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AVALIAÇÃO DE BIOMARCADORES DE GRAVIDADE EM CÃES NATURALMENTE INFECTADOS POR *LEISHMANIA INFANTUM* CLASSIFICADOS CLINICAMENTE E ESTRATIFICADOS QUANTO À CARGA PARASITÁRIA EM ESTUDOS DE CORTE TRANSVERSAL

MANUELA DA SILVA SOLCÀ

Salvador – Bahia
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“Chi nega la ragion delle cose,
pubblica la sua ignoranza”.

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RESUMO

INTRODUÇÃO: A leishmaniose visceral (LV) é, principalmente, causada pelo protozoário *Leishmania infantum* nas Américas, podendo acometer o Homem e animais. Dentre estes, o cão é considerado o principal reservatório doméstico do parasito. O curso da LV canina (LVC) varia entre os animais, podendo alguns se mostrar resistentes à infecção, se mantendo subclínicos, e outros susceptíveis, que irão desenvolver sinais da doença. O estado de resistência ou susceptibilidade à LVC reflete na gravidade da infecção do animal, e não pode ser definido pelo quadro clínico apresentado ou por qualquer parâmetro isolado de resposta imune. **OBJETIVO**: Avaliar a carga parasitária como biomarcador parasitológico, as proteínas LJM11/LJM17 como biomarcadores de exposição à saliva do vetor, e identificar biomarcadores inflamatórios de gravidade da infecção por *L. infantum* em cães. Primeiramente, foi realizada a padronização de uma ferramenta diagnóstica de PCR quantitativa (qPCR), utilizando diferentes amostras biológicas (aspirado esplênico, linfonodos, pele, sangue, medula óssea e swab conjuntival) de cães sintomáticos provenientes da área endêmica de Jequié-BA. A avaliação da carga parasitária de *L. infantum* teve seu desempenho comparado com outras técnicas diagnósticas (cultura de aspirado esplênico, teste rápido e ELISA para LVC) empregando a análise de classe latente (ACL). Para essa análise, foi construída uma variável latente a ser empregada como padrão ouro para avaliação da acurácia desses métodos. Na avaliação inicia dos cães sintomáticos, a qPCR detectou DNA do parasita em 100% dos animais em pelo menos uma das amostras biológicas, sendo que todos foram positivos empregando-se o aspirado esplênico e 70% empregando-se o sangue. Utilizando a variável latente como padrão-ouro, a sensibilidade para a qPCR de aspirado esplênico (95,8%) foi maior do que as obtidas pela qPCR utilizando outras amostras biológicas e por outros testes diagnósticos. Desta forma, em uma segunda etapa, a acurácia da qPCR empregando-se aspirados esplênicos foi avaliada em uma amostra canina obtida de forma randômica (n = 800), coletada durante um estudo de corte transversal na área endêmica de Camaçari-BA. Utilizando a variável latente como padrão-ouro, novamente a sensibilidade para a qPCR de aspirado esplênico foi elevada (95%). Neste estudo de corte transversal foi possível também correlacionar a carga parasitária com a gravidade da infecção, existindo associação positiva significativa entre a intensidade da carga parasitária no baço e o número de sinais clínicos presentes nos cães. Nessa primeira parte do estudo, foi possível demonstrar que i) a utilização de aspirado esplênico na qPCR apresentou maior sensibilidade na detecção de DNA de *L. infantum* do que outras amostras biológicas; ii) a ACL pode ser usada para gerar um padrão ouro adequado para avaliação de técnicas diagnósticas, uma vez que esta técnica oferece uma avaliação mais completa dos resultados obtidos por diferentes métodos diagnósticos para LVC e iii) a carga parasitária se mostrou um bom biomarcador de gravidade clínica nos animais

infectados por *L. infantum*. Posteriormente, após a reação de qPCR ter sido padronizada e validada empregando-se aspirados esplênicos, esta foi aprimorada para utilização em estudos epidemiológicos, sendo padronizada em formato *duplex* tanto na forma líquida como no formato em gel (*ready-to-use*), adicionando-se à reação *primers* específicos para a detecção simultânea de um gene constitutivo canino. Esse protocolo permitiu a detecção de até 0,1 parasitas por amostra. Adicionalmente, a detecção de DNA do hospedeiro na mesma reação simultaneamente fortaleceu o diagnóstico da LVC, agindo como controle interno da reação, reduzindo tempo de execução e diminuindo custos da reação. Após a padronização da qPCR *duplex*, foi realizado um estudo exploratório de uma amostra de cães coletada durante outro estudo de corte transversal, em Camaçari-BA, no qual se objetivou avaliar biomarcadores de exposição à saliva do vetor (proteínas LJM11/LJM17) e identificar biomarcadores inflamatórios, correlacionando-os à gravidade da LVC e à carga parasitária. A análise identificou uma bioassinatura distinta em cães com diferentes manifestações clínicas, caracterizada por uma diminuição dos níveis de LTB4 e de PGE2 e um aumento de CXCL1 e CCL2, de acordo com o agravamento da doença. Além disso, utilizando uma combinação de 3 parâmetros distintos (LTB4, PGE2 e CXCL1) como um marcador, este permitiu a discriminação entre escores clínicos diferentes utilizando uma curva ROC. Foi detectado também, que cães com escores clínicos elevados apresentaram-se, mais frequentemente, positividade para anticorpos anti-saliva e elevadas cargas parasitárias. Este estudo permitiu a avaliação e identificação de vários biomarcadores em cães, que podem ser importantes para auxiliar na avaliação do curso da doença e prognóstico por médicos veterinários, além de futuramente poder ajudar na distinção entre cães resistentes ou susceptíveis direcionando estratégias de controle da LVC em áreas endêmicas. **CONCLUSÃO:** Existem biomarcadores parasitológicos como a carga parasitária, biomarcadores imunológicos e inflamatórios como CXCL1, CCL2, LTB-4, PGE-2, além da produção de anticorpos específicos contra as proteínas LJM 11 e LJM 17 da saliva do vetor, que são capazes de diferenciar animais infectados por *L. infantum* de acordo com seus diferentes quadros clínicos.

Palavras-chave: Leishmaniose Visceral Canina, Biomarcadores, qPCR, Cão

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ABSTRACT

INTRODUCTION: In the Americas, visceral leishmaniasis (VL) is caused by the protozoan *Leishmania infantum*, which can affect humans and animals. Among these, dog is considered the main domestic reservoir of this parasite. Canine VL (CVL) clinical outcome varies among animals, some of which may be resistant to infection remaining subclinical, and others may be susceptible showing signs of the disease. The state of resistance or susceptibility to CVL reflects on the severity of infection in the animal and cannot be defined solely by the clinical condition presented or by any isolated parameter of the immune response. **OBJECTIVE:** Assess parasite load as parasitological biomarkers, LJM11/LJM17 proteins as sandfly saliva exposure biomarkers, and identify inflammatory biomarkers that indicates *L. infantum* infection severity in dogs. Firstly, we performed the standardization of a quantitative PCR diagnostic tool (qPCR) using different biological samples (splenic aspirate, lymph nodes, skin, blood, bone marrow and conjunctival swab) of symptomatic dogs from the endemic area of Jequié-BA. The evaluation of the parasitic load of *L. infantum* had its performance compared to other diagnostic techniques (splenic aspirate culture, rapid test and CVL ELISA) using latent class analysis (LCA). In this analysis, a latent variable was constructed to be used as a gold standard to evaluate the accuracy of these methods. In the initial evaluation of the symptomatic dogs, qPCR detected DNA from the parasite in 100% of the animals in at least one of the biological samples, all of which were positive using the splenic aspirate and 70% using the blood. Employing the latent variable as a gold standard, splenic aspirate qPCR sensitivity (95.8%) was greater than that obtained by qPCR using other biological samples and other diagnostic tests. Thus, in a second stage, the accuracy of qPCR using splenic aspirates was evaluated in a randomly obtained canine sample (n = 800), collected during a cross-sectional study in the endemic area of Camaçari-BA. Using the latent variable as a gold standard, qPCR sensitivity of splenic aspirate was again high (95%). In this cross-sectional study, it was also possible to correlate the parasite load with the severity of the infection, and there was a significant positive association between the intensity of the parasite load in the spleen and the number of clinical signs present in dogs. In this first part of the study, it was possible to demonstrate that i) the use of splenic aspirate in the qPCR presented greater sensitivity in the detection of *L. infantum* DNA than other biological samples; Ii) LCA can be used to generate a suitable gold standard diagnostic techniques assessment, since this technique offers a more complete evaluation of the results obtained by different diagnostic methods for LVC and iii) the parasite load proved to be a good biomarker of clinical severity in animals infected with *L. infantum*. Next, after the qPCR reaction was standardized and validated using splenic aspirates, the same was improved for use in epidemiological studies, being standardized in both liquid and ready-to-use gel format, adding specific primers for the simultaneous detection of a canine constitutive gene. This protocol allowed the detection of up to 0.1 parasites per sample. In addition, the detection of host DNA in

the same reaction simultaneously strengthened the LVC diagnosis, acting as internal reaction control, reducing execution time and reducing reaction costs. After the standardization of the duplex qPCR, we performed an exploratory study of a sample of dogs collected during another cross-sectional study carried out in Camaçari-BA. We aimed to evaluate the biomarkers of vector saliva exposure (LJM11/ LJM17 proteins) and to identify inflammatory biomarkers, correlating them with CVL severity and parasite load. Assessment of protein expression of profile biomarkers identified a distinct biosignature that could cluster separately animal groups with different clinical scores. Increasing severity scores were associated with a gradual decrease of LTB-4 and PGE-2, and a gradual increase in CXCL1 and CCL2. Discriminant ROC analyses revealed that combined assessment of LTB-4, PGE-2 and CXCL1 was able to distinguish dogs with different clinical scores. Dogs with the highest clinical score values also exhibited high parasite loads and positivity for anti-saliva antibodies. This study allowed the evaluation and identification of several biomarkers in dogs, which may be important to assist in the evaluation of the disease course and prognosis by veterinarians, and in the future to be able to help distinguish between resistant or susceptible dogs directing CVL control strategies in endemic areas. **CONCLUSION:** There are parasitological biomarkers such as parasite load, immunological and inflammatory biomarkers such as CXCL1, CCL2, LTB-4, PGE-2, and the production of specific antibodies against LJM 11 and LJM 17 sandfly salivary proteins, which are able to differentiate animals infected by *L. infantum* according to their different clinical condition.

Key-Words: Canine Visceral Leishmaniasis, Biomarkers, qPCR, Dog

LISTA DE ABREVIATURAS

ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
cPCR	Reação em cadeia da polimerase convencional
CCL2	Proteína quimioatratora de monócitos - do inglês: *chemokine (C-C motif) ligand 2*
CXCL1	Proteína ativadora de neutrófilos - do inglês: *chemokine (C-X-C motif) ligand 1*
CXL10	Citocina Induzida por Interferon- do inglês: *chemokine (C-X-C motif) ligand 10*
DPP LVC	Teste rápido para leishmaniose visceral canina do inglês *Dual Path Platform*
DNA	Ácido desoxirribonucléico
DTH	Hipersensibilidade do tipo tardio ao teste cutâneo a antígenos de *Leishmania* – do inglês: *delayed type hypersensitivity*
EIE LVC	Ensaio imunoenzimático para leishmaniose visceral canina
ELISA	Ensaio imunoenzimático
FA	Fosfatase alcalina
GGT	Gamaglutamiltransferase
IC	Intervalo de confiança
IgG	Imunoglobulina G
IgG1	Imunoglobulina G1
IgG2	Imunoglobulina G2
IL	Interleucina
IFN-γ	Interferon gama
kDNA	DNA do cinetoplasto
LJM11	Proteína salivar de *Lutzomyia longipalpis LJM11*
LJM17	Proteína salivar de *Lutzomyia longipalpis LJM11*
LV	Leishmaniose visceral
LVC	Leishmaniose visceral canina
LTB-4	Leucotrieno B4

MS	Ministério da Saúde
OMS	Organização Mundial da Saúde
PCR	Reação em cadeia da polimerase
PGE-2	Prostaglandina E2
qPCR	Reação em cadeia da polimerase quantitativa
RIFI	Reação de imunofluorescência indireta
RNA	Ácido Ribonucléico
rRNA	RNA ribossomal
SOD	Enzima superóxido desmutase
TH1	Linfócitos T auxiliares 1
TH2	Linfócitos T auxiliares 2
TCL	Teste cutâneo a antígenos de *Leishmania*
TGF-β	Fator de transformação do crescimento beta - do inglês: *transforming growth factor beta*
TNF-α	Fator de necrose tumoral alfa – do inglês: *tumor necrosis fator alpha*

SUMÁRIO

1 INTRODUÇÃO

1.1 DISTRIBUIÇÃO DA LEISHMANIOSE VISCERAL

Segundo a Organização Mundial da Saúde (OMS), a leishmaniose é classificada como uma doença negligenciada tropical, presente em cinco continentes, endêmica em 98 países, com mais de 350 milhões de pessoas em risco. Existem três manifestações clínicas diferentes de leishmaniose: cutânea, acometendo a pele; mucosa que acomete mucosas; e visceral, sendo esta a forma mais grave, que afeta os órgãos internos (BRASIL, 2014; WHO, 2017).

A leishmaniose visceral (LV) acomete cerca de 300 mil pessoas mundialmente a cada ano, assim encontra-se entre as seis endemias consideradas prioritárias no mundo pela OMS, com registro de ocorrência em 88 países (DESJEUX, 2004; ALVAR *et al.*, 2012; WHO, 2017). Anualmente, uma média de 4.000 casos de LV são diagnosticados, com uma taxa de mortalidade de 7%. Em 2014, mais de 90% dos novos casos notificados à OMS de LV ocorreram em seis países: Brasil, Etiópia, Índia, Somália, Sudão do Sul e Sudão (WHO, 2017).

Já foi determinado que a presença de LV está diretamente ligada à pobreza, mas outros fatores sociais, assim como alguns ambientais e climatológicos também influenciam diretamente na distribuição da doença (FRANKE *et al.*, 2002; QUINNELL e COURTENAY, 2009; WHO, 2017). Alterações ambientais e demográficas, além de uma intensa tendência migratória para centros urbanos, acarretaram que a partir da década de 70 a ocorrência da LV tenha sido mais frequente em áreas periurbanas e urbanas, com graves consequências para a saúde pública (BEVILACQUA *et al.*, 2001).

Na Região das Américas, foram registrados casos de leishmaniose do sul dos Estados Unidos até o norte da Argentina, com exceção das ilhas do Caribe, Chile e Uruguai. Desta forma, esta doença já foi descrita em pelo menos 12 países, sendo que Brasil, Argentina e Paraguai concentram 78% dos casos (LAINSON e RANGEL, 2005; WHO, 2017). No Brasil, casos de LV já foram notificados em 21 estados, atingindo as cinco regiões do país (MAIA-ELKHOURY *et al.*, 2008; SINAN/SVS/MS, 2017), na região Nordeste localizam-se as principais áreas endêmicas, tendo em 2015, 1.806 casos de LV notificados (SINAN/SVS/MS, 2017).

Na Bahia, em 2015 foram registrados 320 casos de LV, sendo considerada como endêmica em 52% dos municípios, Sua ocorrência na Bahia é elevada, estando abaixo apenas de Maranhão, Ceará e Minas Gerais (SINAN/SVS/MS, 2017). A região central do estado da Bahia historicamente concentrava as taxas de prevalência mais elevadas da enfermidade, porem, há uma década, observou-se uma dispersão geográfica para o litoral e o sul do estado, que eram áreas tradicionalmente indenes, indicando mudanças no perfil ecoepidemiológico da doença (OLIVEIRA *et al.*, 2010).

A mudança no perfil epidemiológico com aumento do número de casos em áreas periurbanas e urbanas (BEVILACQUA *et al.*, 2001) é um fenômeno que pode ser observado em diferentes capitais brasileiras, assim como na Região Metropolitana de Salvador, onde na última década tem se registrado um elevado número de casos humanos e caninos de LV (FRANKE *et al.*, 2002; JULIÃO, 2004). Camaçari, uma das áreas de estudo do presente trabalho, é um município localizado na região metropolitana de Salvador, que foi identificado por estudos anteriores como uma área endêmica para LV e leishmaniose visceral canina (LVC) (CUNHA *et al.*, 1995; JULIÃO *et al.*, 2007.).

1.2 AGENTE ETIOLÓGICO E CICLO BIOLÓGICO DA LV

A LV é causada por protozoários da ordem Kinetoplastida, família Trypanosomatidae, gênero *Leishmania* (MAURICIO *et al.*, 1999), que acomete humanos, animais domésticos e silvestres (LAINSON e SHAW, 1978; KUHLS *et al.*, 2011). Nas Américas, a LV é, principalmente, causada por *Leishmania infantum*, contudo, a espécie *Leishmania amazonensis* já foi reportada como responsável por causar casos raros de LV no Brasil (BARRAL *et al.*, 1991; ALEIXO *et al.*, 2006). Estudos de caracterização molecular do DNA do parasito mostraram que *L. infantum* e *Leishmania chagasi* são organismos indistinguíveis geneticamente, podendo uma espécie ser citada como sinônimo da outra (MAURICIO, STOTHARD e MILES, 2000; KUHLS *et al.*, 2011).

