide (500, 400, 300, 250, 200, 150, 125, 100, 75, 50, 25 µM) and gold ions (0.10, 0.20, 0.25 and 0.30 mM) were diluted in MES buffer (1 mM, pH 6.5) and added to the microtiter plates in triplicate. After 15 min, the absorbance values at 550 nm were determined and photographs were taken showing the growth of the gold nanoparticles in different colors and intensities.

2.3.5 Indirect pELISA protocol

The assay was performed as previously described (DE LA RICA; STEVENS, 2013) with modifications for the detection of anti-*Leishmania* sp. IgG antibodies. A synthetic gene, K28, was generated by fusing multiple tandem repeat sequences of the *L. donovani* haspb1 and k39 kinesin genes to the complete open reading frame of haspb2, thereby increasing antigen epitope density, while providing complementing epitopes in the resulting recombinant protein (PATTABHI et al., 2010) and donated for use in the assay. Briefly, the microtiter plates were sensitized with 100 µl of recombinant antigen rK28 protein diluted in 0.05M carbonate buffer, pH 9.6, at a concentration of 250 ng/mL, as described by Venturin et al. (2015). The plates were then incubated overnight at 4°C, washed three times in PBS containing 0.05% Tween 20, pH 7.4 (washing buffer), and saturated for 1 h with 300 µl per well of PBS-BSA at room temperature. Next, the plates were washed again three times with washing buffer

and 100 µl of serum sample diluted in PBS-BSA (1/50) was added to each well and incubated at room temperature for 3 h, followed by three washes with washing buffer. Subsequently, 100 µl of biotinylated secondary antibody diluted 1/200 in PBS-BSA was added to each well of the microtiter plate, incubated at room temperature for 1 h and washed three times with washing buffer. After that, 100 µl of streptavidin-catalase conjugate diluted in PBS-BSA (1/50) was added to each well and incubated at room temperature for 1 h. The microtiter plate was then washed three times with washing buffer, twice with PBS and at least once with deionized water. This step was meant to remove remaining salts from buffers used in the previous steps. Next, 100 µl of hydrogen peroxide solution (250 µM) was added to each well of the microtiter plate and incubated at room temperature for 30 min. After homogenization, 100 µl of the 0.30mM gold solution was added and incubated at room temperature for 15 to 30 min. When the process is performed correctly, positive controls present blue coloration, while negative controls present red or pink coloration.

2.3.6 Instrumental Analysis

The growth of the gold nanoparticles determined with the naked eye was compared with absorbance obtained by a Tecan microplate reader (Sunrise model ref. 16039400) using a wavelength of 550 nm.

2.4 Results

2.4.1 Optimization of streptavidin-catalase conjugate, hydrogen peroxide and gold ion concentrations

In this experiment, the streptavidin-catalase conjugate was tested at different dilutions (1/10 to 1/2000) and the indirect pELISA signals were recorded (Figure 1). The growth of gold nanoparticles was monitored by measuring the absorbance at 550 nm and the signal shows a dilution-dependent behavior, and therefore that the conjugation was successful (Figure 1A). We verified that the solution changed blue in wells that were sensitized with anti-dog IgG biotinylated antibodies, except dilution 1/2000. Therefore, to obtain a good balance between low nonspecific signal and high detection sensitivity, the optimal dilution of the conjugate was set at 1/50 for the assay (Figure 1B).

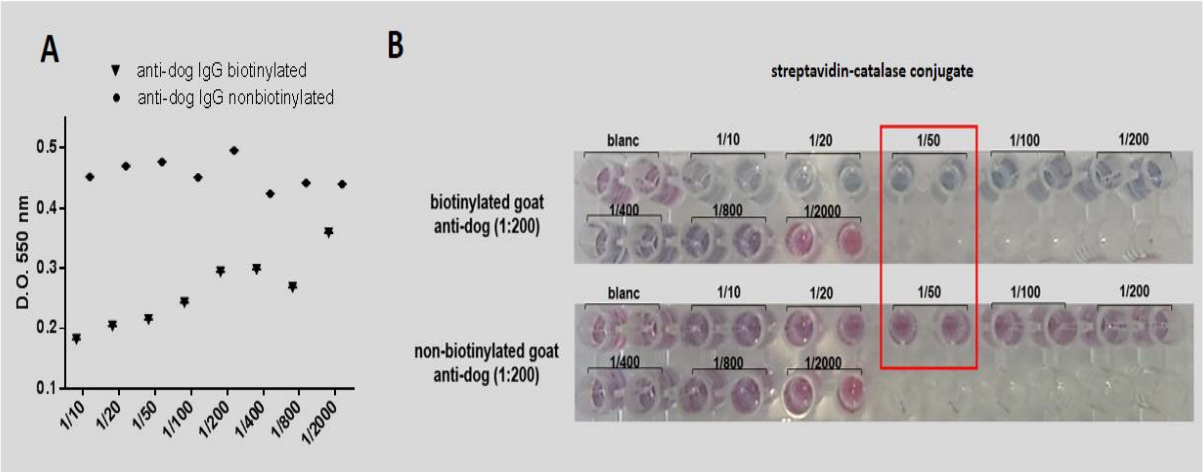

Figure 1. Indirect pELISA in positive sample for CanL with biotinylated and nonbiotinylated anti-dog IgG after incubation with the streptavidin-catalase conjugate at different dilutions. Observation of growth of the gold nanoparticles with the naked (A) and absorbance values at 550 nm for measurements performed (B).

Different concentrations of hydrogen peroxide (500 to 25 μM) were tested at 0.10, 0.20, 0.25 and 0.30 mM of gold ion concentrations. The color tonality of solutions changed from pink to blue with the decrease in hydrogen peroxide concentration (Figure 2). Gold ions at concentrations of 0.10 and 0.20 mM were insufficient to clearly distinguish the color of the solutions with the naked eye (Figure 2A); however, gold ion concentrations of 0.25 and 0.30 μM showed adequate growth of nanoparticles to differentiate the color of the solutions, which changed from pink to blue at a hydrogen peroxide concentration between 250 and 150 μM (Figure 2B).

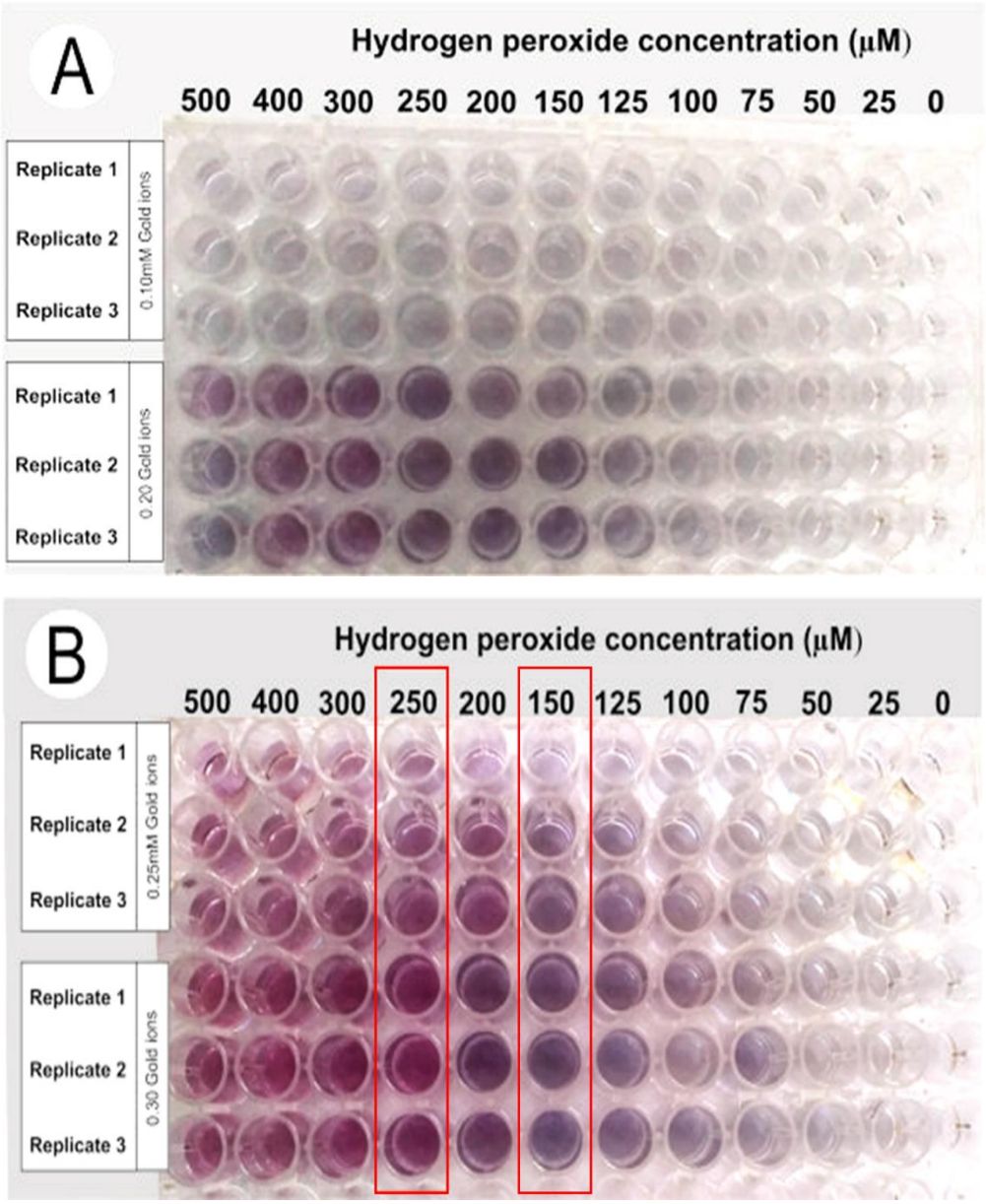

Figure 2. The growth of the gold nanoparticles depends on the concentration of hydrogen peroxide. Gold ion concentrations of 0.10 and 0.20 mM (A) and 0.25 mM and 0.3 mM (B) at different hydrogen peroxide concentrations.

Furthermore, the growth of gold nanoparticles was monitored by measuring the absorbance at 550 nm with different gold ion concentrations (Figure 3A), taken together, these results indicated that the optimal negative control condition of hydrogen peroxide was 250 µM with gold ions at 0.30 mM for indirect pELISA. In the Figure 3B, the growth of gold nanoparticles was monitored at different wavelengths (450 – 700 nm) and a peak shift of absorption spectra with gold nanoparticles growth in an aggregated state when compared to growth of gold nanoparticles in a monodisperse state was observed.

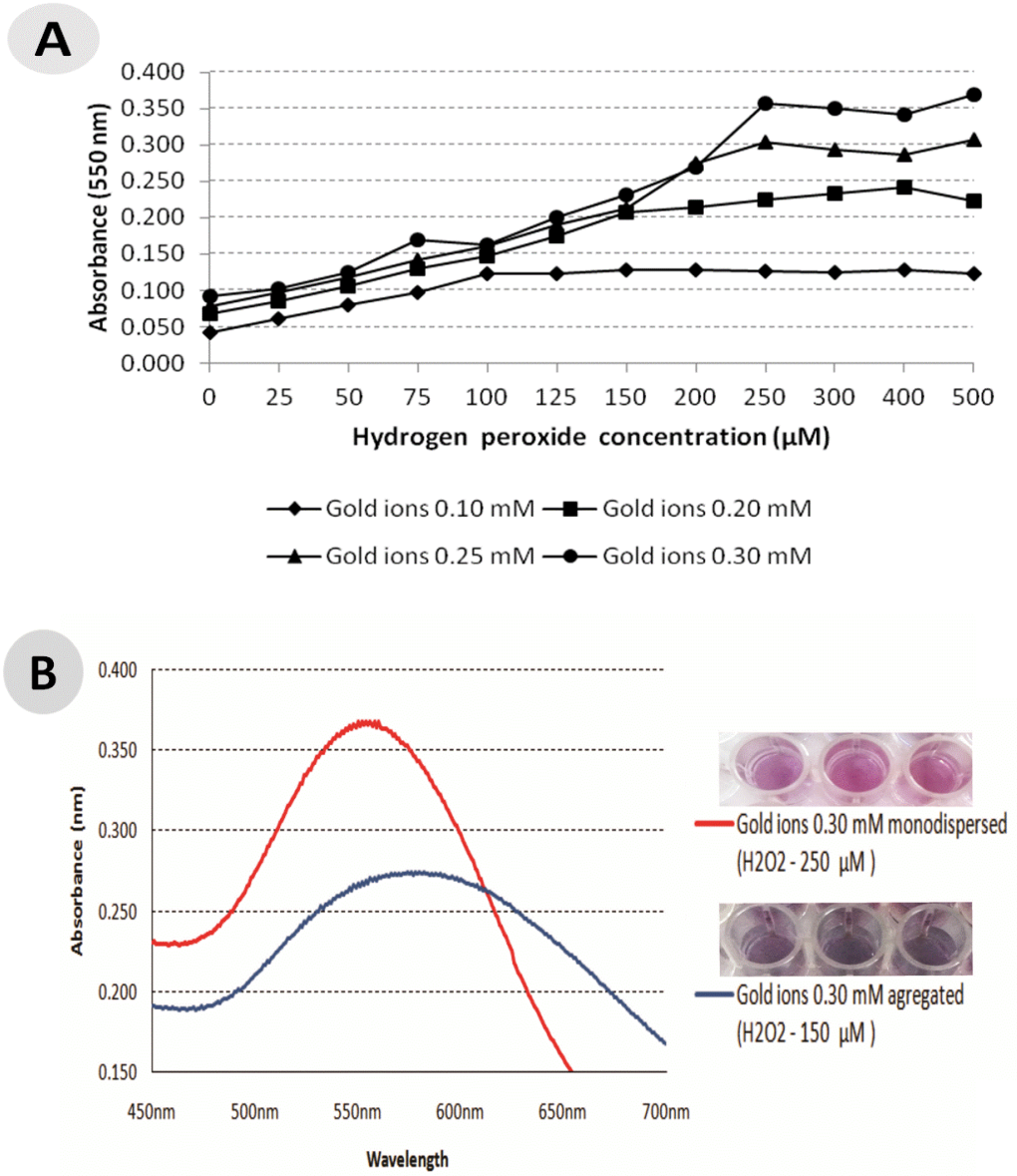

Figure 3. Absorbance curve of gold nanoparticles according to the hydrogen peroxide concentration. (A) Absorbance values at 550 nm. (B) Absorption spectra at different wavelengths (450-700nm) for the growth of nanoparticles gold in the monodispersed (red line) and aggregated (blue line) state.

2.4.2 Assay performance

The performance of the indirect pELISA developed was tested at different dilutions (1/50 to 1/400) of anti-dog IgG biotinylated antibodies (Figure 4, A and B) and at different dilutions (1/2 to 1/400) of a canine serum positive and negative for CanL (Figure 5, A and B). The results were confirmed after visual reading in the laboratory by three observers properly trained.

The concentration of anti-dog IgG biotinylated antibodies was optimized to detect a suitable dilution that yielded the maximum signal with minimal nonspecific interactions. However, no differences between the dilutions were observed with the

naked eye or in absorbance measurements (Figure 4, A and B). Therefore, the dilution of anti-dog IgG biotinylated antibodies was set at 1/200 for the assay.

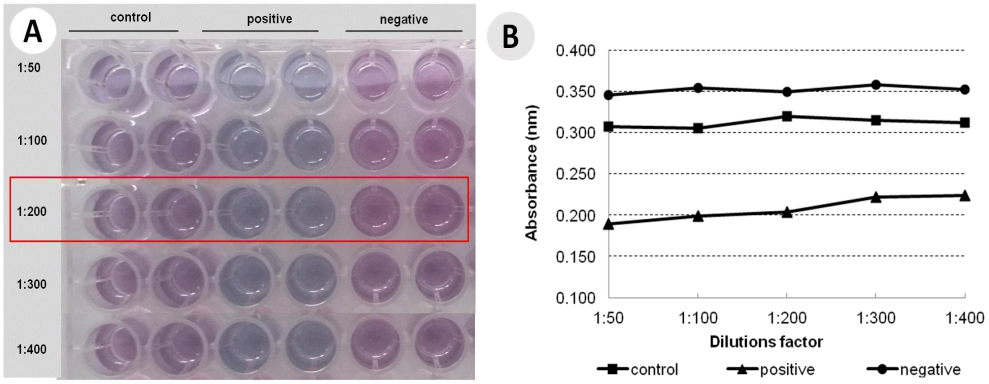

Figure 4. Optimization of the concentration of biotinylated secondary antibody for the assay. (A) The growth of the gold nanoparticles in different biotinylated secondary antibody dilutions using canine serum positive and negative for CanL. (B) Mean absorbance values at 550 nm for measurements performed in duplicate.