L. infantum é um protozoário bifásico, que se apresenta em duas formas distintas durante seu ciclo de vida (ALEXANDER e RUSSELL, 1992). No interior do trato digestório dos vetores flebotomíneos, o parasito encontra-se na forma de

promastigota (KILLICK KENDRICK e RIOUX, 1991). Enquanto que, no hospedeiro vertebrado prevalece a forma intracelular obrigatória, amastigota, encontrada no fagolisossomo de células do sistema fagocítico mononuclear, principalmente, macrófagos (ANTOINE *et al.*, 1990; ALEXANDER, SATOSKAR e RUSSELL, 1999; KAYE e SCOTT, 2011).

Estes protozoários necessitam no seu ciclo biológico de um inseto vetor, sendo transmitidos aos hospedeiros vertebrados pela picada de fêmeas de insetos hematófagos pertencentes à família Psychodidae, chamados flebotomíneos (NEVES, 2006; ROMICH, 2008). No Brasil, a espécie *Lutzomyia longipalpis* é considerada o principal vetor biológico da LV (SHERLOCK, 1996; LAINSON e RANGEL, 2005; MONTEIRO *et al.*, 2005).

O ciclo biológico da LV se inicia quando as fêmeas do inseto vetor alimentam-se de algum hospedeiro vertebrado infectado e tornam-se infectadas (NEVES, 2006; ROMICH, 2008). Na pele do hospedeiro vertebrado, encontram-se células do sistema fagocítico mononuclear albergando formas amastigotas do parasito que são adquiridas durante o repasto sanguíneo pelo inseto vetor (KILLICK-KENDRICK, 1999). No interior do vetor, o sangue infectado ingurgitado passa para o intestino médio, uma vez que *L. infantum* é um parasito cujo desenvolvimento é restrito ao intestino médio do inseto (KAMHAWI, 2006). Posteriormente, os parasitos diferenciam-se em vários estádios de desenvolvimento distintos à medida que migram do intestino médio posterior para a válvula estomodeal, que forma uma junção com o intestino anterior (DESCOTEAUX e TURCO, 1999; KAMHAWI, 2006; BATES, 2007). Após cerca de 6-9 dias, os parasitos completam seu desenvolvimento no tubo digestório dos insetos, transformando-se da forma amastigota em forma promastigota, passando a expressar em sua superfície uma camada de moléculas como lipofosfoglicanos e glicoconjugados, capazes de proteger o parasito da ação das enzimas hidrolíticas presentes no intestino dos flebotomíneos (RUSSELL e WRIGHT, 1988; TALAMAS-ROHANA *et al.*, 1990; ALEXANDER e RUSSELL, 1992). Essas formas infectantes chamadas de metacíclicas encontradas na porção posterior da válvula estomodeal são inoculadas na derme do hospedeiro vertebrado durante o repasto sanguíneo (KILLICK-KENDRICK, 1999; KAMHAWI, 2006).

Durante o repasto sanguíneo, o flebótomo danifica a pele do hospedeiro com sua probóscide, rasgando tecidos, lacerando capilares e criando lagos hemorrágicos

durante sua alimentação. Estes eventos provocam uma resposta imediata do sistema hemostático do hospedeiro, visando evitar a perda de sangue. Contudo, o inseto apresenta componentes farmacologicamente ativos na sua saliva que podem modular o sistema hemostático do hospedeiro (CHARLAB *et al.*, 1999; RIBEIRO e FRANCISCHETTI, 2003; ABDELADHIM, KAMHAWI e VALENZUELA, 2014), como, por exemplo, maxadilan, que apresenta propriedades vasodilatadoras, antiplaquetárias provocando hemorragia e lise tecidual (SAMUELSON *et al.*, 1991; GUILPIN *et al.*, 2002; ANDRADE *et al.*, 2007; DE OLIVEIRA *et al.*, 2009).

Adicionalmente, outras substancias presentes na saliva do vetor, como por exemplo, a proteína amarela LJM11, que age como aglutinante de aminas biogênicas pró-inflamatórias como serotonina, histamina e catecolaminas, são responsáveis por influenciar o desenvolvimento e as funções de leucócitos, como neutrófilos e macrófagos (PRATES *et al.*, 2011; XU *et al.*, 2011; PRATES *et al.*, 2012). Como consequência, estes eventos facilitam a transmissão das formas infectantes de *Leishmania*, uma vez que os vasodilatadores, anticoagulantes e moléculas imuno-moduladoras presentes na saliva promovem a interação inicial do parasito com as células imunes do hospedeiro vertebrado (PRATES *et al.*, 2012). Estas substâncias liberadas pelos flebotomíneos provocam vasodilatação que facilita o contato dos parasitos com as células do hospedeiro, e a quimioatração que atrai os leucócitos para o local do repasto sanguíneo, favorecendo o estabelecimento da infecção no animal picado pelo inseto infectado (ABDELADHIM, KAMHAWI e VALENZUELA, 2014).

Os parasitos inoculados na derme são fagocitados por macrófagos e no interior destas células, as formas promastigotas internalizam seu flagelo, sendo capazes de se adaptarem às condições do vacúolo parasitóforo (KAYE e SCOTT, 2011). No interior desses compartimentos fagolisossomais ácidos e ricos em enzimas hidrolíticas, algumas promastigotas se transformam nas formas amastigotas (NEVES, 2006; ROMICH, 2008), que se multiplicam intensamente por divisão binária simples. O resultante número elevado de parasitos pode levar à lise do macrófago, e uma vez liberadas, as amastigotas poderão ser novamente fagocitadas por outras células do sistema retículo endotelial, podendo se disseminar para outros órgão do sistema fagocítico mononuclear do hospedeiro (NEVES, 2006; REY, 2008).

1.3 PAPEL DO CÃO NO CICLO DA LV

A LV pode acometer humanos, animais silvestres e domésticos (LAINSON e SHAW, 1978), dentre estes, o cão é considerado o principal reservatório doméstico do parasito, além de ser a principal fonte de infecção para o vetor no meio urbano (MOLINA *et al.*, 1994; OLIVEIRA *et al.*, 2001; ALVAR *et al.*, 2004; MARGONARI *et al.*, 2006; MADEIRA *et al.*, 2009; BRASIL, 2014). As evidências que indicam o papel do cão como principal reservatório da LV nas áreas urbanas consistem em: I) presença da LV em 98 países e cerca de 50 desses países possuírem casos de LVC (ALVAR *et al.*, 2012), II) elevado parasitismo na pele de cães infectados que pode funcionar como fonte de infecção para o vetor (MOLINA *et al.*, 1994; MADEIRA *et al.*, 2009); III) ocorrência da enzootia canina, precedendo tanto temporalmente quanto espacialmente a ocorrência da infecção humana (BEVILACQUA *et al.*, 2001; OLIVEIRA *et al.*, 2001; ALVAR *et al.*, 2004; MARGONARI *et al.*, 2006; BRASIL, 2014); IV) observação de elevada prevalência de LVC nas áreas endêmicas para LV, sendo esta superior à prevalência de LV humana (SHERLOCK, 1996; CAMARGO-NEVES *et al.*, 2001; SILVA *et al.*, 2001).

Ademais, apesar de uma parte da população canina ser altamente susceptível à infecção por *L. infantum* e manifestar sinais característicos e clássicos da doença quando infectados (CIARAMELLA *et al.*, 1997; KOUTINAS *et al.*, 1999), a presença de cães sem manifestação clínica da doença que não são facilmente diagnosticados, mas que albergam o parasito na derme, também é frequente em áreas endêmicas, podendo estes animais desempenhar um papel na introdução, dispersão e manutenção da doença em áreas urbanas (MOLINA *et al.*, 1994; GUARGA *et al.*, 2000).

Diante desses fatos, uma das principais medidas de controle da LV no Brasil preconizada pelo Ministério da Saúde (MS) consiste em identificar e eutanasiar os animais sorologicamente positivos (BRASIL, 2014).

1.4 LEISHMANIOSE VISCERAL CANINA

Após a transmissão do parasito pelo inseto vetor, o surgimento dos primeiros sinais clínicos em cães pode ocorrer após semanas ou até alguns anos. Além disso,

uma parcela dos animais infectados pode nunca manifestar sinais característicos de LVC, se mantendo num estado subclínico (CIARAMELLA *et al.*, 1997; KOUTINAS *et al.*, 1999; ALVAR *et al.*, 2004). Até o presente momento, não há uma definição clara de parâmetros que possam distinguir esses animais subclínicos daqueles prepatentes, ou seja aqueles que irão manifestar sinais clínicos decorrentes da infecção por *L. Infantum*, assim como não há uma distinção entre estes cães e aqueles infectados que nunca apresentarão manifestação clínica da doença, os resistentes.

Adicionalmente, não existem evidências claras a respeito de quais desses animais (os que desenvolvem a doença e os que não desenvolvem) são importantes no ciclo de transmissão da doença, mas alguns autores consideram os cães que desenvolvem quadros de doença grave como susceptíveis, enquanto que animais subclínicos são considerados como resistentes à instalação da doença (PINELLI *et al.*, 1994; PINELLI *et al.*, 1995; PINELLI *et al.*, 1999; CORREA *et al.*, 2007; LAGE *et al.*, 2007; REIS *et al.*, 2009; MENEZES-SOUZA *et al.*, 2011).

Após a infecção, a evolução para a doença nos animais depende de diversos fatores como: a virulência do parasito (capacidade de inativar ou resistir ao efeito microbicida dos macrófagos ativados) e a suscetibilidade genética do hospedeiro, dentre outros, que são responsáveis pelo desencadeamento dos mecanismos imunológicos em resposta à infecção (ALVAR *et al.*, 2004; LIPOLDOVA e DEMANT, 2006; BANETH *et al.*, 2008) (GRIMALDI e TESH, 1993).

Uma vez que as formas promastigotas são inoculadas na derme do cão, a infecção pode ser: limitada, sendo controlada localmente pelos macrófagos e outras células de defesa; não disseminada, quando os parasitos conseguem instalar-se apenas na pele e nos linfonodos e no baço, caracterizando um quadro de infecção subclínica; ou ainda disseminada, em caso de falha na resposta imune do hospedeiro, onde os parasitos espalham-se por todo o organismo podendo levar ao aparecimento da doença clínica (BANETH *et al.*, 2008; SARIDOMICHELAKIS, 2009; SOLANO-GALLEGO *et al.*, 2011). Após a picada do inseto vetor, *L. infantum* se distribui rapidamente nos linfonodos e baço, sucessivamente alcançando os rins e o fígado (MOREIRA *et al.*, 2007). Finalmente o parasito se dissemina para os órgãos reprodutivos, pele, bexiga, trato digestório e respiratório do hospedeiro (MOLYNEUX e ASHFORD, 1983). Nos animais susceptíveis a distribuição sistêmica dos parasitos

é extensa, enquanto que nos resistentes ela não é tão disseminada (SARIDOMICHELAKIS, 2009).

Diversos estudos apontam que a população canina em áreas endêmicas é composta por três grupos de animais infectados, sendo estes: i) cães em fase de latência, que não apresentam sinais clínicos e que podem parecer cães resistentes, porém irão desenvolver a doença com o passar do tempo; ii) cães resistentes, que são os animais sem sinais clínicos e que nunca desenvolverão LVC; e iii) cães sintomáticos, que desenvolvem a doença após a infecção ou emergem da população de animais com infecção latente. (DYE, 1996; BURATTINI *et al.*, 1998; NUNES *et al.*, 2008; GRIMALDI *et al.*, 2012b).

Desta forma, a resistência ou a susceptibilidade dos cães à infecção por *L. infantum* não pode ser definida apenas por presença ou ausência de sinais clínicos, sendo indicada a análise conjunta destes com a carga parasitária e titulação de anticorpos anti-*Leishmania* e características relacionadas a diferentes parâmetros da resposta imune humoral ou celular.

1.4.1 Manifestações clínicas

Em cães, a LV pode manifestar-se sob diferentes formas podendo produzir diversas manifestações clínicas, enquanto que no homem o quadro clínico é mais restrito (ALVAR *et al.*, 2004). Os cães podem apresentar sinais que não são específicos, variando desde manifestações cutâneas até manifestações viscerais e sistêmicas (FOGLIA MANZILLO *et al.*, 2013).

Os sinais clínicos iniciais nos cães compreendem apatia, perda de peso, linfadenomegalia, dermatite periocular e nasal, além de alopecia localizada, onicogrifose e edema dos coxins plantares (NICOLE e COMTE, 1908; FERRER, 1999; MENDONCA *et al.*, 1999; GREENE, 2006). Febre, diarreia, epistaxe, hemorragia intestinal, inapetência, hepatoesplenomegalia, hiperqueratose, ulcerações cutâneas e ceratoconjuntivite também são achados clínicos nos animais acometidos (CIARAMELLA *et al.*, 1997; KOUTINAS *et al.*, 1999). Quadros severos se caracterizam por sinais típicos como ulcerações cutâneas disseminadas pelo corpo, cegueira, anorexia, onicogrifose, dermatite, alopecia generalizada, levando o animal à morte (REIS *et al.*, 2009).

Além de manifestar sinais clínicos, cães infectados por *L. infantum* podem apresentar alteração de parâmetros hematológicos e bioquímicos (CIARAMELLA *et al.*, 1997; KOUTINAS *et al.*, 1999), que podem servir como marcadores de gravidade da doença, sendo úteis para avaliação do seu prognóstico (REIS *et al.*, 2006a; DA COSTA-VAL *et al.*, 2007; GEISWEID *et al.*, 2012).

O hemograma fornece subsídios importantes quanto à resposta do cão infectado, pois já foi demostrado que anemia, hiperproteinemia e monocitose estão relacionadas com quadro de LVC disseminada, também chamada de LVC ativa (FEITOSA *et al.*, 2003). Um estudo com animais tratados demonstrou que outros parâmetros hematológicos como a hipoalbuminemia e linfopenia também podem ser úteis para avaliar o prognóstico e prever a sobrevida dos animais (GEISWEID *et al.*, 2012). A gravidade do quadro clínico da LVC também pode ser avaliada pela medida de alanina aminotransferase (ALT), aspartato aminotransferase (AST), fosfatase alcalina (FA) e gamaglutamiltransferase (GGT), enzimas que quando alteradas se relacionam com a gravidade de lesões hepáticas (BENDERITTER *et al.*, 1988; IKEDA-GARCIA *et al.*, 2007), tendo em vista que em quadros de LVC ativa já foi relatado o aumento sérico das enzimas hepáticas (CIARAMELLA *et al.*, 1997). As dosagens séricas de ureia e creatinina também são importantes na LVC, pois são marcadores da redução da taxa de filtração glomerular, podendo seu aumento indicar início de insuficiência renal no animal, que é a principal causa de óbito do cão com LV (BENDERITTER *et al.*, 1988; LOPEZ *et al.*, 1996).

1.4.2 Diagnóstico

Determinar o diagnóstico clínico de LVC é uma tarefa difícil e complexa, uma vez que a maior parte dos sinais associados a esta doença são variáveis e inespecíficos, podendo estar presentes em outras enfermidades que afetam os cães (GRAMICCIA, 2011). Para tentar aumentar a especificidade do diagnóstico clínico, alguns autores empregam técnicas padronizadas de registro clínico, como o cálculo de escore clínico baseado na intensidade e frequência de determinados sinais característicos de LVC apresentados pelos animais (SILVA *et al.*, 2017). Assim, apesar do diagnóstico da LVC ser muitas vezes presumido pela identificação de manifestações clínicas características, este deve ser confirmado por testes

imunológicos ou pela demonstração do parasito utilizando-se técnicas parasitológicas (GRIMALDI e TESH, 1993).

Testes sorológicos permitem a detecção de imunoglobulinas no soro, podendo ser utilizados para o diagnóstico de indivíduos infectados, assim como em estudos de prevalência (GOMES *et al.*, 2008). A depender do tipo de antígeno empregado, esses testes podem apresentar sensibilidade e especificidade variáveis, sendo sujeitos ainda a reações cruzadas (KAR, 1995; SUNDAR e RAI, 2002; FERREIRA *et al.*, 2007; TRONCARELLI *et al.*, 2009; SILVA *et al.*, 2011). As técnicas sorológicas, como o ensaio imunoenzimático (ELISA) e kits imunogromatográficos são os métodos mais empregados para o diagnóstico da LVC nos soroinquéritos promovidos pela saúde pública (ALVES e BEVILACQUA, 2004). Até o ano de 2011, segundo instrução do MS, os inquéritos caninos para triagem e identificação dos animais soropositivos para LVC eram realizados utilizando a técnica de ELISA (EIE-LVC), seguida pela reação de imunofluorescência indireta (RIFI) para confirmação da infecção (BRASIL, 2014). No entanto, a partir de 2011, uma nova instrução normativa foi emitida pelo MS, indicando o uso do teste rápido *dual path platform* (DPP-LVC) para triagem dos animais, e o EIE-LVC como exame confirmatório, eliminando a RIFI da rotina diagnóstica (BRASIL, 2011). A implantação do teste rápido DPP-LVC foi realizada gradativamente em todo o território nacional, abrangendo todos os estados brasileiros até o final de 2012 (BRASIL, 2011). Um estudo de Fraga *et al*., (2016) comparando os diferentes protocolos do MS mostrou que a adoção do novo protocolo (DPP LVC + EIE LVC) melhorou a especificidade no diagnóstico dos animais. Fato bastante relevante e que justifica a troca do método diagnóstico de triagem, uma vez que a eutanásia é a medida empregada para controle da população de reservatórios caninos sendo imprescindível um diagnóstico confirmatório específico do animal suspeito, para que animais sem LVC ou aqueles com outras patologias não sejam eutanasiados erroneamente.

Os métodos parasitológicos como o exame direto de lâminas e o isolamento por cultura de tecidos, permitem a detecção do parasito, e podem ser empregados como métodos diagnósticos confirmatórios para LVC (BARROUIN-MELO *et al.*, 2004). A observação de formas amastigotas ou promastigotas em material obtido de punção de medula óssea, baço ou de outros tecidos consiste no diagnóstico laboratorial definitivo da LV em humanos e caninos (BARROUIN-MELO *et al.*, 2006; REIS *et al.*, 2006a). Assim, as técnicas que permitem essa confirmação são

definidas como padrão-ouro (do inglês *Gold standard*) (DOURADO *et al.*, 2007; GOMES *et al.*, 2008). Seu uso é limitado para avaliação de cães em área endêmica, pois apesar da especificidade destes métodos parasitológicos ser muito elevada, a sensibilidade destas técnicas é baixa, principalmente na detecção de cães pouco parasitados, recém infectados ou subclínicos (PINELLI *et al.*, 1994; SOLANO-GALLEGO *et al.*, 2001).

De tal modo, o resultado de testes sorológicos e parasitológicos deve ser sempre avaliado com cuidado, procurando sempre associar este ao histórico epidemiológico e ao quadro clínico apresentado pelo animal e, quando possível, ao resultado de outro teste diagnóstico mais específico (DA SILVA *et al.*, 2006).

Nas últimas décadas, as técnicas moleculares como a reação em cadeia da polimerase (PCR), foram introduzidas para o diagnóstico da LVC, devido a sua elevada sensibilidade e especificidade (MIRÓ *et al.*, 2008). Estas técnicas detectam o material genético do parasito, podendo ser empregadas como métodos confirmatórios em caso de animais recém infectados ou assintomáticos que, na maioria dos casos, não apresentam soroconversão e apresentam baixa carga parasitária, o que dificulta seu diagnóstico utilizando as técnicas sorológicas e parasitológicas, além de permitir a determinação da carga parasitária (SOLANO-GALLEGO *et al.*, 2001; ALVES e BEVILACQUA, 2004).

1.3.2.1 Diagnóstico Molecular

Técnicas biomoleculares como a reação em cadeia da polimerase convencional (cPCR) e a PCR em tempo real (qPCR), são utilizadas para o diagnóstico da LVC, pois proporcionam elevada sensibilidade e especificidade, superando resultados obtidos pelas técnicas sorológicas e parasitológicas na detecção de animais infectados por *Leishmania* sp. (REALE *et al.*, 1999; SOLANO-GALLEGO *et al.*, 2001; MIRÓ *et al.*, 2008; SOLCÀ *et al.*, 2012).

O método da PCR consiste inicialmente na detecção de uma região específica do DNA do parasito, denominada alvo, por oligonucleotídeos iniciadores (*primers*) que, em condições ideais de temperatura e pH, hibridizam-se especificamente na região escolhida, levando à amplificação exponencial deste fragmento (SUNDAR e RAI, 2002; PAIVA-CAVALCANTI, REGIS-DA-SILVA e GOMES, 2010). A cPCR é uma técnica qualitativa, que apresenta a necessidade de eletroforese em gel de agarose ou poliacrilamida, para visualização do fragmento

amplificado, além de não permitir a quantificação da carga parasitária do animal, que consistem em desvantagens do método diagnóstico (REITHINGER e DUJARDIN, 2007).

A qPCR por sua vez, é uma técnica quantitativa, com capacidade de monitorar e quantificar a amplificação do fragmento de DNA de interesse, utilizando corantes ou sondas fluorescentes, que são consumidas durante a amplificação do produto da qPCR, emitindo uma fluorescência específica ao passo que a reação progride, logo não há a necessidade de visualização de seu produto em gel (PAIVA-CAVALCANTI, REGIS-DA-SILVA e GOMES, 2010). As duas sondas utilizadas são: o SYBR Green (Applied Biosystems, CA, USA), um corante amplamente utilizado porém que tem como desvantagem sua ligação não específica nas sequências de DNA de fita dupla, produzidas durante a amplificação (REITHINGER e DUJARDIN, 2007); e o sistema TaqMan (Applied Biosystems, CA, USA), que consiste em uma sonda que se liga especificamente a sequencia alvo, aumentando a especificidade da qPCR (REITHINGER e DUJARDIN, 2007).

A detecção de DNA de *Leishmania* sp. pelas técnicas de PCR pode ser realizada em uma ampla variedade de amostras clínicas (FISA *et al.*, 2001; MANNA *et al.*, 2004; FRANCINO *et al.*, 2006; MAIA *et al.*, 2009; GALLETTI *et al.*, 2011). Manna *et a*l. (2004), avaliaram através da técnica da cPCR diferentes tecidos de cães naturalmente infectados, obtendo elevada especificidade e sensibilidade: em 99% das amostras de linfonodos, 95% das biópsias de pele e 94% das amostras de sangue.

Os tecidos linfoides estão incluídos dentre os órgãos onde é mais frequente a detecção de *Leishmania* sp. (MARZOCHI *et al.*, 1985), dentre estes, a medula óssea é considerada o maior órgão hematopoiético e um dos principais tecidos linfoides, sendo considerada um local importante de armazenamento do parasito em cães infectados (TROPIA DE ABREU *et al.*, 2011), assim como os linfonodos. Um inquérito realizado por Maia *et al*. (2009) em cachorros com diferentes quadros clínicos, demonstrou que o uso de aspirado de linfonodos pela técnica de cPCR é ideal para o diagnóstico precoce da LVC em cachorros sintomáticos. Todavia, em caso de ausência de linfadenomegalia, é indicado o emprego de aspirado de medula óssea, pois esta apresenta uma maior carga parasitária em relação aos linfonodos (QUARESMA *et al.*, 2009).

O baço é outro tecido linfático muito relevante para o diagnóstico e

patogênese da LVC (SOLCÀ *et al.*, 2012). Reis *et al.* (2006) em um estudo citológico, demonstraram que o baço é um dos principais órgãos onde a densidade parasitária encontra-se elevada durante todo o curso da LVC, tanto em cães assintomáticos como sintomáticos. Saldarriaga *et al.* (2006) demonstraram em um estudo de infecção experimental intradérmica com promastigotas de *Leishmania* sp. em cães, que após 96 horas do inóculo, foi possível identificar o parasito nos linfonodos e baço dos animais. Outro estudo revelou graus variáveis de esplenomegalia na maioria dos cães naturalmente infectados por *L. infantum* sp. (BARROUIN-MELO *et al.*, 2006). Estes achados fazem do baço um tecido importante e interessante para o diagnóstico da LVC (MAIA *et al.*, 2009; CAVALCANTI *et al.*, 2015). Assim como a coleta das amostras de medula óssea e linfonodo (CARVALHO *et al.*, 2009), a de baço é considerada um procedimento invasivo, sobretudo pelo risco de causar lesões internas e, em consequência, hemorragias (LÉVEILLÉ *et al.*, 1993). No entanto, existem descrições de que a coleta de amostras esplênicas é bem tolerada pelos animais (LÉVEILLÉ *et al.*, 1993). Barrouin-Melo *et al*. (2006) descreveram que em um estudo com realização de mais de 257 punções esplênicas, somente em dois animais foi descrito o pinçamento de alça intestinal e hemorragia no local da punção. Estes riscos podem ser minimizados utilizando-se um aparelho de ultrassom que permite a visualização do órgão, favorecendo a realização de uma coleta de material esplênico guiada e segura (WATSON *et al.*, 2011).

Existe uma recomendação para utilização de amostras de sangue e esfregaços conjuntivais no diagnóstico da LVC, por estas serem obtidas de forma menos invasiva, mais fácil e mais rápida, além de terem baixo custo e por sua coleta ser bem aceita pelos proprietários dos animais (AOUN *et al.*, 2009; MAIA *et al.*, 2009; DE ALMEIDA FERREIRA *et al.*, 2012; LOMBARDO *et al.*, 2012). Apesar destas vantagens, alguns estudos evidenciaram que a detecção do parasito em sangue periférico é menos sensível, quando comparado com amostras de outros tecidos como baço, medula óssea, pele e linfonodos (REALE *et al.*, 1999; FISA *et al.*, 2001). No entanto, Francino *et al*. (2006), demonstraram que devido à elevada capacidade da qPCR em identificar DNA do parasito, mesmo que o sangue apresente cargas parasitárias mais baixas, este órgão pode vir a ser empregado para o diagnóstico de infecção por *Leishmania* sp.

Amostras de coleta menos invasiva, como esfregaços conjuntivais podem ser

interessantes para serem empregadas em inquéritos epidemiológicos, uma vez que quase não apresenta risco para os animais, além de não necessitar muito treinamento na realização da técnica de coleta, exemplo dessas amostras podem ser esfregaços conjuntivais (STRAUSS-AYALI *et al.*, 2004; LEITE *et al.*, 2010; DE ALMEIDA FERREIRA *et al.*, 2012; LOMBARDO *et al.*, 2012). Utilizando-se esfregaços conjuntivais, Strauss-Ayali *et al.* (2004) conseguiram detectar pela técnica da cPCR 92% dos animais sintomáticos avaliados no estudo. Lombardo *et al*. (2012) pela técnica de qPCR em esfregaços conjuntivais, obtiveram resultados similares àqueles obtidos com amostras mais invasivas como aspirados de linfonodo. Leite *et al.* (2010) também conseguiram detectar o DNA do parasito a partir de esfregaços conjuntivais de animais assintomáticos pela técnica da cPCR seguida por hibridização. Dentre outras amostras menos invasivas investigadas, outros tecidos vem sendo avaliados como amostras de urina investigadas por Solano-Gallego *et al.* (2007) utilizando a técnica de qPCR, quando os resultados descritos mostraram positividade somente em cães com lesão renal severa; e Naranjo *et al.* (2011) que demonstraram a presença do DNA de *Leishmania* sp. pela qPCR em glândulas lacrimais de cães infectados, obtendo correlação entre positividade e presença de lesão ocular.

Atualmente, o custo da qPCR, quanto aos reagentes e equipamentos utilizados ainda constitui um entrave para seu uso rotineiro. No entanto, esse método apresenta vantagens objetivas sobre a cPCR (MOREIRA *et al.*, 2007; MAIA *et al.*, 2009). Particularmente devido à possibilidade de quantificação do DNA alvo, permitindo monitorar a carga parasitária do animal no decorrer de infecção experimental, ou após tratamento, em países onde este é permitido (PENNISI *et al.*, 2005; MAIA e CAMPINO, 2008; MANNA *et al.*, 2008a; MARTINEZ *et al.*, 2011). Também na qPCR há redução da probabilidade de contaminação e rapidez da reação, uma vez que não é realizada eletroforese para visualização dos resultados (ROLÃO *et al.*, 2004; PAIVA-CAVALCANTI, REGIS-DA-SILVA e GOMES, 2010).

A sensibilidade das técnicas de PCR para detecção de *Leishmania* sp. depende de vários fatores: as condições físico-químicas da reação, a concentração e a natureza do DNA da amostra e os iniciadores e sondas empregados para detecção da região alvo (BASTIEN, PROCOP e REISCHL, 2008). As regiões alvo mais frequentemente empregadas para detecção do DNA de *Leishmania* sp. tendem a ser altamente conservadas e repetitivas como o gene da subunidade do RNA

ribossomal (rRNA) ou o DNA do minicírculo do cinetoplasto (kDNA) (SUNDAR e RAI, 2002; ANTINORI *et al.*, 2007; BASTIEN, PROCOP e REISCHL, 2008; MIRÓ *et al.*, 2008).

Nos últimos anos, diversos métodos de qPCR foram desenvolvidos para a detecção do parasito e o diagnóstico da LVC. No entanto, as metodologias empregadas variaram consideravelmente entre si, comprometendo a validade dos resultados obtidos, principalmente em relação à sensibilidade da técnica. Essas variações compreendem o emprego de diferentes alvos na reação, o uso de diferentes amostras biológicas, diferentes metodologias para o cálculo da carga parasitária dos tecidos de animais infectados (BASTIEN, PROCOP e REISCHL, 2008), além de muitas vezes empregar apenas animais sintomáticos para a análise do desempenho da técnica, superestimando assim sua performance.

Adicionalmente, para evitar resultados falso-negativos é imprescindível a avaliação da qualidade do DNA extraído e usado nas reações de qPCR. A abordagem principal para resolver esta questão consiste em realizar uma PCR com o material empregado para detecção do DNA do parasito, para confirmar a integridade dos genes constitutivos no genoma do hospedeiro, assim como garantir a ausência de inibidores de amplificação. O melhor formato para tal análise é a realização da amplificação do DNA da *Leishmania* sp. e do gene constitutivo dentro da mesma reação (isto é, num formato multiplex). Vários genes domésticos, tais como GAPDH, RNase P e β-actina, já foram utilizados como controles internos para garantir a integridade do molde de DNA e prevenção de resultados falso-negativos melhorando assim a qualidade do resultado (ESPY *et al.*, 2006; PIRON *et al.*, 2007; PELEG *et al.*, 2010; GONCALVES-DE-ALBUQUERQUE SDA *et al.*, 2014).

1.4.3 Resposta imune

Conforme comentado anteriormente, após a infecção, a evolução para a doença nos animais depende de alguns fatores, sendo, os mecanismos imunológicos desencadeados pelo hospedeiro após a infecção, um dos mais importantes (ALVAR *et al.*, 2004; LIPOLDOVA e DEMANT, 2006; BANETH *et al.*, 2008). O curso da infecção por *L. infantum* em cães está ligado à resposta imune do hospedeiro e à persistência e multiplicação do parasito. Os componentes da

imunidade inata e adaptativa envolvem uma gama de interações que é notavelmente diversificada e complexa (REIS *et al.*, 2010).

A resposta imune inata tem um papel relevante na proteção contra o parasito além de ter a capacidade de direcionar a resposta adaptativa, como mostrado por análise da caracterização das células mononucleares do sangue periférico por citometria de fluxo em animais experimentalmente infectados, capazes de controlar a infecção por *Leishmania* sp. sem desenvolver uma imunidade adaptativa específica (GUARGA *et al.*, 2002; MORENO e ALVAR, 2002). Dados sugerem, que da mesma forma que ocorre na infecção humana, cães resistentes desenvolvem e controlam a LV por meio de mecanismos dependentes da resposta imune celular efetora, como foi explorado em diferentes estudos *in vitro* e *in vivo* (PINELLI *et al.*, 1994; VOULDOUKIS *et al.*, 1996; ALVAR *et al.*, 2004); enquanto que, cães susceptíveis apresentam uma resposta imune humoral exacerbada, que é ineficaz no controle da infecção (REIS *et al.*, 2006a). Essa ausência de resposta adequada das células T para controlar o parasito leva ao aparecimento de sinais clínicos, níveis elevados de anticorpos específicos anti-*Leishmania* e alta carga parasitária na pele, medula óssea, baço, fígado e linfonodos (REIS *et al.*, 2006a; GIUNCHETTI *et al.*, 2008; REIS *et al.*, 2010).

Estudos das subclasses de linfócitos presentes no sangue periférico de cães infectados mostraram uma significante redução de populações de células T CD4+ *Leishmania*-específicas e, consequentemente, da imunidade mediada por células nestes animais quando comparados com cães não infectados (MORENO *et al.*, 1999). Além disso, a infectividade a flebótomos por xenodiagnóstico direto aumenta ao passo que ocorre uma redução dos níveis de células T CD4+, confirmando, deste modo, a importância destas células também para diminuição de parasitos circulantes na pele e, como consequência, na transmissibilidade da doença (GUARGA *et al.*, 2000). Por outro lado, as células T CD8+, desempenham um importante papel no desenvolvimento de uma resposta imune efetiva contra *Leishmania* sp., possivelmente, devido a mecanismos citotóxicos durante a LVC. Observa-se que, números elevados de células T CD8+ no sangue periférico de cães assintomáticos estão associados a um baixo parasitismo na medula óssea desses animais (REIS *et al.*, 2006a).

Cães resistentes ao desenvolvimento da forma ativa de LVC, geralmente desenvolvem uma resposta imune celular do tipo helper 1 (TH1), com predominância

de produção de citocinas como IL-12, IFN-γ, IL-2 e fator de necrose tumoral (TNF-α) (PINELLI *et al.*, 1994; PINELLI *et al.*, 1995; PINELLI *et al.*, 1999; CORREA *et al.*, 2007; LAGE *et al.*, 2007; REIS *et al.*, 2009; MENEZES-SOUZA *et al.*, 2011). Estas citocinas aumentam a eficiência das células fagocíticas e linfócitos citotóxicos, ativando uma resposta imune protetora, que leva a uma maior capacidade de eliminação do parasito (MANNA *et al.*, 2006; CAVALCANTI *et al.*, 2015).

Já foi demonstrado que na transição do estado subclínico para aquele em que ocorre aparecimento de sinais, observa-se supressão da resposta proliferativa de linfócitos (MORENO *et al.*, 1999; RHALEM *et al.*, 1999). Desta forma, na ausência de resposta imune celular, pode ocorrer progressão para o desenvolvimento da doença (RHALEM *et al.*, 1999; LEANDRO *et al.*, 2001). Cães susceptíveis tendem a desenvolver uma resposta imune celular do tipo helper 2 (TH2), com predominância de citocinas como IL-4, IL-5, IL-10, IL-13 e TGF-β (PINELLI *et al.*, 1994; PINELLI *et al.*, 1995; PINELLI *et al.*, 1999; CORREA *et al.*, 2007; LAGE *et al.*, 2007; REIS *et al.*, 2009; MENEZES-SOUZA *et al.*, 2011). Além disso, em animais infectados, o aumento do nível de anticorpos da classe IgG é observado e correlaciona-se com a presença de sintomatologia (KEENAN *et al.*, 1984; RAFATI *et al.*, 2005; REIS *et al.*, 2006b). Há controvérsias na literatura relacionadas à classe de anticorpos associada à suscetibilidade e resistência à doença. Alguns relatos apontam IgG1 como associada à suscetibilidade, enquanto outros observam um aumento de IgG2, no decorrer da infecção, independente do quadro clínico do animal (RAFATI *et al.*, 2005; TRAVI *et al.*, 2009). Até a presente data, existem poucos dados que correlacionem o desenvolvimento da infecção por *L. infantum* ou sua gravidade com a presença de quimiocinas específicas como CXCL1, CCL2, CXL10 em cães (MENEZES-SOUZA *et al.*, 2011; MENEZES-SOUZA *et al.*, 2012) (NASCIMENTO *et al.*, 2013), desta forma são necessárias análises mais amplas, de múltiplos marcadores, visando identificar um perfil de resposta e correlaciona-lo com resistência ou susceptibilidade à infecção por *L. infantum*.