In addition, our technique was successfully tested on canine serum positive and negative for CanL. We clearly observed the appearance of a blue colored solution only in the positive samples, while the pink coloration was observed in the negative samples. To test the possibility of nonspecific reactions with other proteins present in the solutions with the plate, we included controls (blank wells without samples) in the microtiter plates that also showed pink colored solutions (Figure 5A). Although we were unable to verify any differences among serum dilutions with the naked eye, the absorbance values at 550 nm determined that 1/50 dilution optimized the assay. In addition, high mean absorbance values (0.340) were determined for negative samples, while positive samples showed low values (0.217) (Figure 5B).

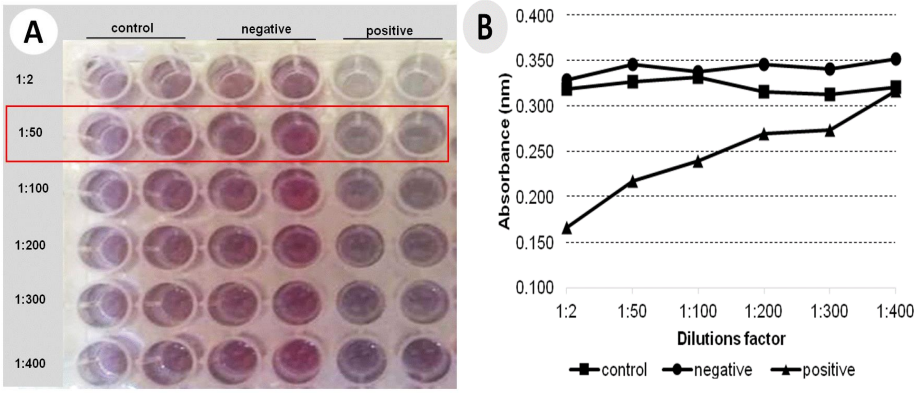

Figure 5. Assay performance and optimization of the concentration of canine serum the assay. (A) The growth of the gold nanoparticles in different dilutions of positive and negative serum for CanL. (B) Mean absorbance values at 550 nm for measurements performed in duplicate (B).

2.5 Discussion

We adapted the analytical principle of the pELISA (DE LA RICA; STEVENS, 2013) to an indirect format (S1 Figure). In the method proposed in this research, the signal generation mechanism is associated with the successive growth of gold nanoparticles that produce blue or red colored solutions in the presence or absence of the anti-*Leishmania* sp. IgG antibodies in canine serum. Briefly, in the absence of the antibodies, the reduction in gold ions due to hydrogen peroxide occurs at a rapid rate, and under these conditions, the solution is expected to present the growth of gold nanoparticles in a monodisperse state, which produces a pink or red solution. Conversely, in the presence of the antibodies, the catalase enzyme consumes the hydrogen peroxide. This slows down the kinetics of gold nanoparticle growth, which results in an aggregated state of gold nanoparticles, producing a blue solution. These blue and red colored solutions are easily distinguishable at a glance, therefore facilitating the detection of positive and negative results with the naked eye.

The optimal concentration of hydrogen peroxide was 250 μM with gold ions at 0.30 mM for pELISA. These concentrations were chosen because together they produced the lowest concentration of hydrogen peroxide without the growth of gold nanoparticles in the aggregated state and, consequently, promoted a change in the color of the solution. The essential step for the success of pELISA is a variation in the concentration of hydrogen peroxide to control the growth and state of aggregation of gold nanoparticles (DE LA RICA; STEVENS, 2013). Therefore, during an actual case assay using indirect pELISA to diagnose CanL, the biocatalytic action of catalase should reduce the hydrogen peroxide concentration to less than 200 μM, producing blue-colored nanoparticle solutions.

The optimal streptavidin-catalase conjugate dilution was 1/50, which was chosen because it presented a good balance between a low nonspecific signal and high detection sensitivity. This is key factor that directly affects the pELISA sensitivity (DE LA RICA; STEVENS, 2013).

We used hydrogen peroxide, gold ions and streptavidin-catalase conjugate at different concentrations than those determined previously (DE LA RICA; STEVENS, 2012; HAN et al., 2018; MOHD BAKHORI et al., 2018). These discrepancies in the concentrations can be explained by environmental factors, including humidity and temperature, that can significantly affect the stability of the nanoparticles. Systematic

experiments to test the ideal hydrogen peroxide and gold ions concentration should be performed and modifications must be adopted in the protocol, where necessary, to ensure the success of the immunoassay (SATIJA et al., 2016). Hence, the reasoning that supports the standardization carried out in our report.

The indirect pELISA was successfully tested on canine sera positive and negative for CanL, in which positive serum yielded aggregated gold nanoparticles growth and solutions clearly distinguished by its blue coloration, whereas negative serum presented monodispersed gold nanoparticles growth and red-colored solution. Similarly, the pELISA was effective at detecting the disease biomarkers with the naked eye. This makes the test particularly attractive because it provides good sensitivity, without requiring expensive equipment to read the results (CHEN et al., 2015; DE LA RICA; STEVENS, 2013; DE LA RICA; STEVENS, 2012; LIANG et al., 2015; MOHD BAKHORI et al., 2018; NIE et al., 2014; SOJINRIN et al., 2017).

Considering that the human VL is associated with high morbidity and mortality, especially in resource-constrained countries (WHO, 2015), and that one of the strategies to reduce the disease in these areas is the monitoring and control of CanL cases (BRASIL, 2014), indirect pELISA could be a useful additional tool in its diagnosis, dispensing the use of sophisticated instruments determine the results, while increasing the practicality of CanL detection in these areas.

2.6 Conclusion

In conclusion, we have demonstrated the detection of anti-*Leishmania* sp. IgG antibodies by indirect pELISA for the first time. The new immunoassay was shown to be an effective method that can be used as an additional tool for the diagnosis of CanL. We hope this report leads to future studies that lead to the validation of pELISA for CanL.

2.7 Acknowledgements

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2.8 Conflict of interest statement

The authors declare that they have no conflicts of interest.

REFERENCE

BRASIL. **Manual de vigilância e controle da leishmaniose visceral.** Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica. 1. ed. [s.l: s.n.]

CHEN, R. et al. Plasmonic Enzyme-Linked Immunosorbent Assay Using Nanospherical Brushes as a Catalase Container for Colorimetric Detection of Ultralow Concentrations of Listeria monocytogenes. **ACS Applied Materials and Interfaces**, v. 7, n. 51, p. 28632–28639, 2015.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. **Nature Nanotechnology**, v. 7, n. 12, p. 821–824, 2012.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the detection of analytes at ultralow concentrations with the naked eye. **Nature Protocols**, v. 8, n. 9, p. 1759–1764, 22 set. 2013. Disponível em: <http://dx.doi.org/10.1038/nprot.2013.085>.

EUSTIS, S.; EL-SAYED, M. A. Why gold nanoparticles are more precious than pretty gold: Noble metal surface plasmon resonance and its enhancement of the radiative and nonradiative properties of nanocrystals of different shapes. **Chemical Society Reviews**, v. 35, n. 3, p. 209–217, 2006.

GUO, L.; KIM, D. H. LSPR biomolecular assay with high sensitivity induced by aptamer-antigen-antibody sandwich complex. **Biosensors and Bioelectronics**, v. 31, n. 1, p. 567–570, 2012. Disponível em: <http://dx.doi.org/10.1016/j.bios.2011.10.047>.

HAN, S. et al. Gold nanoparticle-based colorimetric ELISA for quantification of ractopamine. **Microchimica Acta**, v. 185, n. 4, p. 210, 7 abr. 2018. Disponível em: <http://link.springer.com/10.1007/s00604-018-2736-3>.

LIANG, J. et al. Silver nanoprism etching-based plasmonic ELISA for the high sensitive detection of prostate-specific antigen. **Biosensors and Bioelectronics**, v. 69, p. 128–134, 2015.

LOPES, E. G. et al. Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: One out of five seronegative dogs are infected. **Epidemiology and Infection**, v. 145, n. 12, p. 2436–2444, 2017.

MOHD BAKHORI, N. et al. Immuno Nanosensor for the Ultrasensitive Naked Eye Detection of Tuberculosis. **Sensors (Basel, Switzerland)**, v. 18, n. 6, p. 1–10, 2018.

NIE, X. M. et al. Plasmonic ELISA for the ultrasensitive detection of Treponema pallidum. **Biosensors and Bioelectronics**, v. 58, p. 314–319, 2014. Disponível em: <http://dx.doi.org/10.1016/j.bios.2014.03.007>.

OGISO, M. et al. Carbohydrate immobilized on a dendrimer-coated colloidal gold surface for fabrication of a lectin-sensing device based on localized surface plasmon resonance spectroscopy. **Biosensors and Bioelectronics**, v. 41, n. 1, p. 465–470, 2013. Disponível em: <http://dx.doi.org/10.1016/j.bios.2012.09.003>.

PATTABHI, S. et al. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. **PLoS Neglected Tropical Diseases**, v. 4, n. 9, 2010.

PEROSSO, J. et al. Alteration of sFAS and sFAS ligand expression during canine visceral leishmaniosis. **Veterinary Parasitology**, v. 205, n. 3–4, p. 417–423, out. 2014. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0304401714004919>.

SATIJA, J. et al. Plasmonic-ELISA: Expanding horizons. **RSC Advances**, v. 6, n. 88,

p. 85440–85456, 2016. Disponível em: <http://dx.doi.org/10.1039/C6RA16750K>.

SOJINRIN, T. et al. Plasmonic gold nanoparticles for detection of fungi and human cutaneous fungal infections. **Analytical and Bioanalytical Chemistry**, v. 409, n. 19, p. 4647–4658, 2 jul. 2017. Disponível em: <http://link.springer.com/10.1007/s00216-017-0414-7>.

SOLANO-GALLEGO, L. et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. **Veterinary Parasitology**, v. 165, n. 1–2, p. 1–18, out. 2009. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0304401709003124>.

SOLANO-GALLEGO, L. et al. LeishVet guidelines for the practical management of canine leishmaniosis. **Parasites & Vectors**, v. 4, n. 1, p. 86, 2011. Disponível em: <http://www.parasitesandvectors.com/content/4/1/86>.

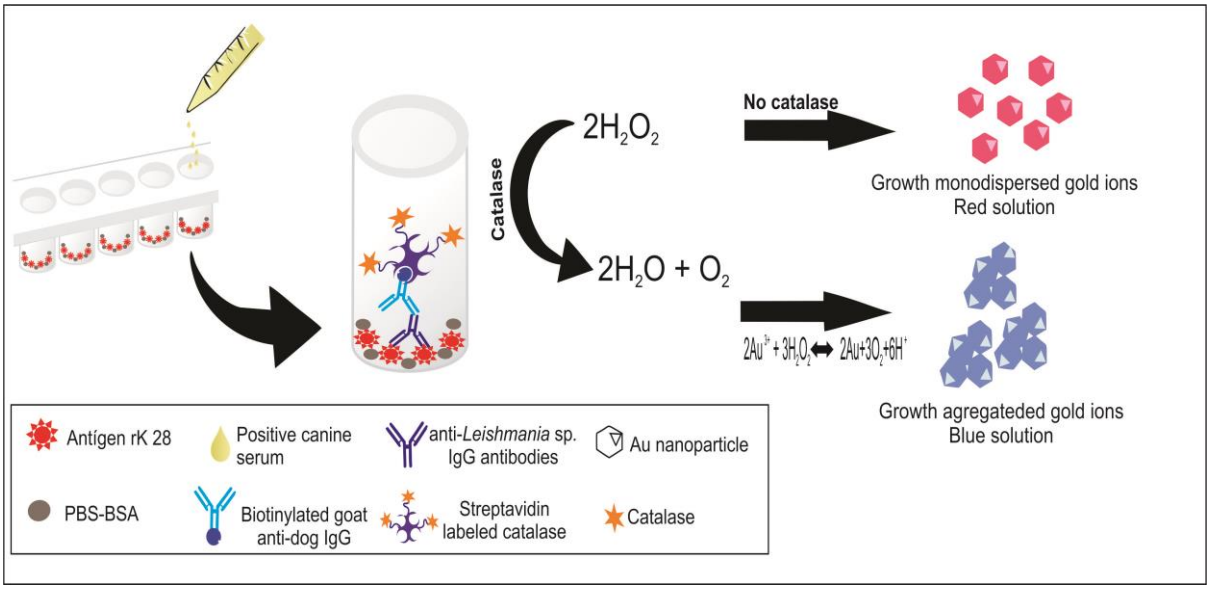
VENTURIN, G. L. et al. Recombinant K28 antigen in ELISA in the diagnosis of canine visceral leishmaniosis. **Parasite Immunology**, v. 37, n. 12, p. 670–673, 2015.

WHO. **WHO bi-regional consultation on the status of implementation of leishmaniasis control strategies and epidemiological situations in Eastern Africa, Addis Ababa, Ethiopia, 9–11 april 2018: summary report**, 2015.

WHO. Epidemiological Report of the Americas. **World Health Organization technical report series**, v. 7, 2019. Disponível em: <http://iris.paho.org/xmlui/bitstream/handle/123456789/50505/Leishreport2019_eng.pdf?ua=1>.

WU, J. et al. Biomedical and clinical applications of immunoassays and immunosensors for tumor markers. **TrAC - Trends in Analytical Chemistry**, v. 26, n. 7, p. 679–688, 2007.

2.9 Supplementary information



S1 Figure. Scheme of analytical principle of the pELISA for detection of anti-*Leishmania* sp. IgG antibodies in canine serum.

3 CAPÍTULO 2 - INDIRECT PLASMONIC ELISA rK28 IMPROVES THE DIAGNOSIS OF LEISHMANIASIS CANINE

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Veterinary Parasitology – ANEXO B

3.1 Abstract

In this study, we evaluated the performance of a new immunoassay “Indirect plasmonic ELISA rK28” (pELISA) for diagnosis of Canine Leishmaniasis (CanL). One hundred seventy serum samples of dogs from endemic area for CanL and 26 of healthy dogs from a nonendemic area were tested by pELISA and compared to indirect ELISA rk28 and imunocromatografic test (Dual Path Platform, TR_DPP®) using as gold standard the real-time PCR in blood samples or conjunctival swab. The TR_DPP®, indirect ELISA rK28 and pELISA presented sensitivity of 79.0%, 89.5% and 94.7% and specificity of 90.1%, 91.4% and 100%, respectively. The highest predictive positive (100%), negative (99.3%) and accuracy (99.4%) value were observed in pELISA. Kappa coefficient between pELISA with real-time PCR showed excellent agreement (0.970), differently of indirect ELISA rK28 and TR_DPP®, which showed good agreement (0.645 and 0.551 respectively). Analysis of the results revealed that the pELISA improved sensitivity and presented high specificity compared to official method recommended by the Ministry of Health in Brazil and may increase the practicality of diagnosis in resource-constrained countries, because it does not require sophisticated instruments to read, suggesting that this method can be used as an additional tool for the diagnosis of CanL in these areas.

Keywords: Visceral Leishmaniasis; Dogs; Enzyme-Linked Immunosorbent Assay; Diagnosis; Metal nanoparticles.

3.2 Introduction

Visceral leishmaniasis (VL) is a zoonosis caused by protozoa of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae). The *Leishmania infantum* is the main etiological agent in Américas and the most common vectors are *Lutzomyia longipalpis* and *Lutzomyia cruzi* (READY, 2014). The VL is associated with high morbidity and mortality and have a wide worldwide distribution, the most cases occur in Africa, Asia and the Americas (WHO, 2019). In endemic areas for VL, infected dogs are the main reservoir for the zoonotic disease and play an important role in human transmission (SOLANO-GALLEGO et al., 2011).

In canine leishmaniasis (CanL) most dogs develop a humoral immune response, with high IgG anti-*Leishmania* sp. and may determine the clinical form of the

disease. However, in endemic areas the disease may remain clinically inapparent for long periods, many infected dogs are “clinically healthy” and have low antibody levels (SOLANO-GALLEGO et al., 2011). In endemic areas, there is a high prevalence of subclinical infection and may represent up to 67% of infected dogs (SOLANO-GALLEGO et al., 2001) and thus, may serve as reservoir for vector transmission, increasing the risk of transmission of disease (DA COSTA-VAL et al., 2007; MICHALSKY et al., 2007; SOARES et al., 2011).

Various techniques are available for diagnosing CanL, which are typically sorted into parasitological, immunological and molecular methods (SOLANO-GALLEGO et al., 2011). In official VL control programs from Brazil, the methods recommended are the imunocromatografic test (Dual Path Platform, TR_DPP®) and indirect Enzyme-Linked Immunosorbent Assay (ELISA) as confirmatory (BRASIL, 2014). The sensitivity and specificity of serological methods depend on the type of antigen used. In addition of total antigen, several *Leishmania* antigens have been characterized, and recombinant technology has been used for the development of recombinant antigens to improve the sensitivity, mainly rK28 antigen (VENTURIN et al., 2015). The rK28 antigen is a synthetic gene, generated by fusing multiple tandem repeat sequences of the *L. donovani* haspb1 and k39 kinesin genes to the complete open reading frame of haspb2, thereby increasing antigen epitope density, while providing complementing epitopes in the resulting recombinant protein (PATTABHI et al., 2010).