1.4.4 Resposta a saliva do vetor

Fatores associados ao vetor podem ter reflexo sobre o curso da infecção nos hospedeiros, dentre estes, a exposição contínua à saliva do inseto pelas repetidas picadas pode influenciar o tipo de resposta do sistema imune (COURTENAY *et al.*,

2002; MORENO e ALVAR, 2002). Os aspectos envolvidos na resposta imune resultando em resistência ou suscetibilidade do hospedeiro dependem amplamente da primeira tentativa de resposta inata do hospedeiro para conter a infecção, que pode influenciar na predominância do tipo de padrão de resposta imune contra *Leishmania* sp. (PRATES *et al.*, 2012).

A constante exposição às picadas do vetor, resultando na produção de anticorpos anti-saliva foi demonstrada em diversas espécies de hospedeiro vertebrado (GOMES *et al.*, 2002; COLLIN *et al.*, 2009; AQUINO *et al.*, 2010). Além disso, a exposição de hospedeiros mamíferos às proteínas salivares do flebotomíneos pode induzir imunidade celular (ANDRADE *et al.*, 2007; VINHAS *et al.*, 2007). Proteínas da saliva do vetor submetidas à sonicação e co-inoculadas em camundongos juntamente com promastigotas metacíclicas do parasito conferiram diminuição da gravidade do quadro clínico de LV (BELKAID *et al.*, 1998). Outros estudos demonstraram o caráter imunogênico da saliva do vetor com estímulo da resposta Th1 e reação de hipersensibilidade tardia em ratos (GOMES *et al.*, 2012) e camundongos (KAMHAWI, 2000).

A exposição a proteínas da saliva do flebotomíneo *Lu. longipalpis* demonstrou um caráter imunogênico e conferiu proteção contra o desenvolvimento de quadro clínico da LV também em humanos. Este achado foi descrito em indivíduos residentes em área endêmica para LV, onde foi demonstrada uma correlação entre os níveis de anticorpos anti-saliva e o desenvolvimento de uma resposta de hipersensibilidade tardia contra o parasito (DTH+), sugerindo um perfil de resistência à infecção (GOMES *et al.*, 2002).

Estudos recentes identificaram duas proteínas presentes na saliva do *Lu. longipalpis*, LJM11 e LJM17, como marcadores específicos à exposição à saliva de *Lu. longipalpis* (SOUZA *et al.*, 2010; TEIXEIRA *et al.*, 2010). Em estudos experimentais com cães, demonstrou-se que as proteínas LJM11 e LJM17 da saliva do vetor também conferem um maior estímulo para resposta imune nestes animais (SOUZA *et al.*, 2010; TEIXEIRA *et al.*, 2010). Até a presente data, não existem dados que correlacionem o desenvolvimento da resposta imune anti-saliva do vetor com os achados clínicos e a gravidade da doença em cães.

1.4.5 Biomarcadores de gravidade

Um biomarcador é uma característica biológica objetivamente medida e avaliada como um indicador de processos biológicos ou patológicos normais, ou uma resposta a uma intervenção terapêutica (BIOMARKERS DEFINITIONS WORKING, 2001). Os biomarcadores podem ser usados para vários propósitos, dependendo da finalidade, do estudo e classificados como: de exposição, de efeito e de suscetibilidade, sendo ainda definidos como instrumentos que possibilitam identificar uma condição adversa antes que sejam evidenciados danos à saúde (AMORIM, 2003).

A identificação de biomarcadores sensíveis e confiáveis de gravidade de doenças é importante para melhorar a qualidade do atendimento ao paciente, para permitir a estimativa mais correta do prognóstico e direcionar e otimizar medidas de tratamento e controle (ANDRADE *et al.*, 2010). Na LVC existe a necessidade da avaliação de um conjunto de marcadores biológicos em estudos integrados para melhorar a compreensão do papel desses marcadores na classificação e predição do desfecho clínico dos cães.

Embora não seja imperativo, um bom marcador deve ter uma estreita relação causal com a patogênese da doença (ANDRADE *et al.*, 2010). O estado de resistência ou susceptibilidade à LVC reflete na gravidade da doença do animal, e não pode ser definido por qualquer parâmetro isolado da resposta imune celular ou humoral, sendo necessária uma análise conjunta. Evidências mostram que padrões de suscetibilidade e resistência (tipo de resposta imune apresentada) à infecção por *L. infantum* podem estar correlacionados ao quadro clínico e a carga parasitária do animal (CAVALCANTI *et al.*, 2015). Adicionalmente, em cães naturalmente infectados com a presença de infecção ativa, definida pelo isolamento de parasitos por aspirado esplênico, existe associação entre altos níveis de anticorpos séricos contra o parasito e maior frequência de emagrecimento (DOS-SANTOS *et al.*, 2008). Esta associação entre parasitismo esplênico e apresentações graves da LVC é intensificada pela ausência de resposta no teste cutâneo a antígenos de *Leishmania* sp. (TCL ou DTH) e pela desestruturação do tecido linfoide esplênico (SANTANA *et al.*, 2008). Adicionalmente, existem evidências de correlação positiva entre sintomatologia manifestada por cães naturalmente infectados, níveis de citocinas produzidas e carga parasitária (MANNA *et al.*, 2006). Não foi encontrada diferença

na produção de citocinas Th1 (como IFN-γ, IL-12 e TNF) no baço de cães com ou sem sinais clínicos (CORREA *et al.*, 2007; LAGE *et al.*, 2007), apesar de haver aumento dos níveis dessas citocinas durante o curso da infecção (LAGE *et al.*, 2007). A predominância da produção de citocinas Th2 reguladoras (como IL-4, IL-10 e TGF-β1) determina o aumento da carga parasitária e a persistência do parasito, contudo sem associação com grupos clínicos de doença (leve, moderada ou grave) (LAGE *et al.*, 2007; STRAUSS-AYALI, BANETH e JAFFE, 2007). No entanto, Correa *et al*. (2007) correlacionaram o aumento de citocinas Th2 reguladoras com a progressão da doença. Cavalcanti *et al*., (2015) descreveram uma diminuição na expressão de citocinas pro inflamatórias como IFN-γ, IL-12, TNF e IL-6 em cães infectados com carga parasitária elevada associada a uma modificação estrutural na micro-arquitetura do tecido linfoide esplênico.

Mais recentemente, tem sido descrita a importância dos eicosanoides na regulação da resposta imune do hospedeiro na infecção por *Leishmania* sp. (DAUGSCHIES e JOACHIM, 2000; FRANCA-COSTA et al., 2015). Porém, existem poucos estudos em cães que avaliaram o comportamento desses marcadores inflamatórios no desenvolvimento e curso da LVC (VENTURIN *et al.*, 2016). Até então, esse comportamento vem sendo analisado em seres humanos em resposta à infecção por *Leishmania* sp. nas formas cutâneas (ARAUJO-SANTOS *et al.*, 2014; KHOURI *et al.*, 2014).

A avaliação da exposição à saliva do vetor pela detecção de anticorpos anti-proteínas salivares pode ser também empregada como um biomarcador em áreas endêmicas (TEIXEIRA *et al.*, 2010). A detecção de anticorpos anti-saliva, além de determinar o grau de exposição ao vetor, parece também influenciar a resposta imune contra o parasito no hospedeiro (PRATES *et al.*, 2012). O grau de exposição ao vetor em cães residentes em áreas endêmicas pode ser determinado através da detecção e determinação da cinética do desenvolvimento de anticorpos específicos anti-saliva do vetor. Contudo, no cão, a correlação entre o grau de exposição ao vetor e o desenvolvimento de resistência ou formas subclínica ou clínica da LV ainda não foi estudada.

2. JUSTIFICATIVA

Embora as medidas preventivas, como o uso de repelentes, possam vir a reduzir a prevalência de LVC, esta doença provavelmente continuará a causar preocupação a profissionais de saúde pública devido ao seu potencial zoonótico, assim como para os veterinários clínicos, principalmente pela falta de uma abordagem diagnóstica direta e de um tratamento eficaz (NOLI e SARIDOMICHELAKIS, 2014). As graves consequências do diagnóstico tardio ou da terapia ineficaz (nos países onde esta é permitida), implicam na manutenção do ciclo de transmissão do parasita, assim como na morte de animais doentes. A utilização de biomarcadores, para mensuração de processos biológicos e avaliação de sua relação com o desfecho clínico é importante para melhorar a abordagem do tratamento, além de promover a expansão do nosso arsenal terapêutico para diferentes doenças, e o aprofundamento de nossa compreensão quanto à fisiologia e patogênese destas enfermidades (STRIMBU e TAVEL, 2010). Assim, a busca e identificação de marcadores de gravidade pode não apenas ajudar a melhorar a compreensão da imunopatogênese da LV no hospedeiro mamífero, assim como direcionar medidas de diagnóstico, prevenção e controle nos animais.

Citocinas circulantes, assim como outros mediadores inflamatórios podem ser empregados como biomarcadores para um diagnóstico precoce, gerador de prognóstico ou ainda como indicadores de falha terapêutica em doenças parasitárias (ANDRADE e BARRAL-NETTO, 2011). A produção de citocinas e quimiocinas que regulam o balanço entre uma resposta pro e anti-inflamatória apresentada pelos animais pode regular o espectro clínico da infecção por *L. infantum*. O espectro clínico pode ser correlacionado também com marcadores parasitológico como a intensidade da carga parasitária em cães infectados. Assim, a determinação da carga parasitária nos hospedeiros possibilita quantificar a intensidade da infecção, que pode ser relacionada com o grau de infectividade destes animais (BORJA *et al.*, 2016), além de servir como uma importante ferramenta para monitorar a eficácia de tratamento, nos países onde este procedimento é permitido (ROURA *et al.*, 2013).

Nossa hipótese é que existem biomarcadores parasitológicos, imunológicos e inflamatórios capazes de predizer a gravidade da infecção por *L. infantum* em cães. Para explorar essa hipótese, objetivamos realizar uma análise exploratória para

avaliar e associar os achados clínicos, parasitológicos e imunológicos em cães naturalmente infectados para identificar dentre esses achados, biomarcadores preditivos quanto à gravidade da infecção por *L. infantum*.

Para alcançar os objetivos propostos, o presente estudo foi realizado em duas etapas. Na primeira etapa, foi desenvolvida e validada uma ferramenta diagnóstica sensível e específica para quantificação da carga parasitária dos animais infectados (Capítulo 01). Na segunda etapa, foi realizado um estudo exploratório de uma amostra de cães coletada durante um estudo de corte transversal de uma área endêmica para LVC, visando avaliar diferentes possíveis biomarcadores e correlacioná-los com a gravidade da infecção por *L. infantum* apresentada pelos animais (Capítulo 02).

3. HIPÓTESE

- Existem biomarcadores parasitológicos, imunológicos e inflamatórios capazes de predizer a gravidade da infecção por *L. infantum* em cães.

4. OBJETIVOS

4.1 OBJETIVO GERAL

- Avaliar a carga parasitária como biomarcador parasitológico, as proteínas LJM11/LJM17 como biomarcadores de exposição à saliva do vetor, e identificar biomarcadores inflamatórios de gravidade da infecção por *L. infantum* em cães

4.2 OBJETIVOS ESPECÍFICOS

- Padronizar uma ferramenta de PCR quantitativa (qPCR) para detecção e quantificação da carga parasitária de *L. infantum*;
- Correlacionar o biomarcador parasitológico (carga parasitária), determinada pela técnica de qPCR padronizada com o quadro clínico dos animais;
- Correlacionar os biomarcadores de exposição à saliva do vetor e biomarcadores inflamatórios com o quadro clínico dos animais e a carga parasitária em um estudo de corte transversal.

CAPÍTULO 1 – Padronização de uma Ferramenta Diagnóstica de qPCR para avaliação da carga parasitária de *L. infantum*

A análise de carga parasitária dos animais pode ser considerada um biomarcador de gravidade, além de funcionar como um parâmetro para acompanhar o curso da infecção e a resposta a tratamento. Desta forma, nesta primeira etapa, um protocolo de qPCR para quantificar a carga parasitária foi desenvolvido e validado tendo se mostrado uma ferramenta diagnóstica para LVC, mais específica e acurada quando comparada com métodos parasitológicos e sorológicos, contribuindo para o alcance dos objetivos propostos na presente tese.

A maioria dos cães expostos em áreas endêmicas, se torna infectada sem demonstrar sinais clínicos específicos e, frequentemente, com pouca ou nenhuma evidência sorológica, dificultando seu diagnóstico pelos testes sorológicos (CAMPINO *et al.*, 2000; BANETH *et al.*, 2008). Os testes sorológicos utilizados atualmente para identificação da infecção na população canina, podem apresentar baixa sensibilidade e especificidade especialmente em cães assintomáticos, recém-infectados, ou ainda em cães que apresentam um quadro clínico inespecífico (OLIVA *et al.*, 2006; COURA-VITAL *et al.*, 2011). Em área endêmica, é comum também à presença de cães com baixa carga parasitária, cujo diagnóstico resulta em resultados falso-negativos, principalmente quando são empregados testes parasitológicos para o diagnóstico (ALVAR *et al.*, 2004; GOMES *et al.*, 2008). Em conjunto, as limitações dos testes sorológicos e parasitológicos, apontam para a necessidade do emprego de técnicas mais sensíveis e específicas como as moleculares para a confirmação da infecção por *Leishmania* sp. em cães (GRIMALDI e TESH, 1993; REALE *et al.*, 1999; SOLANO-GALLEGO *et al.*, 2001; MIRÓ *et al.*, 2008).

A qPCR possibilita a identificação do DNA alvo do parasito, e sua quantificação, permitindo elaborar um diagnóstico de infecção assim como monitorar a carga parasitaria no animal durante o decorrer da infecção natural ou experimental ou, ainda, após o tratamento, em países onde este é permitido (PENNISI *et al.*, 2005; MAIA e CAMPINO, 2008; MANNA *et al.*, 2008b; MARTINEZ *et al.*, 2011). A detecção de DNA de *Leishmania* sp. pode ser realizada em uma ampla variedade de

amostras clinicas (FISA *et al.*, 2001; MANNA *et al.*, 2004; FRANCINO *et al.*, 2006; MAIA *et al.*, 2009; GALLETTI *et al.*, 2011). As amostras biológicas mais amplamente utilizadas para o diagnóstico molecular da infecção por *Leishmania* sp. são baço, medula óssea, linfonodos e pele (MIRÓ *et al.*, 2008; MAIA *et al.*, 2009; SOLCÀ *et al.*, 2012). Todavia a literatura apresenta resultados divergentes e às vezes conflitantes em relação à sensibilidade na detecção do DNA de *Leishmania* sp. pela técnica de qPCR empregando-se diferentes tecidos.

Desta forma, o primeiro capítulo da tese teve como objetivo padronizar uma técnica de qPCR para detecção e quantificação da infecção por *L. infantum* nos cães. Esse trabalho é uma continuidade do projeto desenvolvido anteriormente, no qual foi padronizada uma reação de qPCR e foi avaliado o desempenho diagnóstico de alguns tecidos (baço, medula óssea, sangue e linfonodo poplíteo) empregando-se a cultura esplênica como padrão ouro (SOLCÀ, 2012). Contudo, os dados de acurácia obtidos foram subestimados uma vez que a técnica parasitológica empregada como padrão ouro, embora altamente específica, carece de sensibilidade, prejudicando a análise de acurácia quando empregada como técnica padrão-ouro para avaliação do desempenho de outros métodos diagnósticos.

Assim, no presente trabalho, a qPCR padronizada teve seu desempenho avaliado empregando a análise de classe latente. Várias técnicas diagnósticas foram empregadas para construção de uma variável latente que foi utilizada como padrão-ouro na avaliação da acurácia da qPCR, comparando-a com outros testes diagnósticos e ao desempenho da qPCR. Adicionalmente, com o intuito de definir qual a melhor amostra para a detecção do DNA do parasito, foram empregados seis diferentes tecidos caninos para tal análise. Posteriormente, após a reação de qPCR ter sido validada, esta foi aprimorada para utilização em estudos epidemiológicos, sendo padronizada em formato *duplex* tanto na forma líquida como no formato em gel (*ready-to-use*), adicionando-se iniciadores específicos para a detecção de um gene constitutivo canino à reação padronizada anteriormente.

1.1. OBJETIVO GERAL

- Padronizar uma ferramenta de qPCR para detecção e quantificação da carga parasitária de *L. infantum.*

1.2. OBJETIVOS ESPECÍFICOS

- Avaliar a acurácia da qPCR padronizada para o diagnóstico da LVC empregando análise de classe latente;
- Definir qual a amostra de tecido canino que fornece resultados mais acurados na detecção do DNA do parasito;
- Adaptar a reação de qPCR padronizada para o formato *duplex* visando à detecção simultânea de um gene constitutivo canino e da carga parasitária de *L. infantum*;
- Tornar o protocolo de qPCR *duplex* padronizado para o uso como um produto *point of care* na rotina diagnóstica de LVC.