A novel plasmonic ELISA (pELISA) has emerged that enables the detection of a few molecules with the naked eye (DE LA RICA; STEVENS, 2013). This technique is based on the optical properties (Localized Surface Plasmon Resonance, LSPR) of metal nanoparticles, especially gold, and the successive growth of these nanoparticles mediated by a biocatalytic cycle of the enzymes (OGISO et al., 2013a). Thus, a smaller change in the state of aggregation these metallic nanoparticles will lead to huge changes in their optical properties, offering a potential approach to ultra-sensitive assays of target molecules (SATIJA et al., 2016).

Although, pELISA has been used to investigate different diseases, including cancer and HIV (de La Rica and Stevens, 2012), fungal (SOJINRIN et al., 2017) and bacterial infections (NIE et al., 2014; CHEN et al., 2015; MOHD BAKHORI et al., 2018) there was not been performed in diagnosis of CanL. Recently, our group developed and successfully tested the indirect Plasmonic ELISA rK28 in canine serum

positive and negative for VL, facilitating the detection at the naked eye and may be dispensing the use of sophisticated instruments to read (data not yet publishd).

the present study compared the performance of pELISA in relation to indirect ELISA rK28 and imunocramatografic test (Dual Path Platform TR_DPP®) using as gold standard the real-time PCR in biological samples of dogs from an endemic and nonendemic area for this disease, in order to validate the pELISA as an alternative method for the for diagnosis of CanL.

3.3 Materials and methods

3.3.1 Dogs

The required sample size was calculated and tabulated with marginal errors of 5%, 95% confidence level and expected a proportion of positivity in the canine population (LWANGA; LEMESHOW, 1991). A total of 170 adults dogs (100 females and 70 males) with an average age of 4.6±3.3 years from the different sectors defined by the Epidemiological Surveillance Service of Araçatuba (2°12’32” S and 50°25’58” W), Northwestern São Paulo state, Brazil, a municipality with high endemicity for CanL (S1 Figure) and 26 adult dogs (16 females and 10 males) with an average age of 2.2±3.1 years from the municipality of São Vicente (23°57’48” S and 46°23’32” W), Southern São Paulo state, Brazil, nonendemic area for this disease (group control) were selected for this study (S1 Table). This study was approved by the Brazilian Council on Animal Experimentation (COBEA), with the approval of the Committee for Ethics in Animal Use (CEUA) of the Araçatuba, School of Veterinary Medicine of São Paulo State University (FOA/UNESP) under protocol no. 00766_2017.

3.3.2 Samples and clinical signs

From each dog, 3 ml of blood were collected by venepuncture via femoral or jugular in three aliquots, one in a tube without anticoagulant and the other two in a tube with EDTA (10%). Biological samples were taken at the owner’s residence. EDTA blood was used for hematological examination, DNA extraction, and real-time PCR. The other fraction without anticoagulant was centrifuged at 3000 rpm for 5 min, the serum was transferred to 1.5 ml microtubes and immediately stored at -20°C until the time of serological and biochemical analysis. Sterile swabs were used to scrape the conjunctival mucosa from the lower eyelid of the dog´s left eye, and then the tips of the

swab were broken, transferred to DNase-free microtubes without the addition of any solution, stored at -20°C. Epidemiological records were filled with information such as sex, race, age, and presence of dog symptoms.

3.3.3 DNA extration

The genomic DNA was obtained from peripheric blood samples and conjunctival swab, according to the described by Bashiruddin et al. (1999) and Ferreira et al. (2008), respectively. DNA was extracted using the phenol-chloroform purification method (SAMBROOK et al, 1989) and the concentration of extracted DNA (ng/µl) and degree of purity (coefficient A_{260nm}/A_{280nm}) was determined by spectrophotometer (NanoDrop Technologies ND 1000 UV/VIS, USA).

3.3.4 Molecular diagnosis by real-time PCR in periferic blood and swab conjunctival

The detection of DNA *Leishmania* sp. in peripheric blood samples and conjunctival swab was used as the gold standard. The real-time PCR was performed on a Mastercycler ep Gradient S No. 5345 (Eppendorf North America, Westbury, NY, USA). The reaction was standardized with 50 ng of genomic DNA purified, 4 µl of 5x HOT FIREPol® Evagreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia), 10 pmol (at a final concentration of 500nM) of each primer (ITS1) (5'AGCTGGATCATTTTCCGATG3' and 5'TATGTGAGCCGTTATCCACGC3') (EL TAI et al., 2000) and 13 µl molecular ultrapure water in a final volume of 20 µl. The thermal cycle conditions consisted of a 12 min initial incubation at 95°C, followed by 40 cycles at 95°C for 15 min, 60°C for 20 s and 72°C for 20 s each. The dissociation curve of the amplified fragment was determined at 95°C for 15 sec, 60°C for 15 sec, followed by 20 min until reaching 95°C for 15 s. Standard curves were with DNA from *Leishmania infantum* promastigotes (MHOM/BR00/MER02) with a dilution of 10^7 a 10^2 parasites. The standard curve, calculated by independent experiments was linear over at least 6 log ranges of DNA concentration points. Samples were amplified in a single 96-well plate in triplicates. The samples were considered positive when they presented a threshold cycle (C_t) and melting temperature (T_m) corresponding to the standard curve. The parasitic DNA load was determined in each sample examined by comparison of the data with a specific standard curve. The S18 ribosomal protein (RPS18) was used as the reference gene, for the evaluation of DNA quality. Its expression was determined

by real-time PCR using primers (5'TCCAGCACATTTTGCGAGTA3' and 5'CCACACAGGTTCTTCTTTATTTGG3') (PETERS et al., 2007) at a final concentration of 200nM and the reaction was performed with a final volume of 20 µl. As negative control of the reaction was used only water and the reaction efficiency and correlation coefficient values (R^2) were obtained from 7 serial dilutions of the genomic DNA pool of the standard curve.

3.3.5 Imunocromatografic test (TR-DPP®)

The immunological test performed was Dual Path Platform (TR_DPP®) (Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, Brazil), distributed by the Ministry of Health (Brazil), and executed as recommended by the manufacturer's instructions (BIO-MANGUINHOS, 2011).

3.3.6 Indirect ELISA rK28

The serum of the dogs was analyzed by indirect ELISA rK28 according to the described by Venturin et al. (2015). The results were expressed as the mean optical density obtained from duplicate serum. The absorbance was measured at 490 nm using the automatic reader Spectra Count (Packard Bio Science Company, Meriden, CT, EUA). For the determination of the cut-off value, it was performed a controlled study using 20 serum samples of healthy dogs from the noneendemic area for CanL and indirect ELISA as assay. The point was stipulated from the mean plus three standard deviations of the optical density reading (OD), which was considered to be 0.206 (VENTURIN et al., 2015).

3.3.7 Indirect Plasmonic ELISA rK28

The serum of the dogs were tested by pELISA to the detection of IgG anti-rK28 antibodies with the naked eye. Briefly, in the pELISA, the microtiter plates (1/2 area) (Corning Inc., Corning, NY, USA, Cat. 3690) after sensitized with 50 µl of recombinant antigen rK28 protein diluted in carbonate buffer 0.05M and pH 9.6 in the concentration of 250 ng/mL (VENTURIN et al., 2015). Then washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and pH 7.2 (washing buffer) and saturated for 1h30 with 150 µl by well of PBS-BSA (PBS and BSA to a 1 mg/ml, pH 7.2) at room temperature. Next, the plate was washed again three times with washing buffer. Then 50 µl of serum sample diluted in PBS-BSA (1/50) was added

to each well and incubated at room temperature for 3h, followed by three washes with washing buffer. Subsequently, 50 µl of anti-dog IgG biotinylated antibodies (Sigma, St. Loius, MO, USA) diluted 1/200 in PBS-BSA was added to each well of the microtiter plate, incubated at room temperature for 1h and washed three times with washing buffer. Then, 50 µl of the streptavidin-catalase conjugate (Sigma-Aldrich, cat. no. S4762 and C1345) diluted in PBS-BSA (1/50) was added into each well and incubated at room temperature for 1h. After, the microtiter plate was washed three times with washing buffer, twice with PBS and at least once with deionized water. This step was meant to remove remaining salts from buffers used in the previous steps. Then, 50 µl of hydrogen peroxide solution (Sigma-Aldrich, cat. no. H1009) in the 250 µM concentration was added to each well of the microtiter plate and incubated at room temperature for 30 min. After homogenization, 50 µl of the gold solution (0.30 mM) was added and incubated at room temperature for 15 to 30 min. The blue color should appear on positive control and the red or pink color on negative control. All samples received a different number from the original sample, the assays were performed blind and in duplicate, the results were read with the naked eye for three independent observers properly trained.

3.3.8 Statistical analysis

Statistical analyses were performed using the Biostat program version 4.0, the different methods were submitted to statistical analysis to obtain the sensitivity (SE), specificity (SP), positive and negative predictive values (PV), positive and negative likelihood ratio (LR) and accuracy (AC) considering as references the results obtained in the real-time PCR. The agreement between diagnostic techniques was evaluated by the use the Kappa (κ) concordance index (95% confidence interval) being determined as follows: $\kappa < 0.4$ accepted as weak agreement, $0.4 < \kappa < 0.7$ as good and $\kappa > 0.7$ as optimal (ROSNER, 2006). The McNemar test was used to verify the difference between paired proportions obtained with the diagnostic tests. Differences were considered significant when $p<0.05$.

3.4 Results

3.4.1 Samples

To examine the sensitivity and specificity of new indirect Plasmonic ELISA rk28 (pELISA) and compared with indirect ELISA rk28 and Dual Path Platform (TR_DPP®) 196 dogs were included in the analysis, among these 86.7% (170/196) are dogs from an endemic area and 13.3% (26/196) from nonendemic area. From the 170 samples from endemic area *Leishmania* sp. DNA was detected in 11.2% (19/170) by real-time PCR (using peripheral blood and/or conjunctival swab samples) and was therefore considered infected by *Leishmania* sp. Of the 88.8% (151/170) dogs that were negative by real-time PCR were considered noninfected. Overall, the reactions of real-time PCR presented high efficiency (0.96-1.09) and correlation coefficient (R^2) (0.992-0.999). Moreover, all negative samples presented expression for S18 ribosomal protein (reference gene) excluding the possibilities of negatives false by the gold standard. The real-time PCR was considered the gold standard, because of its high sensitivity and specificity in detecting positive dogs (METTLER et al., 2005).

3.4.2 Clinical signs and laboratory findings

Thirty-one percent (6/19) of the dogs from endemic area were positive by real-time PCR (gold standard) presented three or more clinical signs compatible with CanL. The clinical signs of positive dogs were lymphoadenomegaly (42.1%), onychogryphosis (31.6%) and skin lesions (26.3%) (S2 Table). About of 36% (7/19) of these dogs presented hyperproteinemia (>71g/L) with inversion of the albumin-globulin ratio, characterized by hypergammaglobulinemia (globulin>44g/l) and hypoalbuminemia (albumin <26g/L) (S3 Table). The main hematological alteration of positive dogs was the reduction of MCHC present in 68.4% (13/19) of the dogs, followed by reduction of platelets in 63.2% (12/19) of the dogs (S4 Table). Dogs from the nonendemic area were negative by real-time PCR (gold standard) and no clinical signs compatible with CanL were observed (S2 Table). All dogs from the nonendemic area (26/26) presented biochemical and hematological exams within the parameters of normality (S5 and S6 table).

Dogs from the endemic area were negative real-time PCR (gold standard) showed onychogryphosis (29.8%), lymphoadenomegaly (27.8%), skin lesions and dermatitis both with 15.2% (S7 Table). The main biochemical alterations were hyperproteinemia in 43.3% and the increase of AST protein (4.6%) and urea (2.6%) in the blood (S8Table). The hematological profile of dogs showed an increase WBC in

41.7% (63/151), followed by reduction of platelets in 31.8% (48/163) and PVC in 24.5% (37/151) (S9 Table).

Dogs with VL were staged based on the presence of clinical signs, laboratory findings (complete blood count, biochemical profile) and levels of antibodies (indirect ELISA rK28) (SOLANO-GALLEGO et al., 2011) (Table 1).

Table 1. Clinical staging of positive dogs by real-time PCR (gold standard) based on serological status, clinical signs, and laboratory findings in endemic area for CanL.

Clinical staging	N	(%)	Feactures
"Clinically healthy"	1	5.3	Dogs with negative sorology and no clinicopathological abnormalities, but have a confirmed *L. infantum* infection.
Stage I*	4	21.1	Dogs with negative sorology or low positive antibody levels, no clinicopathological abnormalities observed but mild clinical signs such as peripheral lymphadenomegaly.
Stage II**	13	68.3	Dogs, which apart from the signs listed in stage I, may present cutaneous lesions and weight loss. Positive sorology and of low to high antibody levels, inversion of the albumin globulin ratio, characterized by hypergammaglobulinemia (globulin> 44g/l) and hypoalbuminemia (albumin <26g/l) with renal function still within the parameters of normality (creatinine <1.4 mg/dl).
Stage III***	1	5.3	Dogs, which apart from the signs and clinicopathological abnormalities listed in stage I and II. Positive sorology and medium to high antibody levels, with renal function outside normality parameters (creatinine 1.4-2.0mg /dl).
Total	**19**	**100%**	

* Stage I: mild-moderate disease (level I); ** Stage II: moderate disease; *** Stage III: Severe disease(SOLANO-GALLEGO et al., 2011).

3.4.3 Validation of indirect plasmonic rK28 ELISA

Among the 170 samples from the endemic area, *Leishmania* DNA was detected in 11.2% (19/170) by real-time PCR, which 94.7% (18/19) were positive in pELISA, 89.5% (17/19) in indirect ELISA rK28 and 78.9% (15/19) in TR_DPP® (Table 2).

Considering the clinical staging, none of methods used detected anti-k28 IgG antibodies in the dogs of the group classified as "clinically healthy". In the dogs of the group classified as "sick stage I" of disease, pELISA detected 100.0% (4/4) of

seropositive dogs when compared to indirect ELISA rK28 75.0% (3/4) and to TR_DPP® 50.0% (2/4). In the group of dogs in stage II, the pELISA and indirect ELISA rK28 detected anti-k28 IgG antibodies in 100.0% (13/13) of dog with the disease, whereas the TR_DPP® in detected 92.3% (12/13). In the group of dogs in stage III of disease, all methods were equally efficient in the detection of seropositive dogs (Table 2).

Table 2. Comparison of molecular and serological (rK28 antigen) diagnosis according to the clinical staging of dogs from endemic area for CanL.

Group (N) *	Result serology – N (%)					
	Indirect pELISA rK28		**Indirect ELISA rK28**		**TR_DPP Bio-Manguinhos**	
	+	**-**	**+**	**-**	**+**	**-**
Real-time PCR + (19)	18 (94.7)	1 (5.3)	17 (89.5)	2 (10.5)	15 (78.9)	4 (21.1)
Real-time PCR - (151)	0 (0,0)	151 (100.0)	13 (8.6)	138 (91.4)	15 (9.9)	136 (90.1)
Total (170)	**18**	**152**	**30**	**140**	**30**	**140**
Clinical staging**						
"Clinically healthy" (1)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)
"Sick" - Stage I (4)	4 (100.0)	0 (0.0)	3 (75.0)	1 (25.0)	2 (50.0)	2 (50.0)
"Sick" - Stage II (13)	13 (100.0)	0 (0.0)	13 (100.0)	0 (0.0)	12 (92.3)	1 (7.7)
"Sick" - Stage III (1)	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
Total (19)	**18**	**1**	**17**	**2**	**15**	**4**

** Gold test (real-time PCR in peripheral blood and/or conjunctival swab samples; *** Clinical staging of the disease (SOLANO-GALLEGO et al., 2011).

Values of SE, SP, PPV, NPV, PLR, NLR, AC, k and McN were calculated and used to compare the results between the tests pELISA, indirect ELISA rK28 and TR_DPP® for the diagnostic of CanL in the endemic area (Table 3).

Table 3. Comparison of serological diagnosis of VL in dogs from endemic area in relation to real-time PCR (gold standard).

Sorology	Real-time PCR		Total	K	McN	SE	SP	PPV	NPV	PLR	NLR	AC
	+	-			(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
TR_DPP®												
Positive	15	15	30	0.551	p 0.02	79.0	90.1	50.0	97.1	8.0	0.2	88.2
Negative	4	136	140	good								
Total	**19**	**151**	**170**									
Indirect ELISA rK28												
Positive	17	13	30	0.645	p 0.0098	89.5	91.4	56.7	98.6	10.4	0.1	91.2
Negative	2	138	140	good								
Total	**19**	**151**	**170**									
Indirect pELISA rK28												
Positive	18	0	18	0.970	p 1.00	94.7	100.0	100.0	99.3	$1.4x10^{10}$	0.05	99.4
Negativo	1	151	152	excellent								
Total	**19**	**151**	**170**									

SE, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value; PLR, Positive likelihood ratio; NLR, Negative likelihood ratio; K, Kappa index; McN, McNemar; AC, Accuracy (95% confidence interval).

The immunochromatographic test TR_DPP®, indirect ELISA rK28 and pELISA presented sensitivity of 79.0%, 89.5% and 94.7% and specificity of 90.1%, 91.4% and 100%, respectively (Table 3). The pELISA detected the lowest number of false negative samples (5.26%) and not showed false positive samples. Analysis of the data indicated that pELISA was a more sensitive and specificity test than indirect ELISA rK28 and TR_DPP®. In addition, the highest predictive positive (100%), negative (99.3%) and accuracy (99.4%) value were observed in pELISA (Table 3). The best PLR and NLR value were also obtained by pELISA with $1.4X10^{10}$ and 0.05, respectively.

Based on the interpretation of Kappa by Rosner, 2006, Kappa coefficient from the comparison between pELISA with real-time PCR the panels of positive and negative samples showed excellent agreement, differently of indirect ELISA rK28 and TR_DPP® with real-time PCR which showed good agreement. Detection frequencies of positive and negatives samples were not statistically significant ($P > 0.01$, McNemar test) between pELISA with real-time PCR.

3.5 Discussion

In view of the complexity of the diagnosis of CanL in endemic areas, mainly by serological methods whose efficiency is not yet totally satisfactory (LOPES et al., 2017) and that accurate diagnosis of seroreagent dogs is fundamental in the control of human VL (SOLANO-GALLEGO et al., 2011), this report showing the validation of new pELISA for improve the diagnosis of CanL in this areas.

The samples randomly collected from dogs showed 11,2 % positivity in *Leishmania* sp. DNA by real time PCR (blood samples or conjunctival swab), in an endemic area, previous study using the same method showed 3-32% positivity in an endemic area for canL (LOPES et al., 2017). In this study *Leishmania* sp. positive dogs showed clinical signal and biochemical and hematological alteration compatible with the disease (SOLANO-GALLEGO et al., 2004), the characterization of samples qualify the samples to assay a new diagnostic indirect plasmonic ELISA method.

The pELISA tests improves the sensitivity of the detection of disease biomarkers (Nie et al., 2014), this is crucial for CanL diagnosis, given that the strategies that propose control of CanL as a measure to reduce human VL incidence depend on appropriate identification of infected dogs in situations where the elimination of seropositive dogs already tends to be imperfect (COSTA et al., 2013). Few studies reported the diagnosis for CanL considering the clinical staging of dogs from an endemic area (PEIXOTO et al, 2015). In this report, the clinical staging was performed and the test pELISA correctly showed positive in all dogs in stage I, showing highest sensibility than ELISA indirect rK28 e o TR_DPP®.

The new pELISA showed excellent agreement with real-time PCR. In addition, it decreases false negatives (5.26%) and enables the detection of IgG anti-k28 molecules in canine serum with the naked eye, useful in resource-constrained countries, because it does not require sophisticated instruments to read the results, increasing the practicality of CanL detection in these areas. The pELISA has been used to investigate different diseases diagnoses using capture ELISA protocol, including cancer and HIV (DE LA RICA; STEVENS, 2012), fungal (SOJINRIN et al., 2017) and bacterial infections (CHEN et al., 2015; MOHD BAKHORI et al., 2018) with several advantage in sensitivity, but with indirect assay ELISA protocol only one report for diagnosis was described (NIE et al., 2014) and therefore, the success observed in

indirect plasmonic ELISA to diagnosis in CanL, suggest future investigation in this method to diagnose other diseases.

The pELISA showed high number SE, SP, PPV, NPV, PLR, NLR, and AC, than it was observed in official VL control programs from Brazil, (TR_DPP®) (BRASIL, 2014), that may fail in detect all seropositive dogs (LOPES et al., 2017), and it taken in account the improvement in diagnosis observed in our study, we suggest that this method as a additional tool to diagnostic of the LCan.

3.6 Conclusion

In conclusion, we have validated a novel indirect plasmonic ELISA for the diagnosis of CanL in an endemic area. The pELISA not only improved sensitivity and present high specific compared to official method recommended by the Ministry of Health in Brazil, as increasing the practicality of CanL detection in resource-constrained countries, because it does not require sophisticated instruments to read, suggesting that this method could be used as an additional tool for the diagnosis of CanL in these areas.

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3.8 Conflict of interest statement

The authors declare that they have no conflicts of interest.

REFERENCES

BASHIRUDDIN, J. B.; CAMMÀ, C.; REBÊLO, E. Molecular detection of Babesia equi and Babesia caballi in horse blood by PCR amplification of part of the 16S rRNA gene. **Veterinary Parasitology**, v. 84, n. 1–2, p. 75–83, 1999.

BRASIL. **Manual de vigilância e controle da leishmaniose visceral / Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica.** 1. ed. [s.l: s.n.]

CHEN, R. et al. Plasmonic Enzyme-Linked Immunosorbent Assay Using Nanospherical Brushes as a Catalase Container for Colorimetric Detection of Ultralow Concentrations of Listeria monocytogenes. **ACS Applied Materials and Interfaces**, v. 7, n. 51, p. 28632–28639, 2015.

COSTA, D. N. C. C. et al. Culling Dogs in Scenarios of Imperfect Control: Realistic Impact on the Prevalence of Canine Visceral Leishmaniasis. **PLoS Neglected Tropical Diseases**, v. 7, n. 8, 2013.

DA COSTA-VAL, A. P. et al. Canine visceral leishmaniasis: Relationships between clinical status, humoral immune response, haematology and Lutzomyia (Lutzomyia) longipalpis infectivity. **Veterinary Journal**, v. 174, n. 3, p. 636–643, 2007.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. **Nature Nanotechnology**, v. 7, n. 12, p. 821–824, 2012.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the detection of analytes at ultralow concentrations with the naked eye. **Nature Protocols**, v. 8, n. 9, p. 1759–1764, 22 set. 2013. Disponível em: <http://dx.doi.org/10.1038/nprot.2013.085>.

EL TAI, N. O. et al. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. **Transactions of the Royal**

Society of Tropical Medicine and Hygiene, v. 94, n. 5, p. 575–579, set. 2000. Disponível em: <https://doi.org/10.1016/S0035-9203(00)90093-2>.

FERREIRA, S. de A. et al. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. **Veterinary Parasitology**, v. 152, n. 3–4, p. 257–263, 2008.

LOPES, E. G. et al. Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: One out of five seronegative dogs are infected. **Epidemiology and Infection**, v. 145, n. 12, p. 2436–2444, 2017.

LWANGA, S. K.; LEMESHOW, S. **Sample size determination in health studies: a practical manual.** [s.l: s.n.]

METTLER, M. et al. **EducationVPVol.II**l_. v. 43, n. 11, p. 5515–5519, 2005.

MICHALSKY, É. M. et al. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to Lutzomyia longipalpis phlebotomine sand flies. **Veterinary Parasitology**, v. 147, n. 1–2, p. 67–76, jun. 2007. Disponível em: <https://doi.org/10.1016/j.vetpar.2007.03.004>.

MOHD BAKHORI, N. et al. Immuno Nanosensor for the Ultrasensitive Naked Eye Detection of Tuberculosis. **Sensors (Basel, Switzerland)**, v. 18, n. 6, p. 1–10, 2018.

NIE, X. M. et al. Plasmonic ELISA for the ultrasensitive detection of Treponema pallidum. **Biosensors and Bioelectronics**, v. 58, p. 314–319, 2014. Disponível em: <http://dx.doi.org/10.1016/j.bios.2014.03.007>.

OGISO, M. et al. Carbohydrate immobilized on a dendrimer-coated colloidal gold surface for fabrication of a lectin-sensing device based on localized surface plasmon resonance spectroscopy. **Biosensors and Bioelectronics**, v. 41, n. 1, p. 465–470, 2013. Disponível em: <http://dx.doi.org/10.1016/j.bios.2012.09.003>.

PATTABHI, S. et al. Design, development and evaluation of rK28-based point-of-care

tests for improving rapid diagnosis of visceral leishmaniasis. **PLoS Neglected Tropical Diseases**, v. 4, n. 9, 2010.

PEIXOTO, H. M.; DE OLIVEIRA, M. R. F.; ROMERO, G. A. S. Serological diagnosis of canine visceral leishmaniasis in Brazil: Systematic review and meta-analysis. **Tropical Medicine and International Health**, v. 20, n. 3, p. 334–352, 2015.

PETERS, I. R. et al. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. **Veterinary Immunology and Immunopathology**, v. 117, n. 1–2, p. 55–66, 2007.

READY. Epidemiology of visceral leishmaniasis. **Clinical Epidemiology**, v. 6, n. 1, p. 147–154, 2014.

ROSNER, B. **Hypothesis testing: categorical data. In: Rosner B (ed). Fundamentals of Biostatistics.** [s.l: s.n.]

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS., T. **Molecular cloning: a laboratory manual.** 2. ed. [s.l: s.n.]

SATIJA, J. et al. Plasmonic-ELISA: Expanding horizons. **RSC Advances**, v. 6, n. 88, p. 85440–85456, 2016. Disponível em: <http://dx.doi.org/10.1039/C6RA16750K>.

SOARES, M. R. A. et al. Canine visceral leishmaniasis in Teresina, Brazil: Relationship between clinical features and infectivity for sand flies. **Acta Tropica**, v. 117, n. 1, p. 6–9, jan. 2011. Disponível em: <https://doi.org/10.1016/j.actatropica.2010.08.015>.

SOJINRIN, T. et al. Plasmonic gold nanoparticles for detection of fungi and human cutaneous fungal infections. **Analytical and Bioanalytical Chemistry**, v. 409, n. 19, p. 4647–4658, 2 jul. 2017. Disponível em: <http://link.springer.com/10.1007/s00216-017-0414-7>.

SOLANO-GALLEGO, L. et al. Prevalence of Leishmania infantum Infection in Dogs

Living in an Area of Canine Leishmaniasis Endemicity Using PCR on Several Tissues and Serology. **Journal of Clinical Microbiology**, v. 39, n. 2, p. 560–563, 1 fev. 2001. Disponível em: <http://jcm.asm.org/cgi/doi/10.1128/JCM.39.2.560-563.2001>.

SOLANO-GALLEGO, L. et al. Histological and immunohistochemical study of clinically normal skin of Leishmania infantum-infected dogs. **Journal of Comparative Pathology**, v. 130, n. 1, p. 7–12, 2004.

SOLANO-GALLEGO, L. et al. LeishVet guidelines for the practical management of canine leishmaniosis. **Parasites & Vectors**, v. 4, n. 1, p. 86, 2011. Disponível em: <http://www.parasitesandvectors.com/content/4/1/86>.

VENTURIN, G. L. et al. Recombinant K28 antigen in ELISA in the diagnosis of canine visceral leishmaniosis. **Parasite Immunology**, v. 37, n. 12, p. 670–673, 2015.

WHO. Epidemiological Report of the Americas. **World Health Organization technical report series**, v. 7, 2019. Disponível em: <http://iris.paho.org/xmlui/bitstream/handle/123456789/50505/Leishreport2019_eng.pdf?ua=1>.

3.9 Supplementary information

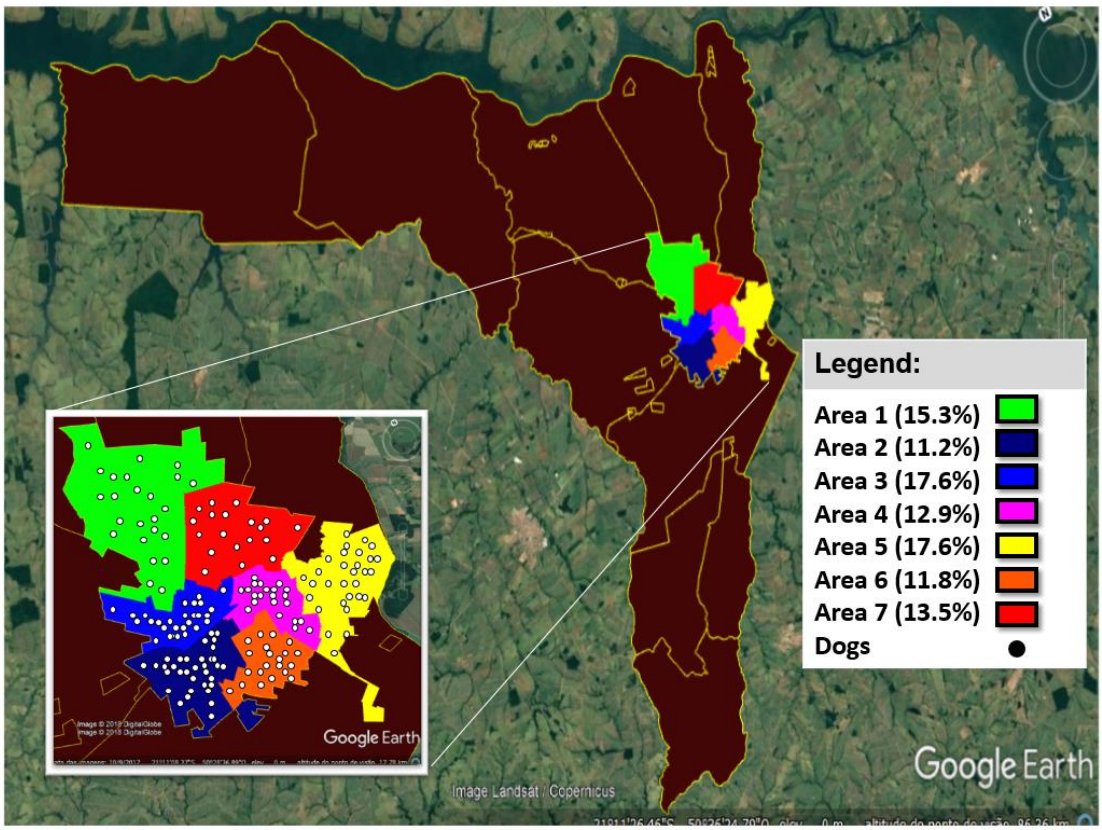

Figure S1. Spatial distribution of dogs of endemic area according to the different different sectors defined by the Epidemiological Surveillance Service of Araçatuba, SP, Brazil, a municipality with high endemicity for CanL.

Table S1. Demographic data and clinical signs of dogs for Araçatuba, SP, Brazil endemic area for CanL and dogs from São Vicente, SP, Brazil (group control), nonendemic area for this disease.

Variable	Endemic area		Nonendemic area	
	n	%	n	%
Sex				
Male	100	58.8	10	38.5
Female	70	41.2	16	61.5
Total	**170**	**100.0**	**26**	**100.0**
Age years				
1-2 years	55	32.4	18	69.2
3-5 years	60	35.3	7	26.9
6-10 years	43	25.3	1	3.8
over 10 years	12	7.1	0	0.0
Total	**170**	**100.0**	**26**	**100.0**
Breed				
Pure	48	28.2	5	19.2
Mixed	122	71.8	21	80.8
Total	**170**	**100.0**	**26**	**100.0**
Clinical signs				
Alopecia	7	3.0	0	0.0
Anemia	15	6.5	0	0.0
Conjutivite	7	3.0	0	0.0
Dermatitis	27	11.6	0	0.0
Skin lesions	29	12.5	0	0.0
Lymphadenomegaly	52	22.4	0	0.0
Pale Mucosa	11	4.7	0	0.0
Onychogrifose	53	22.8	0	0.0
Weight loss	13	5.6	0	0.0
Seborrhea	18	7.8	0	0.0
Total	**232**	**100.0**	**0**	**0.0**

Table S2. Clinical signs of positive dogs from Araçatuba, SP, Brazil, endemic area for CanL and negative dogs from São Vicente, SP, Brazil, nonendemic area for this disease (real-time PCR - gold standard).

Endemic area												Nonendemic area	
Dogs	**LY**	**ON**	**WL**	**SE**	**DE**	**AL**	**SL**	**CO**	**PM**	**AN**	**n° signals**	**Dogs**	**n° signals**
1	X										1	**1**	0
2											0	**2**	0
3											0	**3**	0
4	X						X			X	3	**4**	0
5											0	**5**	0
6	X										1	**6**	0
7											0	**7**	0
8					X						1	**8**	0
9	X								X		2	**9**	0
10	X	X									2	**10**	0
11		X							X	X	3	**11**	0
12	X	X	X	X	X			X	X	X	8	**12**	0
13				X	X						2	**13**	0
14	X			X	X			X			4	**14**	0
15											0	**15**	0
16	X	X					X			X	4	**16**	0
17	X						X				2	**17**	0
18							X				1	**18**	0
19		X	X			X	X				4	**19**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**20**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**21**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**22**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**23**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**24**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**25**	0
-	**-**	-	-	-	-	-	-	-	-	-	-	**26**	0

Lymphoadenomegaly (LY); onychogryphosis (ON); weight loss (WL); seborrhea(SE); dermatitis (DE); alopecia (AL); skin lesions (SL); conjunctivitis (CO); pale mucous (PM); anemia (AN).