1.3. Artigo científico publicado na revista *Plos One*

Manuela da Silva Solcà, Leila Andrade Bastos, Carlos Eduardo Sampaio Guedes, Marcelo Bordoni, Lairton Souza Borja, Daniela Farias Larangeira, Pétala Gardênia da Silva Estrela Tuy, Leila Denise Alves Ferreira Amorim, Eliane Gomes Nascimento, Geraldo Gileno de Sá Oliveira, Washington Luis Conrado dos-Santos, Deborah Bittencourt Mothé Fraga, Patrícia Sampaio Tavares Veras. **Evaluating the Accuracy of Molecular Diagnostic Testing for Canine Visceral Leishmaniasis Using Latent Class Analysis**. 2014.

Este estudo foi realizado para avaliar a acurácia da técnica de qPCR e determinar qual o tipo de tecido fornece a maior taxa de detecção do DNA do parasito. A análise de classe latente foi realizada para avaliar a precisão de diferentes métodos diagnósticos e amostras biológicas avaliadas por qPCR em 51 cães de uma área endêmica. Usando a variável latente como padrão ouro, a qPCR alcançou uma sensibilidade de 95,8% (IC 90,4-100) empregando-se amostras de aspirado esplênico; 79,2% (IC 68-90,3) nos linfonodos; 77,3% (IC 64,5-90,1) na pele e 75% (IC 63,1-86,9) no sangue. A precisão da qPCR utilizando aspirados esplênicos no diagnóstico da LVC foi ainda avaliada utilizando uma amostra aleatória de 800 cães de um estudo de prevalência. A especificidade da qPCR empregando-se os 800 aspirados esplênicos foi de 76,7% (IC 73,7-79,6) e a sensibilidade foi de 95,0% (IC 93,5-96,5). A sensibilidade para a qPCR de aspirado esplênico foi maior do que as obtidas para os outros testes diagnósticos e foi semelhante à observada no estudo inicial com 51 animais. Estes dados reforçam que a qPCR é a técnica mais sensível para o diagnóstico de LVC, e o aspirado esplênico é a amostra biológica mais sensível para detectar a infecção por *L. infantum*. Adicionalmente, foi demonstrado que a análise de classe latente pode ser utilizada para gerar uma variável que funciona como padrão-ouro adequado para avaliação de técnicas diagnósticas de LVC.

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Evaluating the Accuracy of Molecular Diagnostic Testing for Canine Visceral Leishmaniasis Using Latent Class Analysis

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Abstract

Host tissues affected by *Leishmania infantum* have differing degrees of parasitism. Previously, the use of different biological tissues to detect *L. infantum* DNA in dogs has provided variable results. The present study was conducted to evaluate the accuracy of molecular diagnostic testing (qPCR) in dogs from an endemic area for canine visceral leishmaniasis (CVL) by determining which tissue type provided the highest rate of parasite DNA detection. Fifty-one symptomatic dogs were tested for CVL using serological, parasitological and molecular methods. Latent class analysis (LCA) was performed for accuracy evaluation of these methods. qPCR detected parasite DNA in 100% of these animals from at least one of the following tissues: splenic and bone marrow aspirates, lymph node and skin fragments, blood and conjunctival swabs. Using latent variable as gold standard, the qPCR achieved a sensitivity of 95.8% (CI 90.4–100) in splenic aspirate; 79.2% (CI 68–90.3) in lymph nodes; 77.3% (CI 64.5–90.1) in skin; 75% (CI 63.1–86.9) in blood; 50% (CI 30–70) in bone marrow; 37.5% (CI 24.2–50.8) in left-eye; and 29.2% (CI 16.7–41.6) in right-eye conjunctival swabs. The accuracy of qPCR using splenic aspirates was further evaluated in a random larger sample (n = 800), collected from dogs during a prevalence study. The specificity achieved by qPCR was 76.7% (CI 73.7–79.6) for splenic aspirates obtained from the greater sample. The sensitivity accomplished by this technique was 95% (CI 93.5–96.5) that was higher than those obtained for the other diagnostic tests and was similar to that observed in the smaller sampling study. This confirms that the splenic aspirate is the most effective type of tissue for detecting *L. infantum* infection. Additionally, we demonstrated that LCA could be used to generate a suitable gold standard for comparative CVL testing.

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Introduction

Visceral leishmaniasis (VL) is a disease with both medical and veterinary importance that is endemic in Brazil, and in many other countries throughout Latin America, Asia, and Europe [1]. One of the etiological agents of VL is *Leishmania infantum* (syn. *Leishmania chagasi*), which is transmitted to vertebrate hosts through the bites of female sand flies [2–5].

Dogs are considered the main domestic reservoir for this parasite because of their high rates of infection and the high frequency of parasites found in their skin [6–9]. Once infected with *L. infantum*, dogs have clinical manifestations that range from asymptomatic to systemic, including weight loss or cachexia; hypertrophy of the lymph nodes; and changes to the skin such as onychogryphosis, footpad swelling, localized or generalized alopecia, skin ulcers, and nasal or periocular dermatitis. They can also present with pathological alterations such as anemia or hepatic and renal failure [10,11].

Canine visceral leishmaniasis (CVL) can be diagnosed using parasitological, serological, or molecular methods in conjunction with clinical and epidemiological parameters [12]. Serological tests to diagnose CVL are the most common procedures used worldwide [13], however they lack sensitivity and specificity, which makes diagnosing the disease difficult when animals present with low antibody titers or there is cross-reactivity [14–17]. Hence, additional tests could be advantageous for confirming the diagnosis of inconclusive cases. For use as a confirmatory test,

the molecular detection of *Leishmania* spp. provides greater sensitivity and specificity than other diagnostic techniques [8,18].

Numerous studies have described highly sensitive detection of low parasitic loads using quantitative real-time PCR (qPCR) [19–21]. qPCR has also been used to monitor the tissue parasitic load in dogs following anti-*Leishmania* treatment in countries where this procedure is unrestricted [22,23].

Several invasive, and non-invasive, techniques have been used to obtain biological tissue samples to diagnose *Leishmania* infection using conventional PCR and qPCR. The biological samples most widely used for molecular diagnosis of *Leishmania* spp. infection in dogs are the spleen, bone marrow, lymph node, and skin [12,18,24]. However, molecular diagnostic tests in studies using these tissue types have produced variable, and sometimes conflicting results, for identifying *Leishmania*-infected dogs [19,25,26]. This might be because culturing the parasite, which has been used as the gold standard assay [27,28], has a low sensitivity threshold for detecting dogs with a low parasite burden [29,30], which compromises the accuracy evaluation of diagnostic testing.

Therefore, the authors hypothesized that the lack of a reliable gold standard assay could account for the varying accuracy of the molecular diagnostic tests for *Leishmania* infection in different tissues. Latent class analysis (LCA) appraises tests with imperfect reference standards [31–33] using a statistical model to construct the latent class variable. Recently, LCA has been used to accurately evaluate the results of serological tests for diagnosing CVL [34].

The aim of the present study was to determine which type of canine tissue sample in an area with endemic VL provided the highest rate of *Leishmania* DNA detection by qPCR. In addition, qPCR results were compared to parasitological and serological diagnostic tests to determine which test provided the most accurate diagnosis of *L. infantum* infection.

Materials and Methods

1. Ethics Statement

Experimental procedures involving dogs were performed in accordance with Brazilian Federal Law on Animal Experimentation (Law no. 11794), the guidelines for animal research established by the Oswaldo Cruz Foundation [35], and the Brazilian Ministry of Health Manual for the Surveillance and Control of VL [36]. The CPqGM - FIOCRUZ Institutional Review Board for Animal Experimentation approved protocols for both animal euthanasia and sample collection procedures (Permit Number: 015/2009; Permit Number 017/2010).

2. Dogs

As previously described by Lima etal. (2014), over a one week period in July 2010, 51 stray dogs were taken from the streets of Jequié, a municipality located in the State of Bahia, Brazil, which is an area endemic for CVL. These dogs were selected as part of a surveillance and control program for VL that our group conducted in collaboration with the Endemic Diseases Surveillance Program of the State Health Service [37]. A CVL diagnosis was established based on the presence or absence of the following clinical signs: emaciation, alopecia, anemia, conjunctivitis, dehydration, dermatitis, erosion, ulcerations, lymphadenopathy, and onychogryphosis as previously detailed by Lima etal. (2014). Dogs from Jequié were clinically classified as having mild (stage I), moderate (stage II), and severe CVL (stage III) according to Solano-Gallego etal. (2009) [38].

3. Tissue Sampling

Tissue samples were obtained during necropsies as previously described by Lima etal. (2014). Briefly, the dogs were anesthetized and then euthanized by intracardiac injection of a supersaturated solution of potassium chloride (2 mL/kg). Immediately before the lethal injection, 50 mL of blood were collected by intracardiac puncture. Blood samples were preserved in EDTA-2Na tubes (Greiner bio-one, Kremsmünster, Austria) and in blood collection tubes (BD Vacutainer; Becton, Dickinson and Co). During the necropsy, splenic aspirate samples were collected by puncturing the central region of the spleen and bone marrow samples were obtained by puncturing the wing of the ilium, approaching from the dorsal crest. Conjunctival swabs of the right and left eyes were taken by rubbing the swab multiple times against the surface of the lower eyelid. A small fragment of the popliteal lymph node was cut from the whole organ and a skin fragment was collected using a sterile 5 mm punch (Kolplast, Brazil) from the medial portion of the pinna. Tissue samples were collected using sterile needles, swabs, and blades and all of the samples were stored in DNAase- and RNAase-free tubes at $-70^{\circ}C$ until DNA extraction.

4. Hematological and Biochemical Parameters

Hematological and biochemical parameters were evaluated on the day of the necropsy. Total red blood cell and white blood cell counts were determined using an automated cell counter (Pentra 80 counter, ABX Diagnostics, Montpellier, France). Micro-hematocrit tubes containing blood samples were centrifuged at 12,000 rpm for 5 min, and then the hematocrit levels were estimated. Serum was collected by centrifuging the Vacutainer tubes, and was used for the biochemical tests including total protein, globulin, albumin, blood urea nitrogen, and creatinine, using an enzymatic colorimetric method with an A15 auto-analyzer (BioSystems, Barcelona, Spain).

5. Serological and Parasitological Tests

The following serological tests were performed to detect anti-*Leishmania* antibodies: the DPP CVL rapid test which detects rk28-specific antibodies and the EIE CVL with crude *L. major* antigen diagnostic test provided by FIOCRUZ (Bio-Manguinhos Unit, Rio de Janeiro, Brazil). These serum tests were performed in accordance with manufacturer instructions. An in-house ELISA, with crude *L. infantum* antigen was also performed as previously described [39,40]. Parasitological evaluation was performed by culturing part of the splenic aspirate collected during necropsy in Novy–MacNeal–Nicolle (NNN) biphasic medium supplemented with 20% Fetal Bovine Serum (FBS – Gibco BRL, New York, USA) and 100 μg/mL gentamicin to avoid contamination (Sigma Chemical Co., St. Louis, MO) for four weeks at $24^{\circ}C$ [41]. Parasites were detected using microscopy performed at weekly intervals for no less than four weeks. Each splenic culture was prepared in duplicate. All of the culture labels were double-checked to avoid misidentification.

Parasite isolates were randomly selected from five dogs and sent to the national reference laboratory for *Leishmania* typing at the Oswaldo Cruz Institute (CLIOC, Rio de Janeiro, RJ, Brazil). The isolates were typed using monoclonal antibodies and enzyme electrophoresis analysis in order to determine the *Leishmania* species.

6. Control Samples

Splenic aspirate samples from 20 dogs that had previously been identified as *Leishmania*-positive from an endemic area [18] were used as positive controls. Splenic aspirates of 20 healthy dogs from

the municipality of Pelotas, Rio Grande do Sul, Brazil, an area without endemic CVL, were used as negative controls. All of the healthy dogs had no clinical signs of CVL, and tested negative for infection using the in-house ELISA, parasite culturing, and qPCR techniques.

7. Sample Handling and Decontamination Procedures

Due to the high degree of sensitivity inherent in qPCR, exceptional care was taken to avoid cross-contamination during not only the sample collection procedures, but also during DNA extraction and qPCR testing. As previously described [18], all procedures were carried out in an environment that was suitable for sample collection and qPCR procedures. All of the disposable surgical materials were used for a single animal, and the laminar flow hood was decontaminated by UV radiation before each procedure. Filter tips were routinely used throughout all DNA extraction steps and when performing the qPCR [42].

8. DNA Extraction

DNA was obtained from 200 μL of splenic and bone marrow aspirate, 200 μL of blood, 20 mg of lymph node, and 20 mg of a skin fragment using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocols. DNA samples from the conjunctival swabs were purified using a phenol–chloroform method as previously described [42]. The DNA pellets were suspended in 30 μL of Tris–EDTA buffer (10 mmol/L Tris and 1 mmol/L EDTA, pH 8.0). Once extracted, the quality and concentration of each DNA sample were evaluated using a digital spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA) [43]. All of the DNA samples were adjusted to a final concentration of 30 ng/μL, aliquoted, and kept at −20°C until the qPCR assays were performed.

Parasite DNA was extracted from *L. infantum* (MHOM/BR2000/MERIVALDO), *Leishmania amazonensis* (MHOM/Br88/Ba-125), *Leishmania braziliensis* (MHOM/BR/94/H3456), and *Leishmania major* (MHOM/RI//WR-173) promastigotes cultivated at 24°C. For the DNA extraction, the parasites were counted and centrifuged. DNA was extracted from pellets corresponding to a known number of parasites in accordance with the Qiagen protocols.

9. Quantitative PCR (qPCR)

9.1 Inclusion and exclusion criteria. To assess positivity, DNA samples were only included in the analysis if they met the minimum quality criteria: i) the DNA sample concentration was above 30ng/μl; ii) DNA samples amplified with the same efficiency as the DNA curve; and iii) amplification of the 18s rRNA housekeeping gene was successful. Any samples that did not fulfill one or more of the above inclusion criteria were excluded, only 10 out of 51 for skin fragments and 26 out of 51 for bone marrow aspirate. To compare parasitic load in different tissue types, DNA samples were only included in the analysis if they met the minimum quality criteria for all tissue types (samples from 20 dogs out of 51).

9.2 Quantitative PCR Assay. qPCR was used to determine the amount of parasite DNA in canine tissue samples. qPCR assays were performed following an amplification protocol previously described by Francino etal. (2006). The qPCR technique targeted a conserved region of *L. infantum* kDNA to obtain a 120-bp amplicon. All of the reactions were performed in triplicate. The reaction was in a final volume of 25 μL containing: 5 μL (150 ng) of each DNA sample diluted in deionized water and 20 μL of the PCR mixture. The PCR mixture contained: 12.5 μL of Universal Mastermix (Life Technology Corporation, Carlsbad, CA-USA), the forward primer 5′-AACTTTTCTGGTCCTCCG-GGTAG-3′ (LEISH-1) and the reverse primer 5′-ACCCCCA-GTTTCCCGCC-3′ (LEISH-2) both at a final concentration of 900 nM, and a fluorogenic probe 5′-AAAAATGGGTGCAGAA-AT-3′ with a FAM reporter molecule attached to the 5′ end and an MGB-NFQ quencher (200 nM final concentration) linked to the 3′-end (Life Technology Corporation). In order to overcome limitations caused by endogenous PCR inhibitors in the blood, skin fragment, and conjunctival swab samples, all of the steps leading up to DNA amplification were performed in the presence of bovine serum albumin (5 μg/each reaction) (Sigma Chemical) [44].

9.3 Quantification of *Leishmania* kDNA. Quantification of *Leishmania* kDNA was performed using an absolute method based on comparing the cycle threshold (Ct) values from the samples to a standard curve, which was constructed using serial 10-fold dilutions from 10^5 to 10^{-1} parasites performed in triplicate. Reactions were performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technology Corporation). The reaction was carried out under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 two-step cycles, first at 95°C for 15 s and then at 60°C for 1 min. In order to minimize variability between plates, the values from each plate were normalized using a common fluorescence detection baseline. Each sample's Ct value was calculated by determining the point at which its fluorescence signal was above the established detection baseline. The Ct cut-off value was determined using a Receiver-Operator Characteristic (ROC) curve. The optimal Ct cut-off value for the parasite kDNA qPCR assay was determined by calculating sensitivity and specificity for different Ct cut-off points and the ROC curve derived from the amplification values of *Leishmania*-negative samples and *Leishmania*-positive samples (see item 6). Tissue samples were considered positive when the Ct values were equal to or less than the Ct cut-off point determined using the ROC curve analysis. If the standard deviation between triplicates was >0.38, the sample set was reanalyzed by qPCR [45]. The efficiency of the qPCR protocol was evaluated by calculating the slope value of the standard curve for the parasite kDNA. This value, −3.657 (SD = 0.148), was obtained from the mean slope values of nine independent experiments with a correlation coefficient (R^2) of 0.998.

9.4 Assessment of qPCR Analytical Sensitivity and Specificity. Analytical sensitivity was evaluated by determining whether the presence of host tissue interferes with the amplification profiles when using qPCR to detect *L. infantum* DNA in infected dogs. First, a standard curve was constructed using ten-fold dilutions from reference strain *L. infantum* DNA (see item 9.3). Next, a ten-fold dilutions of reference strain *L. infantum* DNA was mixed with the splenic aspirate DNA from negative control animals (see item 6) and another standard curve was constructed from these dilutions. Finally, the amplification profiles of the two curves were compared. The analytical specificity of the qPCR analysis was assessed by comparing the amplification profiles of DNA samples from the *L. infantum* reference strain to profiles from several other *Leishmania* species, including the New World *L. amazonensis* and *L. braziliensis*, and the Old World *L. major*. As described in item 9.3, standard curves for each species were constructed from ten-fold serial dilutions ranging from 10^5 to 10^{-1} parasites performed in triplicate. Analytical specificity was further assessed by evaluating the amplification profiles of DNA obtained from other canine pathogens, such as *Ehrlichia canis* and *Babesia canis*. Briefly, 150 ng of DNA from each pathogen was amplified and compared to the *L. infantum* amplification profile.

9.5 Quantification of 18S rRNA Gene Expression. The expression of the canine housekeeping gene 18S rRNA was measured in order to normalize the concentration of input DNA for each sample and to obtain a reference amplification value to ensure the use of high-quality DNA samples [46]. TaqMan Pre-Developed Assay Reagents (Life Technology Corporation) were used to detect and quantify 18S rRNA gene expression. All of the reactions were performed at a final volume of 25 µL containing: 5 µL of DNA canine tissue sample diluted in deionized water and 20 µL of PCR mixture. The PCR mixture contained: 12.5 µL of Universal Mastermix (Life Technology Corporation), 1.25 µL of 18S GeneEx Assay primer and probe sets (Life Technology Corporation) at a concentration of 20x, and deionized water to obtain the final volume. The positive and negative controls for the housekeeping genes were plated in triplicate and the samples were plated in duplicate. Reactions were performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technology Corporation) using the following protocol: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 10 min; and 40 two-step cycles, first at 95°C for 15 s and then 50°C for 1 min. A seven point standard curve was constructed for the housekeeping gene ranging from 450–18.75 ng. The slope of the standard curve for the 18s rRNA gene was −3.399 (SD = 0.296), which represents the mean slope value of 11 independent experiments with the corresponding coefficient of determination (R^2) of 0.990.

9.6 Parasitic Load in DNA Samples. Samples from 20 of the 51 dogs were used to determine which tissue type harbored the highest parasitic load by comparing the splenic and bone marrow aspirates, blood, conjunctival swab of right and left eyes, lymph node and skin fragments. The parasitic load was expressed as the number of parasites normalized to the established reference amplification value for the 18S rRNA gene in 150 ng of DNA from each tissue sample [47]. Then the value obtained was calculated per 100 mg of host tissue DNA.

10. Evaluation of qPCR accuracy using splenic aspirate samples from a prevalence study

The accuracy of the qPCR assay was evaluated using splenic samples obtained from 800 dogs during a random prevalence study performed in Camaçari, BA, an endemic area for CVL in Brazil. All 800 dogs were clinically evaluated and classified as described in item 2. They were also tested using the following CVL diagnostic methods: DPP CVL rapid test, EIE CVL, our in-house ELISA, and parasite cultures from splenic aspirates as described in item 5. qPCR analysis of splenic aspirate samples was performed as described in item 9.

11. Statistical Analysis

In order to prevent bias, serological, parasitological and molecular techniques were performed and their results were judged without knowledge of the outcome of the other tests.

The ROC curve data analysis described in item 9.3 was performed using GraphPad Prism software v.5.0 (GraphPad Prism Inc., San Diego, CA). Differences in the parasitic load between each type of biological sample were assessed using the Friedman test followed by the Dunn's multiple comparison test. The relationship between parasitic load in the spleen and qPCR positivity in each infected tissue was assessed with the Spearman correlation test using log transformed values for the parasitic load ($p<0.05$).

For the 800 dogs evaluated in the cross sectional study, the intensity of the parasitic load in the spleen (item 9.6) was categorized into three ranges: $<10^4$, 10^4–10^6, and $>10^6$. The number of clinical signs in the dogs (item 2) was stratified into four ranges: 0 (no clinical signs), 1–3, 4–6, and >6 clinical signs. Fisher's exact test was used to evaluate the association between the number of clinical signs and the splenic parasitic load ranges.

LCA was performed using a statistical model to define a latent variable that could be used as a gold standard. To define a latent variable that could accurately identify *L. infantum* infection, three indicators representing serologic (DPP CVL), parasitological (culture from splenic samples), and molecular (splenic aspirate qPCR) diagnostic techniques were included. Animals were grouped into two categories, 'infected dogs', and 'not-infected dogs'. The latent classes were estimated and characterized using two parameters: (a) item-response probabilities and (b) class prevalence, which is the probability of belonging to a latent class according to the response pattern. The estimate was performed using the maximum likelihood with expectation-maximization (EM) algorithm. The goodness of fit of the statistical model was evaluated using entropy, which varied between 0 and 1, with the value 1 indicating that the individuals are perfectly classified into the latent classes. Average probabilities for each latent class, which expresses the uncertainty of global classification, were also assessed *a posteriori,* considering a higher *a posteriori* probability to be a better goodness of fit for the statistical model. The Vuong-Lo-Mendell-Rubin likelihood ratio test was used to choose the number of classes in LCA [48]. The Akaike information criterion (AIC) and Bayes information criterion (BIC) were also evaluated for each model. LCA was performed using the software Mplus 5.2, the syntax for fitting LCA in MPlus program is reported in Appendix S1 [49]. Additionally, the conditional independence was checked by evaluation of significant bivariate residuals [50,51].

The sensitivity and 95% confidence interval (CI) were calculated for each diagnostic technique and each tissue type analyzed, using the LCA latent variable as gold standard. The accuracy (sensitivity and specificity) of the qPCR technique using splenic aspirates was further evaluated with the LCA in a random sample of 800 dogs. Sensitivity of each test was measured as the proportion of positive results, only among those identified as such by the gold standard, while specificity was measured as the proportion of negative results, which were correctly identified as such by the gold standard.

Results

1. Sample description

All 51 dogs from the endemic area of Jequié were mixed-breed, their estimated ages varied from 1–10 years old, the animals weighed 5–30 kg, 45% (23/51) were males, and 55% (28/51) were females. All of the dogs exhibited clinical signs that could be related to CVL including splenomegaly (33/51), emaciation (17/51), hypertrophy of the lymph nodes (46/51), alopecia (21/51), cutaneous alterations (41/51), onychogryphosis (29/51), and ocular alterations (10/51). With respect to clinical pathology, 73% of the dogs presented with anemia (35/48), 98% with hypergammaglobulinemia (49/50), and 98% with hypoalbuminemia (49/50). Using the scale published by Solano-Gallego etal. (2009), all of the dogs were classified as having moderate CVL (stage II), except one animal that also exhibited a creatinine value greater than 1.4 mg/dL and was considered to have severe CVL (stage III).

2. Standardization of the qPCR Protocol

The Ct cut-off value for parasite DNA detection was performed using a ROC analysis. This analysis showed an area under the curve of 1.0, indicating a high probability ($p<0.001$) that a randomly chosen positive sample would be correctly classified.

The Ct cut-off value of 37.0 had prediction rates of 100% sensitivity (CI 83.16–100) and 95% specificity (CI 75.13–99.87) with a likelihood ratio of 20. The analytical sensitivity was then determined. We found that the amplification profile of the reference strain *L. infantum* DNA was similar to that of the reference strain mixed with splenic aspirate DNA from negative control animals. The lower limit of detection was then determined and corresponded to 0.016 parasites per reaction.

In terms of the analytical specificity, the Old World *L. major* parasite DNA samples were remarkably similar to those of *L. infantum* at all of the concentrations tested. In contrast, DNA from *L. amazonensis* and *L. braziliensis* could only be successfully amplified at concentrations of 10^4 and 10^5 parasites per reaction. This corresponded to the same number of cycles needed to amplify DNA from 0.02 parasites per reaction of the *L. infantum* reference strain (Figure S1). *E. canis* and *B. canis* DNA did not amplify using this qPCR protocol (data not shown). With respect to the housekeeping gene, attempts to amplify18S rRNA from DNA samples of *Leishmania* spp. resulted in no detectable qPCR amplification using the same primer set that successfully amplified the gene in canine DNA samples (data not shown).

3. Positivity of diagnostic techniques

Using qPCR, 100% of the dogs from Jequié (51/51) tested positive for parasite DNA in at least one of the tissue types analyzed. Among these, 98% (50/51) tested positive in the splenic aspirate samples; 80.4% (41/51) in blood samples; 68.3% (28/41) in skin fragments; 54.9% (28/51) in lymph node fragments; 35% (7/20) in bone marrow aspirate; 37.3% (19/51) in left eye conjunctival swabs, and 33.3% (17/51) in right eye conjunctival swabs.

Parasites were observed in 35.3% (18/51) of the parasite cultures from splenic aspirate and anti-*Leishmania* antibodies were detected in 43.8% (21/48), 47.1% (24/51), and 66.7% (34/51) of the canine serum samples using the EIE CVL, DPP CVL rapid test, and in-house ELISA, respectively.

4. Accuracy of the diagnostic tests

Latent class was used to provide a reliable estimate of sensitivity and specificity in order to select the tissue that provided the greatest accuracy for qPCR DNA detection. Serological, parasitological, and molecular techniques were used to determine prevalence of the latent classes and conditional probabilities in the LCA model for *L. infantum* infection in dogs. The probability that a dog from Jequié would be classified as infected using the LCA model was 47.1%. Among the animals considered infected by the LCA, the probability that a dog would test positive using qPCR of the splenic aspirate was 95.8%. The probability that a dog tested positive using either DPP CVL or by parasite culture from splenic aspirates was 100.0% or 54.2%, respectively (Table 1).

Entropy was then calculated to assess how well the animals were classified *a posteriori* by the model. The entropy of the Jequié samples was 1.0; indicating accuracy in the classification of dogs using LCA. Moreover, *a posteriori* average probabilities that animals were properly classified into the latent classes "Infected" and "Not Infected" were 100% in both cases in the Jequié animals. The Lo-Mendel-Rubin test indicated that the model with 2 classes was a better fit for the data obtained from the Jequié dogs ($p<0.01$) when compared with the model with only 1 class (data not shown). These results are supported by the analysis of the AIC and BIC (data not shown).

The sensitivity of the tests employed in Jequié to diagnose *L. infantum* infection was assessed employing the latent variable obtained by LCA as the gold standard (Figure 1). Splenic aspirates

Table 1. Prevalence of latent classes and conditional probabilities to the LCA model for *L. infantum* infection detection in dogs.

		Dogs from Jequié n = 51			Dogs from Camaçari n = 800		
			Latent Classes			Latent Classes	
Technique	Result		Infected n = 24 (47.1%)	Not Infected n = 27 (52.9%)		Infected n = 120 (14.5%)	Not Infected n = 680 (85.5%)
		Result Frequency (%)	Conditional Probabilities (%)		Result Frequency (%)	Conditional Probabilities (%)	
DPP CVL	Positive	47.1	100.0	0.0	16.6	82.9	5.5
	Negative	52.9	0.0	100.0	83.4	17.1	94.5
Splenic Aspirate Culturing	Positive	35.3	54.2	18.5	13.2	87.8	0.0
	Negative	64.7	45.8	81.5	86.8	12.2	100.0
Splenic Aspirate qPCR	Positive	98.0	95.8	100.0	34.2	93.3	24.1
	Negative	2.0	4.2	0.0	65.8	6.7	75.9

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provided the highest sensitivity of the available tissues sampled achieving 95.8% (95%CI 90.4–100) of sensitivity. The sensitivity attained in other tissues ranged from 80% to 30% as follows: lymph node fragments 79.2% (95%CI 68–90.3), skin fragments 77.3% (95%CI 64.5–90.1), blood 75% (95%CI 63.1–86.9), bone marrow aspirates 50% (95%CI 30–70), left eye swab 37.5% (95%CI 24.2–50.8), and right eye swab 29.2% (95%CI 16.7–41.6). It was not possible to calculate splenic qPCR specificity since only one sample tested negative in this method. Specificity of the other tissues achieved 66.7% for lymph node fragments (95%CI 53.7–79.6) as well as for bone marrow aspirates (95%CI 47.8–85.6), 63% (95%CI 49.7–76.2) for right and left eye swabs, 42.1% (95%CI 27–57.2) for skin fragments and 14.8% (95%CI 5.1–24.6) for blood. Considering the other diagnostic tests, the sensitivity of the serological tests was 100% for the DPP CVL, followed by 79.2% (95%CI 68–90.3) for the in-house ELISA, 65.2% (95%CI 51.7–78.7) for EIE CVL, while sensitivity for the splenic aspirate culturing was 54.2% (95%CI 40.5–67.8). The specificity was highest for DPP CVL 100%, followed by splenic parasite cultures 81.5% (95%CI 70.8–92.1), EIE CVL 76% (95%CI 63.9–88.1), in-house ELISA 44.4% (95%CI 30.8–58.1).

5. Parasitic load in different tissue types

To further characterize tissue performance for the molecular diagnostic assay, parasitic loads were determined in the different tissues analyzed. As shown in Table 2 a considerable degree of variation was observed among the samples with values ranging from 120 parasites in a splenic aspirate sample up to 186 million parasites found in a bone marrow aspirate sample. However, the median parasitic load was higher in splenic aspirate samples than in the conjunctival swabs from either eye ($p<0.05$) or bone marrow aspirate ($p<0.05$). No statistically significant differences were observed when comparing parasitic loads in the splenic aspirate to the blood or skin tissue samples.

6. Distribution of parasitic load according to number of clinical signs

The distribution of parasitic load according to the number of clinical signs is displayed in Table 3. We observed a significant positive association between the intensity of parasitic load in the spleen and the number of clinical signs present in the dogs. Animals with no clinical signs ($p<0.01$) or those exhibiting 1–3 clinical signs ($p<0.001$) had lower parasitic loads in splenic tissue

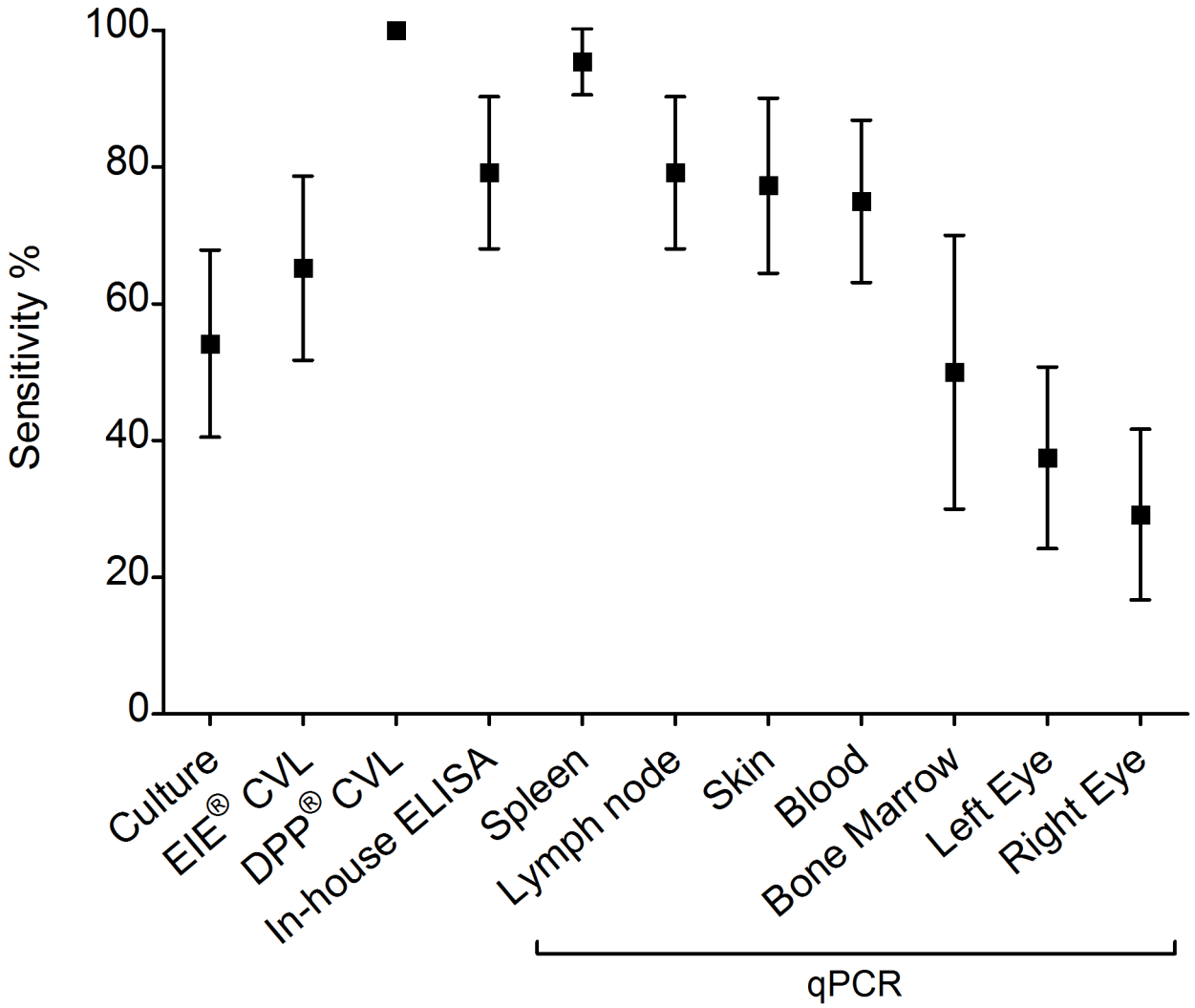

Figure 1. Sensitivity of the different diagnostic techniques employed in the biological samples obtained from Jequié animals (n = 51). Vertical bars represent the 95% confidence intervals. Sensitivity values were obtained using the latent variable as the gold standard.
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Table 2. Parasitic loads detected in different canine tissue types from a total of 20 dogs from the endemic area of Jequié.