Table S3. Biochemical profile of positive dogs by real-time PCR (gold standard) from Araçatuba, SP, Brazil, endemic area for CanL.

Dogs	Biochemical profile								
	Albumin	**Globulin**	**Uric acid**	**ALT**	**AST**	**C**	**TP**	**Urea**	**GGT**
	26-33 (g/L)	27-44 (g/L)	0-2 (mmol/L)	21-102 (U/L)	23-66 (U/L)	0.5-1.5 (µmol/ L)	54-71 (g/L)	1.67-8.33 (mmol/L)	1.2-6.4 (U/L)
1	28.93	32.52	0.62	39.65	36.66	1.08	61.45	3.75	1.16
2	25.10	51.08	3.93	37.24	29.77	0.78	76.18	8.06	2.60
3	37.84	37.41	4.80	44.75	27.58	1.13	70.25	5.64	1.59
4	11.50	73.67	0.53	86.15	132.96	0.83	85.17	4.03	0.00
5	20.24	47.79	0.54	26.31	39.45	1.09	68.03	11.59	2.06
6	26.93	41.07	0.43	54.35	34.38	1.09	71.00	3.58	2.76
7	20.23	55.25	1.04	24.32	60.25	0.97	75.48	3.14	1.25
8	23.1	51.07	0.93	31.2	49.74	2.11	74.17	4.83	5.04
9	26.18	76.86	0.47	24.53	37.51	1.16	93.04	3.81	1.24
10	28.60	46.01	2.65	52.32	39.33	0.73	74.61	5.98	1.66
11	27.29	33.34	1.92	43.15	60.07	1.00	40.63	5.67	4.94
12	26.71	68.18	0.73	22.72	37.5	4.21	84.89	3.32	2.87
13	27.50	63.91	1.74	39.86	74.91	0.68	81.41	4.5	1.16
14	26.82	60.46	0.77	22.21	42.65	1.16	76.28	5.35	1.9
15	27.71	54.37	0.46	47.32	32.25	1.12	79.08	3.89	1.3
16	19.76	49.51	0.53	113.97	95.54	0.70	69.27	5.03	0
17	26.57	63.18	0.58	42.32	39.55	1.23	79.75	5.23	1.56
18	26.84	36.54	0.96	30.79	37.84	1.2	63.38	2.98	1.95
19	32.96	32.55	1.19	27.87	24.46	1.01	65.51	5.16	2.58

Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); Creatinine (C); Gamma glutamyltransferase (GGT); Total protein (TP).
sferase (GGT); Total protein (TP).

Table S4. Complete blood count of positive dogs by real-time PCR (gold standard) from Araçatuba, SP, Brazil, endemic area for CanL.

Dogs	Complete blood count										
	HE	**HB**	**MVC**	**PCV**	**MCHC**	**PL**	**WBC**	**NEU**	**LYM**	**MON**	**EOS**
	5.5-8.5 ($x10^6$/µL)	10-18 (g/dL)	60-77 (fL)	30-36 (g/dL	32-36 (%)	160-430 (x103/µL)	6-17 ($x10^9$/L)	3.0-11.5 ($x10^9$/L)	1.0-4,8 ($x10^9$/L)	0.15-1.35 ($x10^9$/L)	0.15-1.25 ($x10^9$/L)
1	6.09	14.9	72.2	44.0	33.8	209	9.9	5.742	2.48	0.50	1.19
2	4.97	10.4	63.4	31.5	33.0	9	6.8	3.468	2.24	0.75	0.34
3	7.51	18.7	71.5	53.7	34.8	374	12	6.840	2.88	0.84	1.44
4	3.04	5.8	69.9	21.2	27.4	56	6	5.100	0.72	0.18	0.12
5	6.8	13.6	63.4	43.1	31.5	467	13.3	6.650	3.33	1.33	2.00
6	6.62	15.7	71.5	47.3	33.1	170	5.6	3.752	1.18	0.45	0.22
7	7.45	11.9	53.2	39.6	30.0	104	5.8	3.364	1.39	0.58	0.46
8	7.03	16.1	75	52.7	30.5	251	11	6.380	3.85	0.55	0.22
9	3.94	8.1	68.2	26.9	30.2	56	3.8	1.938	1.824	0.00	0.038
10	5.62	11.9	67.4	37.9	31.4	144	8.8	4.048	3.872	0.35	0.528
11	3.06	6.6	66.9	20.5	32.3	59	4.2	2.688	1.344	0.08	0.084
12	3.95	7.7	64.1	25.3	30.4	136	4.5	3.285	0.99	0.05	0.18
13	5.66	11.4	65.3	37.0	30.8	107	6.8	4.080	2.312	0.14	0.272
14	6	11.3	63.2	37.9	29.8	209	8.6	4.558	2.322	0.09	1.634
15	7.63	17.1	69.1	52.7	32.4	155	9.1	5.460	3.003	0.18	0.455
16	4.12	9.2	71.4	29.4	31.2	82	7.3	5.840	1.095	0.22	0.146
17	5.44	10.8	65.9	35.8	30.1	44	8.1	5.832	1.215	0.89	0.162
18	6.44	14.3	70.1	45.1	31.7	230	14.4	9.216	4.32	0.43	0.432
19	2.31	5	71.2	16.4	30.4	218	8.1	2.025	5.346	0.65	0.081

Eosinophils (EOS); Hemoglobin (HB); Lymphocytes (LYM); Neutrophils (NEU); Mean Corpuscular Volume (MVC); Mean Corpuscular Hemoglobin Concentration (MCHC); Monocytes (MON); Platelets (PL); Package Cell Volume Hematocrit (PVC); Red Blood Cells (RBC); White Blood Cells (WBC).

Table S5. Biochemical profile of negative dogs by real-time PCR (gold standard) from São Vicente, SP, Brazil (group control), nonendemic area for CanL.

Dogs	Biochemical profile								
	Albumin	**Globulin**	**Uric acid**	**ALT**	**AST**	**C**	**TP**	**Urea**	**GGT**
	26-33 (g/L)	27-44 (g/L)	0-2 (mmol/L)	21-102 (U/L)	23-66 (U/L)	0.5-1.5 (µmol/L)	54-71(g/L)	1.67-8.33 (mmol/L)	1.2-6.4 (U/L)
1	33.05	36.32	0.69	25.81	46.06	0.97	69.37	7.36	5.33
2	30.70	28.61	0.64	27.44	43.03	1.50	59.31	6.21	1.21
3	31.27	22.04	0.45	64.62	46.12	0.87	53.31	7.86	2.78
4	27.83	36.64	0.53	35.91	41.42	1.21	64.47	5.7	3.54
5	33.17	28.53	0.84	44.66	38.06	1.10	61.70	3.11	1.45
6	25.94	44.40	2.00	30.98	31.26	0.81	70.34	7.72	0.99
7	29.61	28.64	0.70	44.61	51.29	0.94	58.25	7.79	0.61
8	23.67	33.46	0.41	36.92	45.66	0.88	57.13	5.98	2.27
9	16.07	42.63	0.64	24.60	40.54	1.05	58.42	4.82	0.48
10	26.30	44.02	0.70	39.55	48.24	1.13	68.32	7.34	0.00
11	27.78	42.49	0.61	46.32	49.84	0.97	70.27	4.36	0.00
12	28.29	31.22	0.66	33.05	41.95	1.14	59.51	6.38	2.02
13	22.70	35.79	0.45	32.34	40.93	1.09	58.49	6.22	1.41
14	20.94	28.48	0.96	24.78	65.62	0.90	49.42	3.93	0.59
15	27.17	22.76	0.42	52.27	54.40	0.93	49.93	7.68	0.56
16	31.83	21.11	0.33	43.17	39.62	1.21	52.94	5.43	2.84
17	23.03	25.77	0.55	40.43	65.87	0.81	48.8	7.91	4.23
18	27.3	20.68	0.39	37.99	47.25	0.87	47.98	7.21	2.26
19	29.2	28.4	0.38	31.82	30.89	0.96	57.6	6.23	1.3
20	27.34	34.54	0.48	20.15	30.29	0.79	61.88	7.27	3.44
21	28.1	29.78	0.59	35.66	43.77	1.21	57.88	3.93	2.89
22	33.07	33.08	0.54	66.19	30.74	0.85	66.15	6.40	5.95
23	29.21	25.58	0.54	41.20	30.03	1.09	54.79	6.55	2.86
24	27.92	42.98	0.76	42.20	57.53	1.46	70.90	7.22	3.15
25	31.06	31.07	0.93	23.00	39.46	1.11	62.13	9.45	0.00
26	29.25	35.52	0.69	46.53	52.31	1.22	64.77	4.12	0

Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); Creatinine (C); Gamma glutamyltransferase (GGT); Total protein (TP).

Table S6. Complete blood count of negative dogs by real-time PCR (gold standard) from São Vicente, SP, Brazil (group control), nonendemic area for CanL.

	Complete blood count										
	HE	**HB**	**MVC**	**PCV**	**MCHC**	**PL**	**WBC**	**NEU**	**LYM**	**MON**	**EOS**
Dogs	5.5 – 8.5 (x106/µL)	12-18 (g%)	60-77 (U^3)	30-36 (g/dL)	200-500 (x10^3/µL)	5.5-8.0 (g/L)	6000-17000 (uL)	3600-13090 (uL)	720-5100 (uL)	180-1700 (uL)	120-1700 (uL)
1	8.6	18.9	73.3	30.0	301	8.0	11000.0	7810.0	1870.0	880.0	440.0
2	6.97	15.9	76.0	30.0	203	6.6	13300.0	7315.0	3591.0	1596.0	798.0
3	7.84	17.8	75.3	30.2	310	5.6	9000.0	6570.0	1800.0	450.0	180.0
4	8.16	19.1	77.2	30.3	204	6.8	14300.0	8580.0	3003.0	1287.0	1430.0
5	7.28	17.0	78.3	29.8	237	6.4	17900.0	12530.0	2327.0	1969.0	1074.0
6	6.64	14.3	76.8	28.0	200	7.4	8800.0	6424.0	1408.0	528.0	440.0
7	7.08	14.8	73.4	28.5	259	6.6	12300.0	8733.0	2706.0	615.0	246.0
8	5.24	11.6	78.2	28.3	599	6.0	18100.0	11222.0	3439.0	1810.0	1629.0
9	6.89	11.0	58.1	27.5	511	7.2	14700.0	7938.0	3087.0	1911.0	1764.0
10	6.21	14.0	77.3	29.2	296	7.6	16300.0	9128.0	2608.0	1956.0	2445.0
11	6.53	13.0	70.4	28.3	305	7.8	17400.0	12702.0	2436.0	2088.0	174.0
12	6.92	15.5	75.1	29.8	302	7.2	3400.0	2516.0	238.0	544.0	102.0
13	6.61	14.2	74.1	29.0	408	6.6	15200.0	9272.0	2888.0	1520.0	1520.0
14	5.80	12.3	74.1	28.6	296	6.0	15100.0	9815.0	1812.0	2718.0	755.0
15	7.30	16.0	75.3	29.1	244	5.8	7400.0	3848.0	2294.0	814.0	444.0
16	6.94	15.8	76.4	29.8	203	5.8	13500.0	9315.0	2700.0	675.0	810.0
17	6.89	15.0	75.5	28.8	562	7.0	15300.0	8874.0	3672.0	918.0	1836.0
18	7.23	14.8	70.5	29.0	445	5.2	16500.0	11715.0	2640.0	1320.0	660.0
19	7.38	13.7	74.5	24.9	620	7.0	20100.0	14070.0	2613.0	2211.0	1206.0
20	7.22	14.0	67.9	28.6	540	7.2	12300.0	8487.0	1722.0	984.0	1107.0
21	6.51	14.6	76.8	29.2	637	7.0	14000.0	11200.0	1820.0	560.0	420.0
22	6.93	15.1	75.0	29.0	305	7.2	10500.0	6930.0	2625.0	840.0	105.0
23	6.63	14.7	76.9	28.8	279	6.2	10400.0	5304.0	3744.0	832.0	520.0
24	7.78	17.3	78.4	28.4	260	7.2	19300.0	12352.0	3281.0	1158.0	2509.0
25	6.41	14.7	81.1	28.3	201	6.8	9800.0	5586.0	2646.0	882.0	686.0
26	8.07	17.3	73.1	29.3	311	7.4	14700.0	9702.0	3822.0	588.0	588.0

Eosinophils (EOS); Hemoglobin (HB); Lymphocytes (LYM); Neutrophils (NEU); Mean Corpuscular Volume (MVC); Mean Corpuscular Hemoglobin Concentration (MCHC); Monocytes (MON); Platelets (PL); Package Cell Volume Hematocrit (PVC); Red Blood Cells (RBC); White Blood Cells (WBC).

Table S7. Clinical signs of negative dogs by real-time PCR (gold standard) from Araçatuba, SP, Brazil, endemic area for CanL.

Dogs	Endemic area										n° signals
	LY	ON	WL	SE	DE	AL	SL	CO	PM	AN	
1										X	1
2	X										1
3							X				1
4											0
5											0
6											0
7				X	X						2
8											0
9											0
10	X									X	2
11		X									1
12											0
13											0
14											0
15	X										1
16											0
17	X										1
18	X	X		X	X						4
19	X	X									2
20	X	X									2
21											0
22		X									1
23											0
24	x						X			X	3
25											0
26	X	X									2
27											0
28											0
29		X									1
30											0
31		X									1
32											0
33											0
34											0
35											0
36											0
37					X						1
38	X									X	2
39	X									X	2
40				X			X				2

41										0
42										0
43	X		X							2
44	X				X					2
45						X				1
46									X	1
47										0
48										0
49										0
50	X									1
51							X			1
52		X								1
53				X		X				2
54	X	X								2
55										0
56										0
57										0
58							X			1
59										0
60										0
61				X	X					2
62				X	X					2
63										0
64		X								1
65	X	X								2
66	X									1
67	X									1
68	X						X		X	3
69	X	X			X		X			4
70		X								1
71	X	X			X					3
72	X								X	2
73										0
74					X					1
75				X						1
76		X								1
77										0
78		X								1
79										0
80			X	X	X			X		X	5
81			X				X	X	X	X	5
82		X									1
83										0
84										0
85										0
86										0

87										0
88		X			X			X		3
89										0
90										0
91		X								1
92										0
93		X								1
94		X								1
95		X								1
96		X								1
97			X							1
98		X				X	X			3
99		X								1
100										0
101										0
102										0
103										0
104	X	X								2
105										0
106	X						X			2
107										0
108		X								1
109		X								1
110		X								1
111	X	X	X	X	X	X	X			7
112	X	X		X	X	X	X	X		7
113										0
114									X	1
115	X	X	X				X	X	X	7
116	X						X		X	3
117	X									1
118							X			1
119	X				X		X			3
120										0
121	X	X					X			3
122	X	X					X			3
123		X			X					2
124										0
125	X			X	X					3
126	X		X							2
127										0
128	X		X						X	3
129	X	X			X					3
130										0
131										0
132		X	X				X		X	5

133		X		X	X			3
134		X						1
135		X			X			2
136	X		X			X		3
137	X					X		2
138							X	1
139		X			X			2
140				X	X	X		3
141								0
142								0
143								0
144	X							1
145	X				X			2
146		X						1
147	X	X						2
148		X			X	X		3
149	X							1
150								0
151								0

Lymphoadenomegaly (LY); onychogryphosis (ON); weight loss (WL); seborrhea(SE); dermatitis (DE); alopecia (AL); skin lesions (SL); conjunctivitis (CO); pale mucous (PM); anemia (AN).

Table S8. Biochemical profile of negative dogs by real-time PCR (gold standard) from Araçatuba, SP, Brazil, endemic area for CanL.

Dogs	Biochemical profile								
	Albumin	**Globulin**	**Uric acid**	**ALT**	**AST**	**C**	**TP**	**Urea**	**GGT**
	26-33 (g/L)	27-44 (g/L)	0-2 (mmol/L)	21-102 (U/L)	23-66 (U/L)	0.5-1.5 (µmol/L)	54-71 (g/L)	1.67-8.33 (mmol/L)	1.2-6.4 (U/L)
1	17,51	44,70	0,53	37,31	31,04	1,19	62,21	4,38	0,20
2	28,88	40,60	1,68	49,56	52,60	0,95	69,48	5,01	0,00
3	23,74	47,92	0,32	31,69	99,74	1,06	71,66	3,90	1,30
4	36,29	40,84	0,62	37,35	49,51	1,20	77,13	7,38	1,40
5	43,14	44,00	8,85	52,89	28,45	1,00	87,14	4,56	2,01
6	34,00	33,90	1,26	19,70	37,09	1,04	67,90	4,13	0,66
7	25,59	49,71	0,56	41,31	36,25	1,29	75,30	8,41	1,00
8	26,48	44,51	0,72	56,58	34,43	1,33	70,99	4,24	1,01
9	32,46	36,84	0,55	60,31	39,12	1,03	69,30	5,04	1,11
10	13,66	72,44	0,25	21,14	37,19	1,17	86,10	5,81	0,59
11	37,38	35,68	1,65	19,86	31,53	1,04	73,06	5,30	0,14
12	18,18	50,82	1,32	30,04	70,23	1,03	69,00	6,41	1,75
13	25,66	42,41	0,78	44,75	43,10	1,07	68,07	5,19	0,43
14	23,45	48,56	0,89	58,65	47,16	1,27	72,01	6,79	1,21
15	21,49	62,58	0,32	21,95	29,13	1,11	84,07	5,06	0,10
16	26,58	37,32	0,35	28,25	27,50	0,99	63,90	4,70	0,42
17	29,19	35,21	0,34	23,85	39,96	0,86	64,40	5,55	4,28
18	24,51	52,72	0,62	53,41	52,48	1,10	77,23	5,40	0,00
19	30,88	25,95	0,39	54,49	25,17	0,94	56,83	3,37	3,94
20	33,12	34,95	1,22	77,53	62,21	0,88	68,07	2,59	1,65
21	29,57	43,13	1,73	74,96	48,19	1,28	72,70	7,73	1,41
22	22,34	59,06	0,98	16,04	53,58	1,20	81,40	7,28	0,00
23	30,67	42,69	0,25	24,90	23,96	1,09	73,36	4,03	6,58
24	24,83	52,74	0,64	48,25	43,39	0,99	77,57	4,57	1,87
25	24,47	55,37	1,27	47,96	57,47	0,91	79,84	9,12	2,74
26	30,16	38,04	2,08	49,78	37,56	0,82	68,20	5,22	1,55
27	20,50	37,04	0,42	39,87	44,02	1,35	57,54	3,76	3,17
28	21,52	42,42	0,33	58,19	39,81	1,38	63,94	2,90	2,13
29	29,99	68,15	0,83	52,18	49,61	1,05	98,14	4,01	3,38
30	12,37	49,16	1,48	15,99	44,63	0,86	61,53	7,95	0,00
31	21,97	23,22	0,97	93,26	48,89	0,92	45,19	7,53	0,78
32	31,41	39,60	1,59	29,84	43,85	1,08	68,31	6,02	5,26
33	19,29	45,17	0,92	26,88	35,70	1,04	64,46	3,11	3,30
34	31,33	29,60	1,81	63,55	83,85	1,04	50,93	6,60	1,30
35	34,43	28,95	1,55	43,94	36,81	0,87	63,38	7,19	5,79
36	32,72	28,54	0,74	33,26	38,07	1,23	61,26	5,62	9,37
37	32,86	33,20	1,46	34,36	37,39	0,98	66,06	7,14	3,81
38	15,22	38,50	1,19	50,80	89,87	0,70	53,72	5,32	2,15
39	19,21	52,32	0,63	32,14	32,99	0,87	71,53	5,70	1,46

40	22,43	29,33	0,71	43,19	64,36	0,87	51,76	5,01	1,45
41	22,91	56,47	0,67	23,92	33,31	1,12	79,38	6,04	1,24
42	27,38	63,46	2,86	46,36	48,54	0,97	91,93	4,28	0,72
43	29,35	39,67	1,20	36,87	49,46	0,92	69,02	4,06	5,48
44	25,56	35,39	0,52	62,01	35,00	1,18	60,95	5,69	6,06
45	28,72	33,73	0,51	29,04	31,94	0,96	62,45	2,87	3,60
46	23,82	82,42	1,10	28,05	34,13	1,24	96,24	7,49	1,00
47	26,97	68,82	1,18	55,23	38,00	1,32	85,79	8,68	1,06
48	26,54	45,96	0,42	69,23	36,36	0,98	71,50	4,44	6,08
49	31,74	30,91	0,86	31,32	50,57	1,12	62,55	3,79	5,96
50	29,21	35,63	1,86	32,78	74,66	1,39	64,84	6,34	3,06
51	28,96	50,65	2,77	44,02	38,68	0,94	71,61	3,82	1,37
52	30,92	30,69	0,80	45,56	39,44	1,31	61,61	4,52	5,15
53	28,67	45,79	0,71	41,81	45,15	1,36	74,46	6,23	4,42
54	33,16	33,10	0,51	31,66	35,55	1,10	66,26	4,19	4,70
55	30,99	32,68	1,51	20,12	29,47	1,00	63,67	4,56	2,67
56	27,03	46,40	1,05	28,98	38,35	0,84	71,63	5,80	2,29
57	30,26	29,48	1,51	82,63	64,74	0,94	59,74	7,65	5,15
58	31,35	30,91	0,82	41,84	49,54	0,68	62,26	3,65	3,68
59	34,35	39,14	1,76	28,82	44,65	1,16	71,49	5,64	3,66
60	31,45	27,97	1,32	22,84	33,90	1,07	56,42	3,83	5,08
61	28,10	28,07	1,03	36,10	27,41	0,95	56,17	3,51	2,86
62	28,93	26,55	1,40	30,32	27,94	1,07	55,48	5,21	4,77
63	26,90	46,03	1,07	29,84	37,17	0,91	72,93	3,57	1,02
64	34,14	38,08	1,97	41,54	60,92	0,67	72,22	8,00	2,97
65	26,05	39,04	1,99	26,32	27,31	0,79	65,09	1,73	3,42
66	32,68	31,36	1,26	74,40	51,09	1,05	64,04	5,45	2,42
67	26,94	27,56	0,65	29,59	33,41	0,84	54,50	1,95	2,43
68	23,92	36,99	0,53	27,91	33,57	1,16	60,91	2,06	1,02
69	26,41	39,06	0,64	94,15	46,93	0,95	64,47	8,08	2,65
70	31,60	26,70	2,02	20,35	35,43	0,96	58,30	4,37	1,32
71	30,72	37,32	1,39	72,58	35,05	1,02	68,04	4,24	3,44
72	37,08	39,89	1,11	37,83	46,18	1,25	76,97	7,00	1,30
73	26,27	54,93	0,54	22,75	26,50	1,27	80,20	1,79	1,75
74	18,34	81,21	1,21	43,98	51,19	1,10	99,55	3,66	6,27
75	26,68	35,61	0,51	78,42	46,51	1,16	62,29	3,94	4,25
76	18,83	36,05	0,79	49,38	44,32	0,87	52,88	3,59	6,76
77	29,64	34,71	1,70	38,49	49,35	1,21	64,35	8,26	4,88
78	26,12	40,79	1,25	93,60	24,19	0,88	62,91	5,11	5,82
79	32,39	32,45	0,61	97,16	39,09	1,31	64,84	5,84	3,22
80	27,33	37,91	0,38	21,06	24,20	0,82	53,24	6,03	2,23
81	26,54	60,70	0,79	44,31	68,44	1,04	77,24	5,53	2,20
82	26,80	67,93	0,57	21,41	29,96	1,13	90,73	3,33	1,08
83	26,64	72,12	0,97	22,94	29,66	1,09	92,76	2,98	2,50
84	28,50	30,42	0,60	34,62	30,58	1,12	58,92	3,22	3,55
85	80,95	92,33	49,48	142,2	43,25	1,11	173,2	6,68	0,00

86	34,53	29,29	2,11	23,59	29,10	1,30	63,82	7,45	1,59
87	28,01	35,55	0,35	53,03	25,05	0,95	63,56	6,09	2,14
88	27,76	77,22	0,77	31,72	34,46	0,93	88,98	3,11	2,39
89	26,98	27,55	0,99	32,77	37,30	0,75	53,53	5,62	1,69
90	30,57	27,31	0,57	6,64	34,06	1,07	57,88	6,31	3,11
91	31,94	34,55	0,59	46,61	57,79	1,01	66,49	5,66	0,00
92	36,21	51,74	1,20	29,56	40,60	0,93	87,95	4,29	1,20
93	29,30	43,21	0,44	41,23	28,24	1,53	72,51	5,11	1,81
94	31,45	30,75	1,63	62,50	39,08	1,09	62,20	6,91	1,93
95	32,25	32,58	0,42	66,44	35,78	1,05	64,83	5,10	6,32
96	26,73	56,88	0,32	67,51	25,03	1,03	58,61	5,61	3,74
97	26,58	31,17	0,32	69,03	23,83	1,13	57,75	5,17	3,24
98	30,62	51,19	1,32	31,36	38,28	1,26	81,81	5,61	1,14
99	31,08	27,85	0,63	37,52	33,29	1,11	56,93	5,96	3,57
100	32,02	28,17	0,57	62,17	39,07	1,23	60,19	4,35	2,04
101	26,32	36,82	0,47	31,24	36,86	1,33	62,14	2,39	1,53
102	27,31	28,31	0,68	27,00	33,97	1,65	55,89	9,59	5,03
103	27,73	31,53	1,18	32,46	31,58	1,31	59,26	6,12	2,93
104	29,53	31,77	0,45	35,21	26,26	1,31	61,30	5,50	3,01
105	33,32	35,16	0,87	66,08	34,30	0,93	68,48	4,01	2,26
106	29,61	34,24	0,59	37,53	34,19	1,20	63,85	6,16	3,54
107	27,96	61,74	0,61	59,85	36,54	1,04	83,70	4,49	1,18
108	35,77	34,65	1,02	24,32	42,02	1,21	70,42	5,32	1,10
109	33,26	26,45	0,77	33,61	46,82	1,20	59,71	5,34	4,78
110	30,10	32,49	1,53	44,71	38,33	1,25	62,59	6,98	2,21
111	22,99	32,80	0,62	48,95	32,60	1,03	55,79	5,99	2,36
112	27,28	65,16	0,88	25,95	43,79	0,79	77,44	5,24	1,18
113	25,55	31,50	1,02	34,20	59,89	0,96	57,05	8,68	1,36
114	18,90	63,03	0,40	22,23	50,88	1,49	81,93	3,31	0,00
115	13,37	73,02	0,41	22,74	32,27	1,07	86,39	8,02	1,19
116	21,51	42,50	0,33	31,73	27,09	2,48	64,01	8,96	1,80
117	25,79	49,78	0,26	21,01	24,54	1,14	75,57	6,63	3,47
118	26,96	53,57	1,56	20,54	30,01	0,91	75,53	5,89	1,00
119	20,73	62,35	1,93	27,67	26,28	0,99	83,08	7,05	1,99
120	32,36	33,91	0,99	45,15	34,82	1,11	66,27	5,91	2,89
121	15,06	64,60	2,00	40,45	55,73	1,13	79,66	8,21	0,00
122	22,32	43,27	0,52	28,97	23,77	1,04	62,59	2,62	2,16
123	19,21	32,96	0,45	39,18	25,08	0,91	52,17	3,05	2,53
124	28,68	37,20	2,40	54,71	44,91	1,07	65,88	6,44	2,91
125	37,70	33,40	1,68	44,11	62,84	1,13	71,10	3,83	6,49
126	24,01	45,30	1,26	32,96	40,21	0,92	69,31	6,60	3,10
127	32,27	34,16	1,29	44,04	30,59	1,15	66,43	8,84	1,72
128	31,67	32,08	0,71	40,84	35,74	0,97	63,75	4,30	2,51
129	22,60	43,47	1,11	25,72	25,78	0,96	66,07	5,60	2,03
130	10,14	81,41	0,89	24,87	72,17	0,80	91,55	5,07	0,00
131	29,67	29,51	1,69	67,30	21,38	1,11	59,18	7,97	1,94

132	13,79	56,45	0,55	21,93	37,18	0,92	70,24	1,87	1,91
133	31,27	33,13	1,80	62,92	53,43	0,85	64,40	4,39	0,00
134	26,13	54,34	0,86	26,11	39,35	1,12	80,47	2,41	1,51
135	37,92	26,65	1,28	33,15	52,56	0,98	64,57	3,52	1,92
136	23,69	63,08	2,77	31,98	23,61	0,82	86,77	6,87	1,21
137	20,78	57,30	0,68	47,24	50,34	0,92	78,08	1,88	1,13
138	33,13	29,85	1,47	30,23	42,06	1,06	62,98	4,77	1,69
139	29,51	40,18	2,07	50,10	37,66	0,87	69,69	6,67	1,83
140	26,76	55,70	0,84	21,65	27,27	1,20	82,46	4,30	0,00
141	30,04	32,52	0,47	36,93	32,77	1,27	62,56	2,60	3,38
142	31,71	29,00	0,87	57,56	33,85	1,26	60,71	4,51	6,56
143	32,66	29,78	0,55	63,98	38,53	1,08	62,44	3,76	3,83
144	32,27	44,98	1,59	25,03	62,46	1,05	77,25	3,52	0,00
145	20,62	39,99	0,64	37,30	41,13	0,81	60,61	1,74	3,67
146	32,88	34,36	1,91	37,68	37,47	1,62	67,24	8,93	5,47
147	26,15	59,93	1,64	33,64	46,37	1,14	86,08	4,39	2,22
148	28,74	50,42	0,40	32,51	28,61	0,94	79,16	6,14	1,28
149	30,09	28,34	1,56	29,88	40,97	0,95	58,43	7,13	1,57
150	30,81	25,73	2,70	30,83	73,33	0,97	56,54	7,80	1,76
151	27,92	26,44	1,94	29,82	41,96	1,14	54,36	7,85	1,52

Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); Creatinine (C); Gamma glutamyltransferase (GGT); Total protein (TP).

Table S9. Complete blood count of negative dogs by real-time PCR (gold standard) from Araçatuba, SP, Brazil, endemic area for CanL.

Dogs	Complete blood count										
	HE	**HB**	**MVC**	**PCV**	**MCHC**	**PL**	**WBC**	**NEU**	**LYM**	**MON**	**EOS**
	5.5-8.5 x10^6/µL	10-18 (g/dL)	60-77 (fL)	30-36 (g/dL	32-36 (%)	160-430 (x103/µL)	6-17 (x10^9/L)	3.0-11.5 (x10^9/L)	1.0-4,8 (x10^9/L)	0.15-1.35 (x10^9/L)	0.15-1.25 (x10^9/L)
1	4,63	9,80	68,70	31,81	30,80	34,00	7,00	4,62	1,47	0,63	0,28
2	5,93	14,90	73,50	43,59	34,20	170,00	11,90	5,71	4,88	0,48	0,83
3	7,03	17,20	73,40	51,60	33,30	358,00	13,60	11,29	1,09	0,54	0,82
4	6,77	19,10	71,10	48,13	39,70	242,00	8,80	5,28	2,38	0,26	0,88
5	7,18	17,20	72,80	52,27	32,90	424,00	12,20	9,76	1,46	0,61	0,37
6	6,45	15,00	70,50	45,47	33,00	101,00	19,60	12,15	2,94	1,37	3,14
7	5,79	14,10	70,30	40,70	34,60	59,00	14,10	9,45	2,40	0,99	1,27
8	7,37	18,90	74,20	54,69	34,60	121,00	10,80	6,16	2,59	1,19	0,86
9	7,40	17,10	74,10	54,83	31,20	309,00	9,50	6,08	2,19	0,48	0,76
10	4,91	8,60	57,30	28,13	30,60	68,00	9,80	5,68	3,43	0,49	0,20
11	6,76	16,90	75,20	50,84	33,20	282,00	11,70	6,90	3,04	0,59	1,17
12	6,28	13,10	69,60	43,71	29,90	66,00	20,20	9,29	7,27	2,02	1,62
13	7,23	16,30	72,30	52,27	31,20	87,00	15,30	10,71	3,06	1,07	0,46
14	6,57	13,20	67,80	44,54	29,60	80,00	6,90	4,35	1,93	0,55	0,69
15	5,80	11,30	71,10	41,24	29,10	113,00	13,30	10,51	0,53	1,06	1,20
16	6,38	14,90	70,30	44,85	33,20	79,00	18,00	11,16	4,14	0,90	1,80
17	7,26	18,90	74,80	54,30	34,80	311,00	7,50	4,95	1,20	0,68	0,68
18	5,59	13,30	71,70	40,08	33,20	67,00	12,90	5,68	4,90	0,65	1,68
19	6,89	16,90	70,00	48,23	35,00	306,00	12,10	7,50	2,30	0,48	1,82
20	8,69	22,30	69,30	60,22	36,90	197,00	16,80	13,27	1,01	2,35	0,17
21	7,33	17,20	68,50	50,21	34,20	153,00	8,00	3,28	3,84	0,32	0,56
22	6,38	14,00	69,00	44,02	31,80	309,00	19,00	12,16	4,94	0,95	0,95
23	7,09	14,80	65,70	46,58	31,80	135,00	14,80	10,36	2,66	0,59	1,18
24	2,96	7,00	71,40	21,13	33,10	23,00	4,20	3,36	0,63	0,08	0,13
25	6,36	14,60	71,60	45,54	32,00	172,00	10,00	7,80	1,70	0,10	0,40
26	6,44	14,80	70,60	45,47	32,50	203,00	11,80	4,96	6,14	0,12	0,59
27	6,72	16,10	73,50	49,39	32,60	37,00	16,70	12,02	2,67	1,00	1,00
28	7,31	18,20	75,20	54,97	33,10	121,00	15,20	9,27	2,89	1,52	1,52
29	6,42	18,60	80,90	51,94	35,80	152,00	6,40	4,74	0,90	0,58	0,19
30	8,10	20,20	72,50	58,73	34,40	249,00	14,60	11,10	1,75	1,02	0,73
31	7,81	19,60	71,10	55,53	35,30	217,00	12,20	8,05	2,93	0,61	0,61
32	7,96	18,00	75,50	60,10	30,00	50,00	7,70	4,85	2,23	0,23	0,39
33	7,00	18,30	79,10	55,37	33,00	340,00	9,10	3,61	4,26	0,33	0,16
34	8,43	20,80	73,70	62,13	33,40	396,00	9,90	6,24	3,56	0,00	0,10
35	5,57	14,40	73,60	41,00	35,20	228,00	16,70	8,02	8,52	0,00	0,17
36	7,99	19,70	71,70	57,29	34,40	206,00	10,20	5,20	4,59	0,31	0,10
37	8,26	19,60	71,20	58,81	33,30	82,00	7,30	4,23	2,77	0,07	0,22
38	1,62	4,10	84,30	13,66	30,10	90,00	11,00	5,72	5,17	0,00	0,11
39	3,89	8,70	74,60	29,02	30,00	29,00	6,90	4,55	2,07	0,07	0,21
40	4,79	11,00	73,60	35,25	31,20	37,00	7,30	3,80	3,29	0,07	0,15
41	5,98	13,30	71,40	42,70	31,20	90,00	16,20	6,97	9,07	0,00	0,16