Tissue type	Positivity	Parasitic loads[a]				
		Minimum	25% Percentile	Median	75% Percentile	Maximum
Splenic Aspirate	100% (20/20)	120	1,088	4,365	14,325	74,000,000
Blood	70% (14/20)	0	0	7,960	19,800	228,000
Skin Fragment	60% (12/20)	0	0	1,870	21,500	32,400,000
Lymph node Fragment	60% (12/20)	0	0	830.5	9,288	7,800,000
Bone Marrow Aspirate	35% (07/20)	0	0	0.0*	28,275	186,000,000
Left Eye Swab	50% (10/20)	0	0	645.0*	2,073	240,000
Right Eye Swab	35% (07/20)	0	0	0.0*	3,141	147,000

[a]number of parasites normalized by the established reference amplification value for the housekeeping gene 18S rRNA in 100 mg of host tissue DNA.
*$p<0.05$ Friedman's together with Dunn's multiple comparisons test of splenic aspirates and swab of right or left eye and splenic aspirates and bone marrow.
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($<10^4$). In contrast, animals with >6 clinical signs ($p<0.01$) showed relatively higher loads ($>10^6$). The dogs presenting with 4–6 clinical signs were homogeneously distributed throughout the three ranges.

7. Accuracy of qPCR using splenic aspirate samples from a prevalence study

Splenic aspirate samples collected from a random study conducted in the endemic area of Camaçari were used to evaluate the high sensitivity observed for the qPCR technique developed using convenience sampling from Jequié. Positive diagnoses in the samples from Camaçari varied according to diagnostic test. In this sample, 34.2% were positive using qPCR, 24.4% using EIE CVL, 19.8% using the in-house ELISA, and 16.6% using DPP CVL.

Similar to the samples from Jequié, LCA was used to analyze the results from the Camaçari samples. Reliability of the LCA model was evaluated and the probability of an animal being infected with *L. infantum* was calculated. The response patterns obtained from the latent class model that were used are listed in Table 4. Animals from Camaçari that had at least two positive test results were classified by the LCA model as 'Infected'. However, the presence of a positive result from the splenic aspirate parasite culture implied a 100% probability of being infected with *L. infantum*, regardless of the DPP CVL and splenic aspirate qPCR results. When dogs from this endemic area tested negative by all three diagnostic techniques, the probability that the animal was infected with *L. infantum* was 0%. Furthermore, the probability of animals being infected was still very low when only splenic aspirate qPCR (2.7%) or DPP CVL (1.4%) tested positive according to this LCA model.

The entropy of the Camaçari samples was 0.934, and the *a posteriori* average probabilities of being correctly classified as "Infected" and "Not Infected" were, respectively, 92.4% and 99.3%. Similar to the analysis performed with samples from Jequié, using random samples, the Lo-Mendel-Rubin test indicated that the model with 2 classes was optimal and was supported by the analysis of the AIC and BIC (data not shown).

Using LCA, the sensitivity of the splenic aspirate qPCR (95%; 95%CI 93.5–96.5) was higher than for the other diagnostic tests: DPP CVL (86.4%; 95%CI 84.1–88.8), splenic parasite cultures (83.5%; 95%CI 80.8–86.2), the in-house ELISA (78.3%; 95%CI 75.5–81.2), and EIE CVL (72.5%; 95% CI 69.4–75.6) (Figure 2A). However, the specificity was highest for splenic parasite cultures (100%), followed by DPP CVL (95.6%; 95%CI 94.2–97), the in-house ELISA (90.6%; 95%CI 88.6–92.6), EIE CVL (84.1%; 95%CI 81.6–86.6), and splenic aspirate qPCR (76.7%; 95%CI 73.7–79.6) (Figure 2B).

Discussion

The present study found that a qPCR protocol targeting *Leishmania* kDNA provided the highest diagnostic sensitivity in dogs from Jequié when compared to standard serological and parasitological methods. In this endemic area, the DPP CVL rapid test and EIE CVL were able to detect infection in 47.1% and 43.8%, respectively, of a population of symptomatic dogs. Interestingly, 100% of these dogs tested positive with respect to at least one of the tissue types analyzed using qPCR. Similar results have been obtained by other studies, in which high sensitivity was achieved using molecular techniques [14,16,52]. Together these results reinforce the notion that the number of

Table 3. Distribution of parasitic load according to number of clinical signs in dogs from the prevalence study.

Number of Clinical Signs	Splenic Parasitic Load Ranges			Fisher Exact Test
	$<10^4$	10^4–10^6	$>10^6$	
0	8 (57.1%)	5 (35.7%)	1 (7.1%)	$p<0.01$
1–3	55 (42%)	49 (37.4%)	27 (20.6%)	$p<0.001$
4–6	37 (39.4%)	27 (28.7%)	30 (31.9%)	$p=0.11$
>6	5 (16.1%)	9 (29.0%)	17 (54.8%)	$p<0.01$
Total	105	90	75	

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Table 4. Response patterns[a] of Camaçari dogs for LCA model with 2 latent classes for diagnosis of CVL.

Response pattern					
DPP CVL	**Splenic Aspirate Culturing**	**Splenic Aspirate qPCR**	**Frequency Observed % (n)**	**CVL Probability *a posteriori* (%)**	**Result Based on LCA**
N	N	N	60.1 (429)	0.0	Not infected
N	N	P	20.5 (146)	1.4	Not infected
P	N	N	3.6 (26)	2.7	Not infected
N	P	N	0.1 (01)	100.0*	Infected
P	N	P	2.7 (19)	54.7	Infected
N	P	P	2.1 (15)	100.0	Infected
P	P	N	0.7 (05)	100.0	Infected
P	P	P	10.2 (73)	100.0	Infected

[a]Response patterns of all samples tested using the three techniques.
*Estimation based on only one animal sample presenting this pattern.
N: Negative; P: Positive.
doi:10.1371/journal.pone.0103635.t004

infected dogs detected by serological surveys in endemic areas is severely underestimated [53,54].

Several methods have been recently developed for the molecular detection of *Leishmania* spp. [20,21,55], that provide divergent results when used in a variety of clinical canine samples [54]. Among the tissues analyzed, the authors observed that splenic aspirate samples provided the highest detection rate, successfully identifying 98% of the samples that tested positive. This result is supported by the fact that the spleen is a key site for parasite multiplication in naturally infected dogs [24,56]. Interestingly, following splenic aspirate samples, 80.4% of blood samples tested positive using qPCR. In addition, we found that the parasitic loads achieved were similar in the blood and splenic aspirate samples. These are promising results given that drawing blood is a much less invasive sampling technique to detect *Leishmania* infection in dogs than obtaining splenic aspirates. In contrast, several other studies have found that bone marrow and lymph node tissues offered a higher number of positive results than

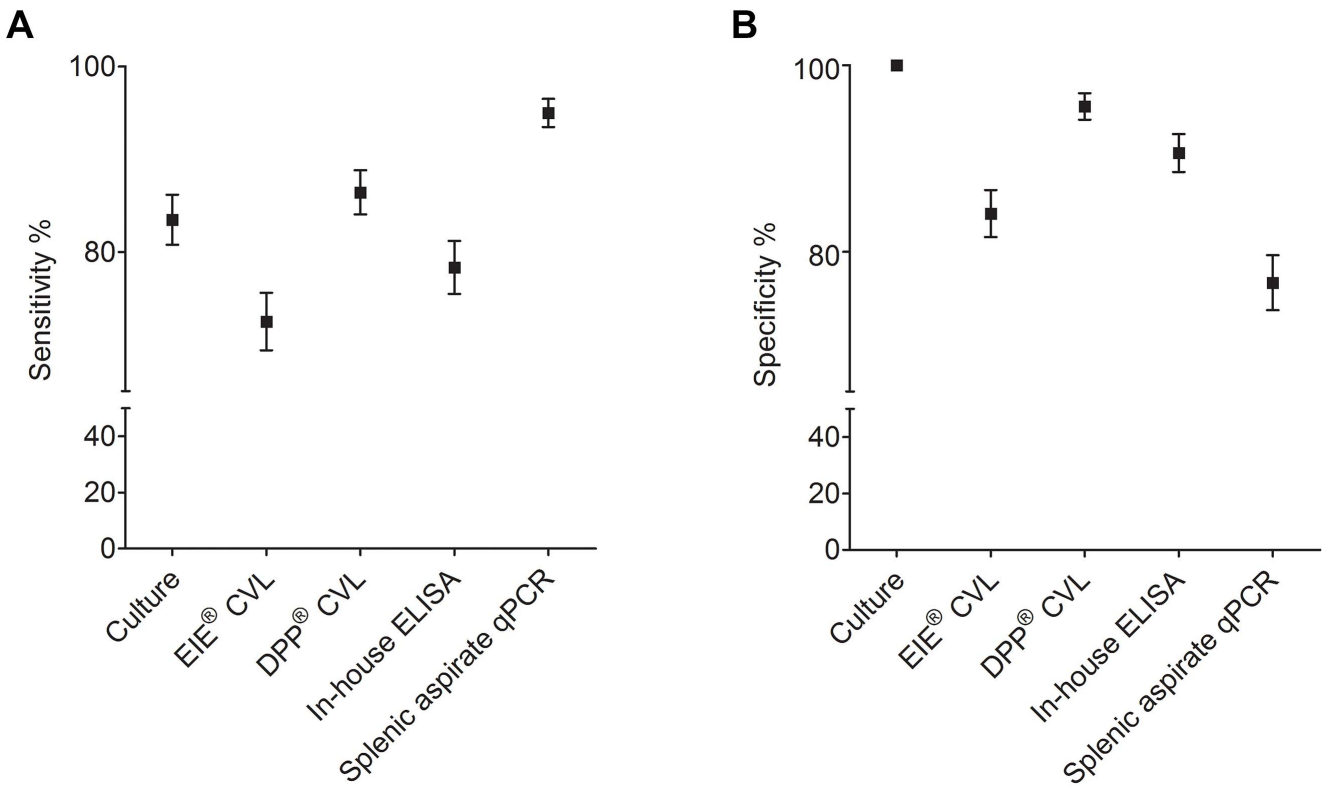

Figure 2. Sensitivity and specificity of the different diagnostic techniques employed in the biological samples obtained from Camaçari animals (n = 800). Vertical bars represent the 95% confidence intervals. **A)** Sensitivity and **B)** Specificity values obtained using the latent variable as the gold standard.
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blood [46,55,57,58]. Francino etal. (2006) suggested that using qPCR to detect *Leishmania* parasites in blood samples might be sufficient to diagnose infection given the technique's ability to quantify extremely low parasitemia. However, other authors consider the blood to be a poor source of *Leishmania* DNA [59], mostly because blood samples do not have satisfactory detection rates using conventional PCR. The underlying cause of these poor results may be the high frequency of PCR inhibitors found in blood, in addition to low parasitic loads, which could lead to false negatives especially in asymptomatic dogs [52]. Serum albumin can be added to avoid any potential inhibiting effects in qPCR reaction [44]. In the present study we added serum albumin to blood, skin, and conjunctival swab samples. Our results demonstrate that splenic aspirates or blood can be effectively used to detect parasite DNA using qPCR [18,19].

The analytical specificity of the qPCR technique was also evaluated in the present study by comparing the amplification profiles of *L. infantum* DNA to other Old and New World *Leishmania* species. The amplification profile of the Old World species *L. major* was remarkably similar to that of *L. infantum* (Figure S1). This corroborates other studies that have shown a great deal of similarity between the genomes of these species [60]. To the best of our knowledge, *L. major* is not known to be a causative agent of CVL, nor have any cases linked to this parasite been reported in Latin America [61]. kDNA from New World parasites, such as *L. amazonensis* and *L. braziliensis*, was successfully amplified using this protocol, but only at high concentrations of 10^4 and 10^5 parasites per reaction (Figure S1). Protocols capable of distinguishing between *Leishmania* species are preferable in endemics areas for both cutaneous and visceral forms of the disease [62]. In this study, five *Leishmania* species isolated from the dogs were identified by multilocus enzyme electrophoresis as *L. infantum*. Nonetheless, the use of splenic aspirate samples can avoid misleading diagnostic results since visceralization of *L. braziliensis* has not been reported and visceralization of *L. amazonensis* is a relatively rare event both in humans or dogs [62–64].

Regrettably, an ideal gold standard is still lacking for CVL diagnosis [65]. Historically, parasite culturing and immunofluorescence antibody test (IFAT) have been abundantly used. However, culturing is shown to have low sensitivity, while IFAT low specificity [65]. An alternative to using a single technique as the gold standard is to utilize LCA, once this method defines a latent variable to be used as gold standard, considering all diagnostic tests impartially. Indeed, LCA has been proved to successfully estimate the sensitivities and specificities of different diagnostic tests for several diseases [34,66–69]. LCA has been an useful tool for validating serological diagnostic methods for VL, since this analysis provides more realistic estimates of diagnostic test performance [34,67]. In the scientific community still exist concerns regarding the high sensitivity of qPCR results, especially when this technique is able to detect very low parasitic loads. In addition, some authors state that is impossible for qPCR to differentiate between the DNA of a living parasite and a dead one. Otherwise, Prina etal. (2007) [70] were the only ones that proved that as soon as 1 h after exposure to a substance able to kill the parasites, only less than 1% of the initial *Leishmania* DNA could be detected by qPCR. No other group demonstrated these results, especially using invivo experiments. Thus, in the present study, we have decided not to consider all the dogs as infected, even if they displayed parasite in at least one tissue by the qPCR, and perform the qPCR accuracy evaluation using the latent variable.

Employing the latent class variable as the gold standard, we found that the sensitivity for splenic aspirate qPCR and DPP CVL were 95.8% and 100% respectively, in a population of symptomatic dogs in Jequié. However, these results were limited since it was a small sample size. To address this, the results of the qPCR testing were evaluated using a larger random sampling of dogs that consisted of a population of positive and negative dogs, which are representative of the population of an endemic area for VL. In this random population survey using 800 dogs, the high sensitivity of splenic aspirate qPCR was confirmed achieving 95% of sensitivity, while the DPP CVL sensitivity was corrected to 83.5%. Despite the high sensitivity of the splenic aspirate qPCR, the specificity was relatively low (76.7%). This could be due to the large number of dogs from the randomly sampled population that tested positive only by splenic aspirate qPCR (20.5%) and were considered as 'Not infected' by the LCA. These animals were likely misclassified by LCA as false negatives, since the splenic aspirate qPCR is known to be the most sensitive diagnostic technique for CVL, most likely more sensitive than the variables used to define the variable latent class.

Several studies have demonstrated a positive correlation between clinical manifestations of CVL and parasitic load in the spleen, lymph nodes and skin using several techniques [20,41,56,71]. Using qPCR of splenic aspirate in dogs, we also found a positive association between parasitic load and clinical manifestations of CVL, reinforcing the notion that can be used not only for detection of infection but also to monitor disease severity in dogs.

Although splenic aspirate collection is considered an invasive procedure by many dog owners [27,72], Barrouin-Melo etal. (2006) noted that minor complications were observed in only three out of 257 dogs that underwent splenic aspiration. Complications can be further minimized by visualizing the spleen using an ultrasound device to guide splenic aspiration [72,73]. In our experience, during the prevalence study in the municipality of Camaçari, the splenic aspirate procedure assisted by ultrasonography was well tolerated in all 800 dogs without any reported complication.

In conclusion, the authors found that, the splenic aspirates and blood, provided the greatest sensitivity for detecting *Leishmania* DNA using qPCR. In addition, the results indicated that LCA could be used to create a suitable gold standard for diagnosis, since this technique offers a more comprehensive evaluation of the results obtained using different diagnostic testing methods for CVL.

Supporting Information

Figure S1 Amplification profiles of DNA samples from *Leishmania* spp. A) *L. infantum*; **B)** *L. major*; **C)** *L. amazonensis*; **D)** *L. braziliensis*. DNA samples derived from the *L. infantum* reference strain, and several other *Leishmania* species, including New World *L. amazonensis* and *L. braziliensis*, and Old World *L. major*. Standard curves were constructed using amplification patterns from ten-fold serial dilutions performed in triplicate ranging from 10^5 to 10^{-1} parasites per reaction.
(TIF)

Appendix S1 Syntax for fitting LCA in MPlus program.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MSS CESG DBMF PSTV. Performed the experiments: MSS LAB MB LSB DFL. Analyzed the data: MSS CESG PGSET LDAFA WLCS DBMF PSTV. Contributed reagents/materials/analysis tools: DFL GGSO EGN PGSET LDAFA. Wrote the paper: MSS CESG LDAFA DBMF PSTV.