42	6,21	15,90	75,70	47,01	33,80	59,00	10,50	6,30	3,78	0,11	0,32
43	5,05	12,50	74,20	37,47	33,40	192,00	12,80	8,19	4,48	0,00	0,13
44	5,66	12,80	70,20	39,73	32,20	96,00	18,40	13,43	4,60	0,00	0,37
45	5,57	12,40	67,40	37,54	33,00	248,00	12,40	7,94	3,72	0,12	0,62
46	3,80	9,40	76,80	29,18	32,30	44,00	19,80	10,69	8,91	0,00	0,20
47	3,65	19,60	68,70	25,08	38,40	72,00	10,30	7,31	2,37	0,21	0,41
48	6,13	17,50	73,60	45,12	34,50	226,00	10,40	6,97	3,02	0,31	0,10
49	5,74	18,20	73,80	42,36	31,20	181,00	9,80	6,47	1,76	0,59	0,98
50	7,10	16,20	75,90	53,89	33,00	411,00	17,40	10,79	6,09	0,00	0,52
51	7,35	15,70	70,00	51,45	25,30	33,00	9,20	5,52	3,59	0,00	0,09
52	6,43	15,70	71,50	45,97	34,10	374,00	20,40	10,81	8,57	0,20	0,82
53	6,75	16,10	73,90	49,88	32,50	373,00	16,70	10,35	5,85	0,00	0,50
54	6,18	16,50	76,40	47,22	34,30	355,00	15,00	10,95	3,00	0,45	0,60
55	6,54	12,50	74,30	48,59	32,00	318,00	18,10	11,77	4,89	0,18	1,27
56	6,92	14,10	69,60	48,16	32,10	149,00	9,60	5,09	4,42	0,00	0,10
57	7,43	12,40	74,50	55,35	31,30	428,00	10,50	7,25	2,42	0,42	0,42
58	5,59	16,40	72,80	40,70	32,10	481,00	10,00	7,90	2,00	0,00	0,10
59	7,89	15,40	73,20	57,75	34,70	256,00	11,70	7,72	3,86	0,00	0,12
60	5,38	13,10	73,70	39,65	33,00	69,00	3,90	2,11	1,56	0,12	0,12
61	5,48	12,00	72,50	39,73	32,90	337,00	9,00	6,03	2,16	0,00	0,81
62	6,62	16,70	72,80	48,19	34,70	215,00	14,70	8,23	6,03	0,29	0,15
63	6,85	16,80	72,20	49,46	34,00	226,00	8,70	4,18	4,35	0,09	0,09
64	6,97	17,60	72,10	50,25	35,00	351,00	10,30	5,36	3,91	0,21	0,82
65	6,65	15,20	67,90	45,15	33,70	197,00	13,50	5,94	5,13	1,08	1,35
66	7,49	18,60	74,00	55,43	33,50	281,00	9,20	6,62	1,84	0,37	0,37
67	5,65	12,80	69,10	39,04	32,80	369,00	12,90	7,35	3,87	0,13	1,55
68	5,53	12,20	65,60	36,28	33,70	268,00	17,40	13,40	3,48	0,35	0,17
69	6,55	13,60	67,80	44,41	30,60	169,00	18,00	9,54	5,22	0,90	2,34
70	7,75	17,70	73,00	56,58	31,30	339,00	9,50	6,08	2,47	0,38	0,57
71	7,85	17,20	66,90	52,52	32,70	336,00	11,10	5,66	4,66	0,22	0,56
72	8,27	18,80	69,40	57,39	32,80	348,00	12,80	6,91	4,22	0,13	1,54
73	7,45	16,10	70,40	52,45	30,70	198,00	12,40	3,72	7,69	0,12	0,87
74	5,91	12,80	68,60	40,54	31,60	257,00	15,80	7,90	7,74	0,00	0,16
75	5,94	13,90	71,20	42,29	32,90	140,00	13,80	6,90	6,62	0,00	0,28
76	4,77	11,10	70,60	33,68	33,00	29,00	14,00	6,44	6,86	0,56	0,14
77	6,40	15,90	75,10	48,06	33,10	481,00	16,70	6,68	9,69	0,00	0,33
78	5,47	12,80	73,60	40,26	31,80	470,00	16,70	8,68	6,68	0,33	1,00
79	9,26	21,20	68,20	63,15	33,50	75,00	8,70	4,87	2,52	0,17	1,13
80	4,28	9,20	67,70	28,98	31,80	90,00	7,30	3,21	3,21	0,58	0,29
81	4,54	9,20	62,90	28,56	32,20	39,00	8,90	5,43	3,12	0,18	0,18
82	6,28	14,20	67,60	Z	33,40	213,00	12,50	7,88	3,00	0,63	1,00
83	6,23	13,50	69,20	43,11	31,30	75,00	5,10	2,35	2,24	0,15	0,36
84	6,95	16,50	72,90	50,67	32,60	85,00	8,20	4,84	2,05	0,16	1,15
85	7,17	20,30	72,50	51,98	39,10	414,00	9,00	7,02	1,89	0,00	0,09
86	7,46	17,30	69,30	51,70	23,10	236,00	7,70	5,39	1,93	0,00	0,39
87	7,88	17,10	67,40	53,11	32,20	70,00	5,90	3,66	1,77	0,41	0,06
88	5,53	11,50	63,10	34,89	33,00	79,00	5,60	2,35	2,80	0,11	0,34
89	6,18	14,30	69,50	42,95	33,30	278,00	12,20	5,49	5,37	0,24	1,10
90	7,27	17,60	70,70	51,40	34,30	270,00	9,70	4,75	4,46	0,29	0,19
91	7,78	18,70	73,90	57,49	32,50	229,00	9,50	5,32	3,90	0,19	0,10

92	6,89	16,60	72,20	49,75	33,40	186,00	8,70	5,66	2,35	0,00	0,70
93	5,19	13,00	74,30	38,56	33,70	545,00	20,60	15,45	3,50	0,41	1,24
94	7,67	18,60	68,30	52,39	35,50	180,00	10,70	6,10	4,28	0,21	0,11
95	7,56	18,50	69,70	52,69	35,10	266,00	9,90	5,54	3,47	0,20	0,69
96	6,57	12,00	69,50	45,66	69,50	365,00	10,00	6,20	2,70	0,70	0,40
97	5,01	13,30	76,20	38,18	34,90	215,00	12,10	7,26	4,48	0,24	0,12
98	6,59	14,90	67,20	44,28	33,70	254,00	10,50	5,78	4,31	0,21	0,21
99	7,12	17,90	70,40	50,12	35,70	292,00	12,90	8,90	2,84	1,03	0,13
100	6,56	16,70	79,40	52,09	32,10	302,00	10,90	6,98	2,51	0,44	0,98
101	6,76	14,40	68,20	46,10	31,20	308,00	19,60	10,19	4,31	1,18	3,92
102	5,18	10,80	65,50	33,93	31,80	269,00	10,90	4,14	5,45	0,33	0,98
103	6,44	13,80	63,50	40,89	33,80	170,00	20,60	10,51	5,77	0,62	3,71
104	6,64	16,00	73,60	48,87	32,70	95,00	11,40	7,07	3,08	0,46	0,80
105	7,26	16,10	67,60	49,08	32,80	221,00	9,40	6,86	2,26	0,19	0,09
106	7,94	17,10	67,30	53,44	32,00	295,00	7,90	5,61	1,98	0,24	0,08
107	8,66	19,10	70,00	60,62	31,50	145,00	9,20	5,70	2,85	0,46	0,18
108	7,41	17,70	76,10	56,39	31,40	545,00	11,30	8,59	1,92	0,57	0,23
109	8,11	17,80	67,80	54,99	32,40	124,00	10,30	6,18	2,88	0,21	1,03
110	7,82	17,50	68,80	53,80	32,50	273,00	7,20	3,17	3,24	0,22	0,58
111	6,90	16,60	71,80	49,54	24,00	115,00	9,20	4,69	3,40	0,18	0,92
112	6,86	17,40	76,50	52,48	33,20	181,00	16,60	5,15	6,14	3,15	2,16
113	5,19	10,70	65,50	33,99	31,50	117,00	13,70	4,25	6,03	2,74	0,69
114	5,39	10,60	65,40	35,25	30,10	108,00	5,10	2,45	1,99	0,36	0,31
115	5,24	9,90	59,50	31,18	31,80	48,00	7,70	4,93	1,46	0,39	0,92
116	4,66	10,60	69,40	32,34	32,80	88,00	5,90	2,36	2,07	0,89	0,59
117	6,25	11,90	61,70	38,56	30,90	165,00	11,70	4,68	5,85	0,00	1,17
118	4,96	11,00	69,50	34,47	31,90	246,00	16,20	8,26	5,83	6,48	1,46
119	5,33	11,10	64,80	34,54	64,80	109,00	9,20	5,43	2,94	0,18	0,64
120	7,53	16,60	66,80	50,30	33,00	274,00	9,80	7,45	1,76	0,29	0,29
121	6,20	13,30	69,50	43,09	30,90	318,00	15,70	9,58	3,14	1,41	1,57
122	6,02	14,10	69,00	41,54	33,90	232,00	9,30	7,07	1,30	0,28	0,65
123	7,21	15,70	69,40	50,04	31,40	314,00	18,40	13,06	2,58	2,39	0,37
124	7,44	18,10	74,70	55,58	32,60	258,00	10,50	6,83	3,36	0,21	0,11
125	6,80	14,00	71,50	48,62	31,60	351,00	16,50	9,90	4,79	0,99	0,83
126	7,87	19,70	73,90	58,16	33,90	193,00	9,00	5,58	2,34	0,45	0,36
127	7,00	18,10	75,90	53,13	34,00	215,00	12,10	7,14	2,90	0,61	1,45
128	5,93	13,60	68,30	40,50	33,50	127,00	9,10	3,91	3,09	1,91	0,18
129	6,30	14,90	72,60	45,74	32,60	380,00	11,10	8,55	1,55	0,56	0,44
130	7,71	17,80	66,50	56,16	34,70	345,00	14,50	10,59	3,77	0,00	0,15
131	7,36	19,00	76,30	56,16	33,80	149,00	14,60	6,86	5,40	0,88	1,46
132	4,68	6,90	46,40	21,72	31,70	59,00	9,00	2,88	3,60	1,62	0,90
133	7,49	17,70	69,60	52,13	33,90	199,00	8,70	5,82	2,09	0,61	0,17
134	6,59	16,50	69,50	45,80	36,00	127,00	9,80	4,80	2,25	1,67	1,08
135	6,84	16,70	75,00	51,30	32,50	258,00	18,10	8,87	5,79	1,27	2,17
136	6,97	16,70	70,20	48,93	34,10	261,00	18,30	9,33	4,39	1,65	2,93
137	7,64	16,50	67,30	51,42	32,10	154,00	8,70	4,52	3,48	0,61	0,09
138	6,94	17,30	74,00	51,36	24,90	326,00	9,30	5,12	1,67	0,93	1,58
139	6,82	18,30	72,20	49,24	37,10	245,00	18,00	9,18	6,12	1,80	0,90
140	6,18	15,00	69,50	42,95	34,90	138,00	13,70	5,34	4,66	2,19	1,51
141	7,17	17,40	71,40	51,19	34,00	260,00	15,40	8,16	5,39	0,31	1,54

142	6,93	17,00	76,90	53,29	31,90	60,00	9,20	5,52	2,39	0,28	1,01
143	6,79	16,50	73,10	49,63	33,20	340,00	11,70	5,62	3,74	0,94	1,40
144	6,65	16,10	71,90	47,81	33,60	27,00	24,50	12,74	6,37	2,45	2,94
145	5,29	11,90	69,80	36,92	32,40	371,00	7,20	5,69	0,72	0,14	0,65
146	6,86	17,70	72,60	49,80	35,50	280,00	9,50	5,04	3,33	0,86	0,29
147	7,58	17,00	69,70	52,83	32,10	124,00	9,00	3,87	2,52	2,16	0,45
148	6,98	15,60	70,30	49,07	31,80	136,00	10,70	8,03	1,39	0,54	0,75
149	6,63	14,30	68,80	45,61	31,30	345,00	11,20	7,95	1,68	1,23	0,34
150	6,01	14,50	70,30	42,25	34,30	315,00	9,40	5,55	3,20	0,56	0,09
151	5,37	12,70	69,30	37,21	34,10	318,00	10,90	8,72	1,53	0,55	0,11

Eosinophils (EOS); Hemoglobin (HB); Lymphocytes (LYM); Neutrophils (NEU); Mean Corpuscular Volume (MVC); Mean Corpuscular Hemoglobin Concentration (MCHC); Monocytes (MON); Platelets (PL); Package Cell Volume Hematocrit (PVC); Red Blood Cells (RBC); White Blood Cells (WBC).

REFERÊNCIAS DA INTRODUÇÃO GERAL

ALVAR, J. et al. Canine Leishmaniasis. In: **Advances in Parasitology**. [s.l: s.n.]57p. 1–88.

ARAÚJO, V. E. M. de et al. Relative Risk of Visceral Leishmaniasis in Brazil: A Spatial Analysis in Urban Area. **PLoS Neglected Tropical Diseases**, v. 7, n. 11, p. e2540, 7 nov. 2013. Disponível em: <https://dx.plos.org/10.1371/journal.pntd.0002540>.

BHATIA, A. et al. Cloning, characterization and serological evaluation of K9 and K26: Two related hydrophilic antigens of Leishmania chagasi. **Molecular and Biochemical Parasitology**, v. 102, n. 2, p. 249–261, 1999.

BOGGIATTO, P. M. et al. Transplacental Transmission of Leishmania infantum as a Means for Continued Disease Incidence in North America. **PLoS Neglected Tropical Diseases**, v. 5, n. 4, p. e1019, 12 abr. 2011. Disponível em: <http://dx.plos.org/10.1371/journal.pntd.0001019>.

BOURDOISEAU, G. et al. Lymphocyte subset abnormalities in canine leishmaniasis. **Veterinary Immunology and Immunopathology**, v. 56, n. 3–4, p. 345–351, 1997.

BOUYAHIA, N. et al. Impedance spectroscopy and conductometric biosensing for probing catalase reaction with cyanide as ligand and inhibitor. **Bioelectrochemistry**, v. 80, n. 2, p. 155–161, fev. 2011. Disponível em: <http://dx.doi.org/10.1016/j.bioelechem.2010.07.006>.

BRASIL. **Manual de vigilância e controle da leishmaniose visceral / Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica.** 1. ed. [s.l: s.n.]

BURNS, J. M. et al. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. **Proceedings of the National Academy of Sciences**, v. 90, n. 2, p.

775–779, 2006.

CAMARGO-NEVES, V. L. F. de et al. Utilização de ferramentas de análise espacial na vigilância epidemiológica de leishmaniose visceral americana – Araçatuba , São Paulo , Use of spatial analysis tools in the epidemiological surveillance of American visceral leishmaniasis ,. **Caderno de Saúde Pública**, v. 17 (5), n. 5, p. 1263–1267, 2001.

CÂNDIDO, T. C. et al. 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. **Veterinary Parasitology**, v. 157, n. 3–4, p. 175–181, 2008.

CARDIM, M. F. M. et al. Introduction and expansion of human American visceral leishmaniasis in the state of Sao Paulo, Brazil, 1999-2011. **Revista de Saude Publica**, v. 47, n. 4, p. 1–9, 2013.

CLARK, M. F.; LISTER, R. M.; BAR-JOSEPH, M. **ELISA techniques.** In: [s.l: s.n.]871p. 742–766.

COURTENAY, O. et al. Heterogeneities in Leishmania infantum Infection: Using Skin Parasite Burdens to Identify Highly Infectious Dogs. **PLoS Neglected Tropical Diseases**, v. 8, n. 1, p. 26, 2014.

COUTINHO, M. T. Z. et al. Participation of Rhipicephalus sanguineus (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. **Veterinary Parasitology**, v. 128, n. 1–2, p. 149–155, mar. 2005. Disponível em: <https://doi.org/10.1016/j.vetpar.2004.11.011>.

COUTINHO, M. T. Z.; LINARDI, P. M. Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? **Veterinary Parasitology**, v. 147, n. 3–4, p. 320–325, jul. 2007. Disponível em: <https://doi.org/10.1016/j.vetpar.2007.04.008>.

DANTAS-TORRES, F. et al. Detection of Leishmania infantum in Rhipicephalus sanguineus ticks from Brazil and Italy. **Parasitology Research**, v. 106, n. 4, p. 857–860, 3 mar. 2010. Disponível em: <http://link.springer.com/10.1007/s00436-010-1722-4>.

DANTAS-TORRES, F. et al. Further thoughts on “Asymptomatic dogs are highly competent to transmit Leishmania (Leishmania) infantum chagasi to the natural vector”. **Veterinary Parasitology**, v. 204, n. 3–4, p. 443–444, 2014. Disponível em: <http://dx.doi.org/10.1016/j.vetpar.2014.04.018>.

DANTAS-TORRES, F.; OTRANTO, D. When is an “asymptomatic” dog asymptomatic? **Veterinary Parasitology**, v. 202, n. 3–4, p. 341–342, 2014. Disponível em: <http://dx.doi.org/10.1016/j.vetpar.2014.02.008>.

DE ALMEIDA LEAL, G. G. et al. Immunological profile of resistance and susceptibility in naturally infected dogs by Leishmania infantum. **Veterinary Parasitology**, v. 205, n. 3–4, p. 472–482, out. 2014. Disponível em: <https://doi.org/10.1016/j.vetpar.2014.08.022>.

DE ASSIS, J. et al. Estudo comparativo dos métodos diagnósticos para leishmaniose visceral em cães oriundos de Ilha Solteira, SP. **Revista Brasileira de Parasitologia Veterinaria**, v. 19, n. 1, p. 17–25, 2010.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. **Nature Nanotechnology**, v. 7, n. 12, p. 821–824, 2012.

DE LA RICA, R.; STEVENS, M. M. Plasmonic ELISA for the detection of analytes at ultralow concentrations with the naked eye. **Nature Protocols**, v. 8, n. 9, p. 1759–1764, 22 set. 2013. Disponível em: <http://dx.doi.org/10.1038/nprot.2013.085>.

DE QUEIROZ, N. M. G. P. et al. Canine Visceral Leishmaniasis diagnosis by immunohistochemistry and PCR in skin tissues in association with RIFI and ELISA-test. **Revista Brasileira de Parasitologia Veterinaria**, v. 19, n. 1, p. 34–40, 2010.

DESJEUX, P. The increase in risk factors for leishmaniasis worldwide. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 95, n. 3, p. 239–243, 2001.

DESJEUX, P. Leishmaniasis: current situation and new perspectives. **Comparative immunology, microbiology and infectious diseases**, v. 27, n. 5, p. 305–18, 2004. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15225981>.

DO ROSÁRIO, E. Y. et al. Evaluation of enzyme-linked immunosorbent assay using crude Leishmania and recombinant antigens as a diagnostic marker for canine visceral leishmaniasis. **Memorias do Instituto Oswaldo Cruz**, v. 100, n. 2, p. 197–203, 2005.

DOS-SANTOS, W. L. C. et al. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. **Veterinary Immunology and Immunopathology**, v. 123, n. 3–4, p. 251–259, 2008.

EUSTIS, S.; EL-SAYED, M. A. Why gold nanoparticles are more precious than pretty gold: Noble metal surface plasmon resonance and its enhancement of the radiative and nonradiative properties of nanocrystals of different shapes. **Chemical Society Reviews**, v. 35, n. 3, p. 209–217, 2006.

FEITOSA, MM, IKEDA FA , LUVIZOTTO MCR, P. S. Aspectos clínicos de cães com leishmaniose visceral no município de Araçatuba–São Paulo (Brasil). **Clínica Veterinária**, v. 5, n. 28, p. 36–44, 2000.

FERREIRA, M. G. P. A. et al. Potential role for dog fleas in the cycle of Leishmania spp. **Veterinary Parasitology**, v. 165, n. 1–2, p. 150–154, out. 2009. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0304401709003689>.

FERREIRA, S. de A. et al. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. **Veterinary Parasitology**, v. 152, n. 3–4, p. 257–263, 2008.

GONTIJO, C. M. F.; MELO, M. N. Visceral Leishmaniasis in Brazil: current status, challenges and prospects. **Revista Brasileira de Epidemiologia**, v. 7, n. 3, p. 338–349, 2004. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1415-790X2004000300011&lng=pt&tlng=pt>.

GUO, L.; KIM, D. H. LSPR biomolecular assay with high sensitivity induced by aptamer-antigen-antibody sandwich complex. **Biosensors and Bioelectronics**, v. 31, n. 1, p. 567–570, 2012. Disponível em: <http://dx.doi.org/10.1016/j.bios.2011.10.047>.

IKONOMOPOULOS, J. et al. Molecular diagnosis of leishmaniosis in dogs. **Veterinary Parasitology**, v. 113, n. 2, p. 99–113, abr. 2003. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S030440170300061X>.

LACHAUD, L. et al. Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis. **Journal of Clinical Microbiology**, v. 40, n. 1, p. 210–215, 1 jan. 2002. Disponível em: <http://jcm.asm.org/cgi/doi/10.1128/JCM.40.1.210-215.2002>.

LAURENTI, M. D. et al. Asymptomatic dogs are highly competent to transmit Leishmania (Leishmania) infantum chagasi to the natural vector. **Veterinary Parasitology**, v. 196, n. 3–4, p. 296–300, 2013. Disponível em: <http://dx.doi.org/10.1016/j.vetpar.2013.03.017>.

LAURICELLA, M. A. et al. An rK28-based immunoenzymatic assay for the diagnosis of canine visceral leishmaniasis in Latin America. **American Journal of Tropical Medicine and Hygiene**, v. 95, n. 1, p. 92–98, 2016.

LEITE, R. S. et al. PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. **Veterinary Parasitology**, v. 170, n. 3–4, p. 201–206, 2010. Disponível em: <http://dx.doi.org/10.1016/j.vetpar.2010.02.020>.

LIMA, V. M. F. de et al. Apoptosis in T lymphocytes from spleen tissue and peripheral

blood of L. (L.) chagasi naturally infected dogs. **Veterinary Parasitology**, v. 184, n. 2–4, p. 147–153, 2012.

LIMA, V. M. F. et al. Anti-leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. **Brazilian Journal of Medical and Biological Research**, v. 36, n. 4, p. 485–489, 2003.

LIMA, V. M. F. et al. Evidence of Leishmania spp. Antibodies and DNA in Bush Dogs (Speothos venaticus) in Brazil. **Journal of Zoo and Wildlife Medicine**, v. 40, n. 1, p. 91–94, mar. 2009. Disponível em: <http://www.bioone.org/doi/full/10.1638/2008-0043.1>.

LIMA, W. G. et al. Canine visceral leishmaniasis: a histopathological study of lymph nodes. **Acta Tropica**, v. 92, n. 1, p. 43–53, set. 2004.

LOMBARDO, G. et al. Detection of Leishmania infantum DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. **Veterinary Parasitology**, v. 184, n. 1, p. 10–17, 2012. Disponível em: <http://dx.doi.org/10.1016/j.vetpar.2011.08.010>.

LOPES, E. G. et al. Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: One out of five seronegative dogs are infected. **Epidemiology and Infection**, v. 145, n. 12, p. 2436–2444, 2017.

MANNA, L. et al. Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis. **Veterinary Parasitology**, v. 125, n. 3–4, p. 251–262, 2004.

MOLYNEUX, D. H.; ASHFORD, R. W. **The biology of Trypanosoma and Leishmania, parasites of man and domestic animals**. [s.l: s.n.]

MORENO, J.; ALVAR, J. Canine leishmaniasis: epidemiological risk and the experimental model. **Trends in Parasitology**, v. 18, n. 9, p. 399–405, set. 2002. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S1471492202023474>.

NIE, X. M. et al. Plasmonic ELISA for the ultrasensitive detection of Treponema pallidum. **Biosensors and Bioelectronics**, v. 58, p. 314–319, 2014. Disponível em: <http://dx.doi.org/10.1016/j.bios.2014.03.007>.

NUNES, C. M. et al. Avaliação da reação em cadeia pela polimerase para diagnóstico da leishmaniose visceral em sangue de cães. **Revista brasileira de parasitologia veterinária = Brazilian journal of veterinary parasitology : Órgão Oficial do Colégio Brasileiro de Parasitologia Veterinária**, v. 16, n. 1, p. 5–9, 2007.

OGISO, M. et al. Carbohydrate immobilized on a dendrimer-coated colloidal gold surface for fabrication of a lectin-sensing device based on localized surface plasmon resonance spectroscopy. **Biosensors and Bioelectronics**, v. 41, n. 1, p. 465–470, 2013a. Disponível em: <http://dx.doi.org/10.1016/j.bios.2012.09.003>.

OGISO, M. et al. Carbohydrate immobilized on a dendrimer-coated colloidal gold surface for fabrication of a lectin-sensing device based on localized surface plasmon resonance spectroscopy. **Biosensors and Bioelectronics**, v. 41, n. 12, p. 465–470, mar. 2013b. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0956566312006094>.

OWENS, S. D. et al. Transmission of visceral leishmaniasis through blood transfusions from infected English Foxhounds to anemic dogs. **Journal of the American Veterinary Medical Association**, v. 219, n. 8, p. 1076–1083, out. 2001. Disponível em: <https://doi.org/10.2460/javma.2001.219.1076>.

PALATNIK-DE-SOUSA, C. B. et al. Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. **The American Journal of Tropical Medicine and Hygiene**, v. 65, n. 5, p. 510–517, 1 nov. 2001. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/11716106>.

PATTABHI, S. et al. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. **PLoS Neglected Tropical Diseases**, v. 4, n. 9, 2010.

PORROZZI, R. et al. 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. **Clinical and Vaccine Immunology**, v. 14, n. 5, p. 544–548, 2007.

QUARESMA, P. F. et al. Molecular diagnosis of canine visceral leishmaniasis: Identification of Leishmania species by PCR-RFLP and quantification of parasite DNA by real-time PCR. **Acta Tropica**, v. 111, n. 3, p. 289–294, 2009.

READY. Epidemiology of visceral leishmaniasis. **Clinical Epidemiology**, v. 6, n. 1, p. 147–154, 2014.

REALE, S. et al. Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. **Journal of Clinical Microbiology**, v. 37, n. 9, p. 2931–2935, 1999.

RISSIN, D. M. et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. **Nature Biotechnology**, v. 28, n. 6, p. 595–599, 2010. Disponível em: <http://dx.doi.org/10.1038/nbt.1641>.

ROSATI, S. et al. Prokaryotic Expression and Antigenic Characterization of Three Recombinant Leishmania Antigens for Serological Diagnosis of Canine Leishmaniasis. **Clinical and Vaccine Immunology**, v. 10, n. 6, p. 1153–1156, 2003.

ROSYPAL, A. C. et al. Serological survey of Leishmania infantum and Trypanosoma cruzi in dogs from urban areas of Brazil and Colombia. **Veterinary Parasitology**, v. 149, n. 3–4, p. 172–177, 2007.

SADICK, M. D. et al. Cytokine regulation of murine leishmaniasis: Interleukin 4 is not sufficient to mediate progressive disease in resistant C57BL/6 mice. **Infection and Immunity**, v. 59, n. 12, p. 4710–4714, 1991.

SANTA ROSA I.C.A.; OLIVEIRA I.C.S. Leishmaniose visceral: breve revisão sobre

uma zoonose reemergente. **Clinica Veterinária**, v. 2, n. 11, p. 24–28, 1997.

SANTANA, C. C. et al. Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: a study on naturally infected dogs. **Parasite immunology**, v. 30, n. 10, p. 515–24, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18665902%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2592477>.

SATIJA, J. et al. Plasmonic-ELISA: Expanding horizons. **RSC Advances**, v. 6, n. 88, p. 85440–85456, 2016. Disponível em: <http://dx.doi.org/10.1039/C6RA16750K>.

SILVA, F. L. et al. Venereal transmission of canine visceral leishmaniasis. **Veterinary Parasitology**, v. 160, n. 1–2, p. 55–59, mar. 2009. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0304401708006031>.

SOLANO-GALLEGO, L. et al. Prevalence of Leishmania infantum Infection in Dogs Living in an Area of Canine Leishmaniasis Endemicity Using PCR on Several Tissues and Serology. **Journal of Clinical Microbiology**, v. 39, n. 2, p. 560–563, 1 fev. 2001. Disponível em: <http://jcm.asm.org/cgi/doi/10.1128/JCM.39.2.560-563.2001>.

SOLANO-GALLEGO, L. et al. Histological and immunohistochemical study of clinically normal skin of Leishmania infantum-infected dogs. **Journal of Comparative Pathology**, v. 130, n. 1, p. 7–12, 2004.

SOLANO-GALLEGO, L. et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. **Veterinary Parasitology**, v. 165, n. 1–2, p. 1–18, out. 2009. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0304401709003124>.

SOLANO-GALLEGO, L. et al. LeishVet guidelines for the practical management of canine leishmaniosis. **Parasites & Vectors**, v. 4, n. 1, p. 86, 2011. Disponível em: <http://www.parasitesandvectors.com/content/4/1/86>.

STRAUSS-AYALI, D. et al. Polymerase Chain Reaction Using Noninvasively Obtained

Samples, for the Detection of Leishmania infantum DNA in Dogs . **The Journal of Infectious Diseases**, v. 189, n. 9, p. 1729–1733, 2004.

TAFURI, W. L. et al. An alternative immunohistochemical method for detecting Leishmania amastigotes in paraffin-embedded canine tissues. **Journal of Immunological Methods**, v. 292, n. 1–2, p. 17–23, 2004.

TEIXEIRA-NETO, R. G. et al. Canine visceral leishmaniasis in an urban setting of Southeastern Brazil: An ecological study involving spatial analysis. **Parasites and Vectors**, v. 7, n. 1, p. 1–10, 2014.

VAISH, M. et al. Evaluation of rK28 antigen for serodiagnosis of visceral Leishmaniasis in India. **Clinical Microbiology and Infection**, v. 18, n. 1, p. 81–85, 2012.

VENTURIN, G. L. et al. Recombinant K28 antigen in ELISA in the diagnosis of canine visceral leishmaniosis. **Parasite Immunology**, v. 37, n. 12, p. 670–673, 2015.

WHO. **Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases**. Disponível em: <http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf>. Acesso em: 15 fev. 2019.

WHO. Epidemiological Report of the Americas. **World Health Organization technical report series**, v. 7, 2019. Disponível em: <http://iris.paho.org/xmlui/bitstream/handle/123456789/50505/Leishreport2019_eng.pdf?ua=1>.

WU, J. et al. Biomedical and clinical applications of immunoassays and immunosensors for tumor markers. **TrAC - Trends in Analytical Chemistry**, v. 26, n. 7, p. 679–688, 2007.

ANEXO

ANEXO A – NORMAS PARA PUBLICAÇÃO NA REVISTA JOURNAL OF IMMUNOLOGICAL METHODS

DESCRIPTION

The Journal of Immunological Methods is devoted to covering techniques for: (1) Quantitating and detecting antibodies and/or antigens. (2) Purifying immunoglobulins, lymphokines and other molecules of the immune system. (3) Isolating antigens and other substances important in immunological processes. (4) Labelling antigens and antibodies. (5) Localizing antigens and/ or antibodies in tissues and cells. (6) Detecting, and fractionating immunocompetent cells. (7) Assaying for cellular immunity. (8) Documenting cell-cell interactions. (9) Initiating immunity and unresponsiveness. (10) Transplanting tissues. (11) Studying items closely related to immunity such as complement, reticuloendothelial system and others. (12) Molecular techniques for studying immune cells and their receptors. (13) Imaging of the immune system. (14) Methods for production or their fragments in eukaryotic and prokaryotic cells.

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