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Supporting Information Solcà et al., 2014

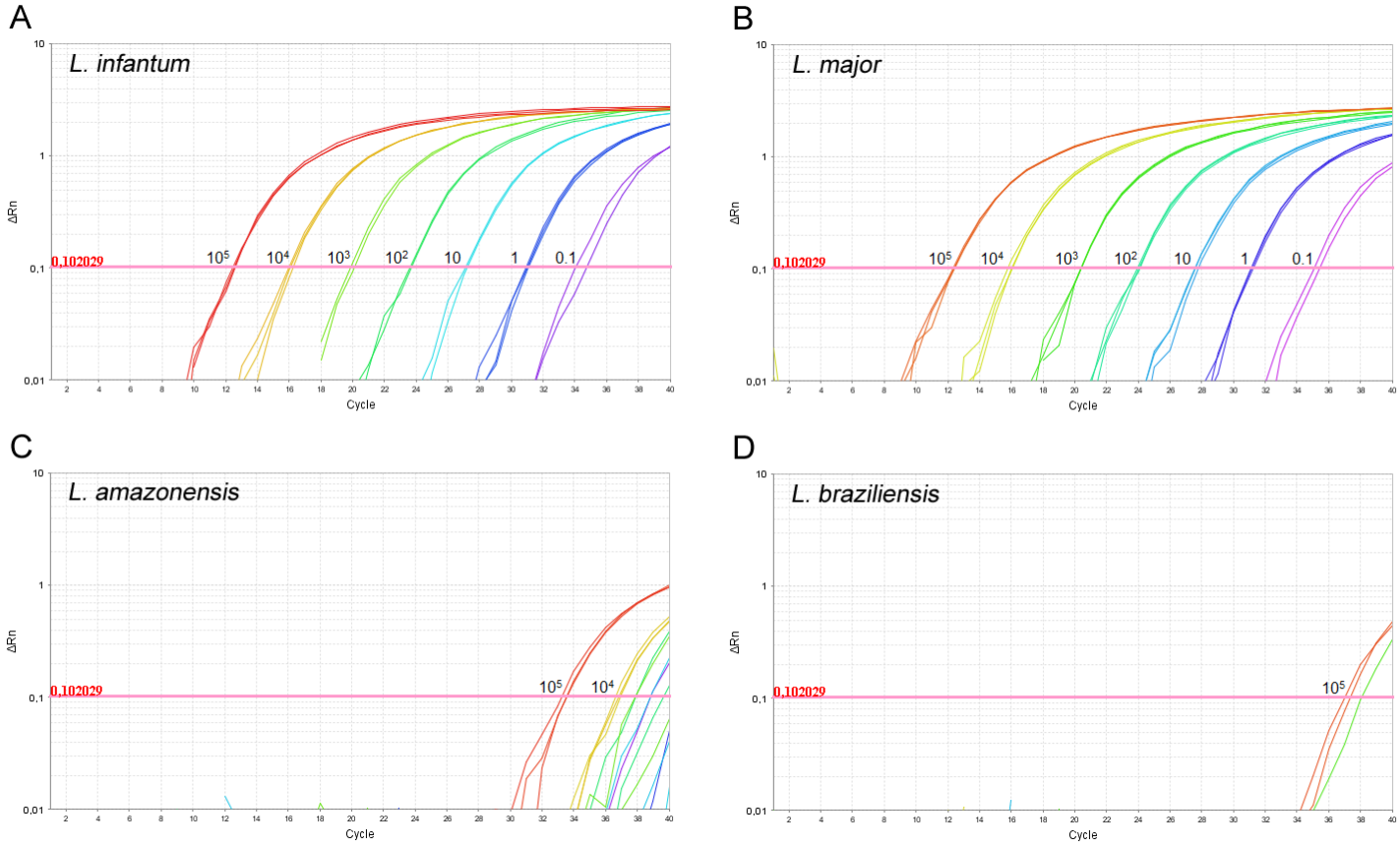

Figure S1.
Amplification profiles of DNA samples from *Leishmania* spp. A) *L. infantum*; **B)** *L. major*; **C)** *L. amazonensis*; **D)** *L. braziliensis*. DNA samples derived from the *L. infantum* reference strain, and several other *Leishmania* species, including New World *L. amazonensis* and *L. braziliensis*, and Old World *L. major*. Standard curves were constructed using amplification patterns from ten-fold serial dilutions performed in triplicate ranging from 10^5 to 10^{-1} parasites per reaction.
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Appendix S1.
Syntax for fitting LCA in MPlus program.
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APPENDIX

Mplus Commands for LCA

```
TITLE: LCA with binary latent class indicators
DATA:   FILE IS "dogs.ascii";
VARIABLE:
        NAMES ARE id DPPCVL EIECVL ELISA culture
spleen blood limph righte lefte skin bonemar;
        USEVARIABLES = DPPCVL culture spleen;
        CATEGORICAL= DPPCVL culture spleen;
        CLASSES = c (2);
        MISSING = .;
        AUXILIARY = id;
ANALYSIS:
        TYPE = MIXTURE;
        STARTS = 100 10;
        STITERATIONS = 20;
OUTPUT:
        TECH11 TECH14 TECH10;
PLOT:
         TYPE IS plot3;
        SERIES IS DPPCVL (1) culture (2) spleen (3);
SAVEDATA:
        FILE IS LCA_dogs.txt;
        SAVE IS cprob;
        FORMAT IS free;
```

1.4. Artigo científico submetido à revista *Diagnostic Microbiology and Infectious Disease*

Rita de Cássia Pontello Rampazzo*, Manuela da Silva Solcà*, Liliane Celestino Sales Santos, Lais de Novaes Pereira, José Carlos Oliveira Guedes Junior, Deborah Bittencourt Mothé Fraga, Patrícia Sampaio Tavares Veras, Marco Aurélio Krieger, Alexandre Dias Tavares Costa. **Improvements on the detection of *Leishmania* spp. in naturally infected dogs by real time PCR: a duplex and ready-to-use reaction format**. 2017.

O presente estudo teve como objetivo padronizar uma reação de qPCR *duplex* gelificada para a identificação de animais infectados por *L. infantum*. Este protocolo foi desenvolvido para detectar e quantificar simultaneamente uma sequência conservada do kDNA de *L. infantum* bem como uma sequência conservada do gene do rRNA canino. A detecção simultânea do gene 18S canino reforça a validade dos resultados da qPCR, principalmente, os resultados negativos por confirmar a presença do DNA genômico nas amostras caninas, excluindo a possibilidade de resultados falso negativos. Assim, quando ocorrer à amplificação do DNA do hospedeiro, associado à inexistência de amplificação do DNA de *Leishmania* pode-se concluir que há ausência de DNA do parasito. Ademais, a realização da qPCR em formato *duplex*, reduz o tempo de execução da técnica, combinando duas reações em uma, além de diminuir gastos com reagentes. O formato em gel (*ready-to-use*) permite que o usuário adicione apenas água e o DNA extraído das amostras, reduzindo ainda mais o tempo de execução, tornando-a mais fácil e evitando erros durante a manipulação de reagentes. Estas duas características são altamente importantes em um laboratório de diagnóstico de rotina podendo facilitar e acelerar o diagnóstico da LVC. A reação de qPCR em formato *duplex* foi avaliada em diferentes tecidos de cães com diferentes manifestações clínicas de LVC, provenientes de uma área endêmica. A sensibilidade da reação de qPCR em formato *duplex* foi testada em relação à sensibilidade da reação de qPCR *singleplex*, assim como, foi comparada à performance de detecção do DNA de *L.*

infantum no formato líquido e gelificado da reação *duplex*. A padronização da qPCR em formato *duplex* gelificado garante especificidade e diminuição dos gastos com reagentes, sem perdas significativas de sensibilidade, que comprometam o diagnóstico de LVC.

Improvements on the detection of *Leishmania* spp in naturally infected dogs by real time PCR: a duplex and ready-to-use reaction format

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Abstract

Background. Canine visceral leishmaniasis is usually diagnosticated by serological tests, but these methods present limitations in accuracy. Thus, improvement in assessing *Leishmania* infection in dogs is needed. Quantitative PCR (qPCR) is a sensitive and specific techinique which presents limitations that hinder its implementation in diagnostic routines. Absolute requirement for freezer storage of qPCR reagents is one of them. Furthermore, internal controls used in the qPCR reaction for *Leishmania* detection have also not been developed, their use concomitantly would avoid false-negative results. The aim of this study was to develop a gelified duplex qPCR for the identification of infected animals. We combined a new reaction for detection of the 18S canine gene into a duplex qPCR that concomitantly amplifies a conserved sequence of *L. infantum* DNA in canine blood, spleen or skin tissue samples. The duplex reaction was compared to the traditional singleplex format. A gelified, freezer-free format was also produced and compared to the regular, liquid/frozen format. For comparison, samples were characterized by standard parasitological and serological protocols.

Results. The new duplex qPCR exhibited the same detection limit of 0.1 parasites/reaction as the singleplex reaction format. Such limit was observed for samples from all three tissues studied, using *L. infantum* DNA as target. We used samples of 82 asymptomatic and symptomatic dogs from an endemic area to compare our duplex qPCR to other diagnostic methods, and observed that spleen aspirates samples provided higher rate of positivity (92.9%), followed by skin (50%) and blood samples (35.7%). The gelified format showed a limit of detection of 1 parasite/reaction without affecting the reaction efficiency.

Conclusions. Our results show that amplification of *Leishmania* and canine DNA in a single reaction exhibits the same sensitivity and specificity as amplification of *Leishmania* DNA alone. Detection of host genes strengthen qPCR results by confirming DNA presence in the samples and the absence of polymerase inhibitors. We also present a qPCR format that allows reagents to be stored on plate, which shows similar clinically-relevant performance as the regular format. Thus, the new format reduces the hand-on-time to set up a test while reducing the chance of operator's mistakes.

(350/350 words)

Keywords: Canine visceral leishmaniasis; diagnosis; duplex; qPCR

Background

Leishmania infantum is the causative agent of visceral leishmaniasis (VL), a neglected tropical disease found throughout Europe and Latin America. VL, or kalazar, is considered the most serious presentation among the various clinical forms of leishmaniasis [1]. In Brazil, natural transmission occurs via the bite of infected female phlebotomine sandflies [2]. Dogs are considered the main urban reservoir of VL, mostly due to the high rate of canine infection in endemic areas and the intense parasitism in canine skin [3,4]. Since these animals live in close contact with humans and insect vectors, they are believed to be important to the maintenance of the *L. infantum* transmission cycle [2,5–7].

In South America, Brazil has several endemic areas for VL, as well as a large and widespread canine population [8]. One of the strategies for the

surveillance and control of VL recommended by the Brazilian Ministry of Health is to monitor canine visceral leishmaniasis (CVL) by detection and removal of seropositive dogs [9]. Currently, CVL diagnostic protocol established by Brazilian Ministry of Health employs the Dual Path Platform (DPP® CVL) as a screening method and the enzyme-linked immunosorbent assay (EIE® CVL) as a confirmatory test [9]. These serological tests rely on antibody detection and carry important limitations, such as: i) cross-reactions with antibodies produced against other canine pathogens, ii) fluctuations in antibody titers during *Leishmani*a infection that may affect the sensitivity of these diagnostic methods, and iii) no or low production of antibodies by some infected animals that hampers accurate detection by serological testing [10–12]. Both conventional (cPCR) and quantitative (qPCR) PCR have proven to be capable of complementing the clinical diagnosis of leishmaniasis [13,14]. Therefore, VL diagnosing would greatly be improved by the inclusion of PCR as a confirmatory test, since it offers higher specificity and sensitivity than EIE® CVL. Additionally, qPCR has the advantage of parasite load quantification, allowing therapeutic monitoring of infected humans and dogs [13,15–17].

Different biological tissues have been used to carry out molecular tests to detect *L. infantum* DNA, such as splenic, bone marrow or lymph node aspirates, skin fragments, blood, and conjunctival swabs [10]. At the time of tissue selection, factors such as the invasiveness of the protocol, the biology of *L. infantum*, and the animal's clinical condition must be considered. Symptomatic animals tend to present higher parasite burden throughout their organism when compared to asymptomatic ones [18]. Less invasive collection protocols are preferable to epidemiological studies, such as blood, conjunctival swab or skins

fragments [10,19]. However, in order to confirm *Leishmania* infeccion, the spleen is considered a key site for the accumulation and multiplication of *L. infantum* even in the early infection, since the parasite exhibits a known tropism to lymphoid tissues [19,20]. Thus, even though splenic aspiration is an invasive procedure, it is a safe sampling technique for the parasitological detection of *Leishmania infantum* [21], being a suitable sample to be used by veterinary practitioners, since splenic parasite density is high throughout the course of CVL in both symptomatic and asymptomatic dogs [19,20].

Despite the accuracy of cPCR and qPCR in *Leishmania* DNA detection, some questions regarding the validity of test results still linger, mainly arising from the quality of the extracted DNA used in reactions with false-negative results. The main approach to address these issues is to perform a parallel PCR to confirm the integrity of constitutive genes in the host genome, preferably within the same reaction (i.e., in a multiplex format). Several housekeeping genes, such as GAPDH, RNase P, and β-actin, have already been used as internal controls to ensure the DNA template's integrity and prevention of false-negative results [22–26], improving the quality of the result.

However, a major drawback of the PCR technique is the requirement for a controlled, stable temperature of -20 ^{o}C during transport and storage of reagents (a.k.a. "cold chain"), which steeply increases the costs and in extreme cases precludes the adoption of qPCR as a diagnostic tool. In the last years, several technologies have emerged trying to solve this issue. Among them, gelification is particularly useful for its easiness of use at the laboratory bench as well as introduction into a production line. The gelification process stabilizes the qPCR reagents allowing the complete, ready-to-use reaction to be

transported at room temperature and stored at 4 °C for extended periods [27–29].

In order to improve the diagnostic protocols used to assess *Leishmania* infection in dogs, the present study aimed to develop a gelified duplex qPCR for the identification of infected animals. The gelified duplex qPCR was designed to simultaneously identify both a conserved sequence of the host, as well as a conserved repetitive sequence of *L. infantum* parasites in three clinically-important canine tissues (blood, spleen and skin). We also show that the duplex qPCR can be pre-loaded onto the 96-well plates in a ready-to-use format. This format allows the user to add only water and the extracted DNA, preventing mistakes during reagents manipulation as well as decreasing the hands-on time needed for launching a reaction. These two features are highly praised in a routine diagnostic laboratory and can expedite the sample-to-answer process, while also increasing the robustness of the test. Furthermore, since the ready-to-use format can be transported and stored at above-freezing temperatures, users can expect a reduction in transportation/storage associated costs.

Methods

1. Serological and Parasitological Tests

We employed the serodiagnostic protocol recommended by the Brazilian Ministry of Health to detect anti-*Leishmania* antibodies, consisting of a rapid test (DPP® CVL, Bio-Manguinhos Unit, Rio de Janeiro, Brazil) followed by a confirmatory test (EIE® CVL, Bio-Manguinhos Unit, Rio de Janeiro, Brazil). Serological tests were performed in accordance with manufacturer instructions. DPP® CVL consist in a chromatographic immunoassay for antibodies detection,

based on Dual-Path Platform technology in which rK28 *Leishmania* antigens are impregnated on a nitrocellulose membrane strip [30]. EIE CVL is an ELISA immunoassay to detect anti-Leishmania antibodies which uses crude *Leishmania major* extracts as antigen (as described in the test´s manual).

Parasitological evaluation of splenic aspirate was performed as previously described [31,21]. Briefly, part of the splenic aspirate collected was cultured in Novy–MacNeal–Nicolle biphasic medium supplemented with 20% Fetal Bovine Serum (Gibco BRL, New York, USA) and 100 mg/mL gentamicin to prevent contamination (Sigma Chemical Co., St. Louis, MO) during four weeks at 24°C . Parasites presence was assessed using microscopy during four weeks. Each splenic culture was cultured in duplicate. All of the culture labels were double-checked to avoid misidentification.

2. Canine Tissue Sampling

A random sample of 82 dogs was selected from a cross-sectional study conducted in the municipality of Camaçari, located in the State of Bahia, North-eastern Brazil (latitude: 12º 41' 51" S; longitude: 38º 19' 27" W) during 2015. Dogs were examined and classified according to presence of clinical signs such as: emaciation; anemia; periocular dermatitis; crusts or ulcers in the ears; despigmentaion, hyperkeratosis or lesions in the muzzle; splenomegaly; linfoadenomegaly; alopecia; exfoliative dermatitis; and onychogryphosis. Each clinical sign was given a grade of 0, 1 or 2 depending on the intensity of the clinical manifestation. Clinical score was calculated as the sum of grades for each clinical sign, and could then range from 0 to 24 points. Infected animals with a clinical score of ≤ 3 were classified as asymptomatic dogs and a clinical

score of ≥ 4 were categorized as symptomatic dogs [32]. As controls dogs, three healthy soronegative CVL dogs resident in an non endemic area for CVL were used.

All samples were previously characterized by splenic aspirate culturing and were used to validate the new duplex qPCR. The canine tissue samples were collected from all 82 dogs as follows: 10 mL of blood by cephalic puncture, 500 µL of splenic aspirate by ultrasound guided aspiration, and a skin fragment from the lateral scapular region using a sterile 3 mm punch (Kolplast, Brazil). Both skin and splenic samples were collected after performing trichotomy under cutaneous anesthesia with 500 µL of 1% lidocaine hydrochloride (Hypofarma, São Geraldo, MG - Brazil). Samples of blood, splenic aspirate and skin were obtained from CVL negative dogs and used as negative reaction controls. Blood samples were preserved in EDTA-2Na tubes (Greiner bio-one, Kremsmunster, Austria) and all other samples were stored in DNAase- and RNAase-free tubes at -70 ^{o}C until DNA extraction.

3. Sample preparation

DNA from blood, splenic aspirate and skin samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. *L. infantum* (MHOM/BR2000/MERIVALDO) or *L. major* (MHOM/RI/WR-173) DNA was obtained from promastigotes cultivated in Schneider's Complete Medium supplemented with 20% bovine fetal serum at 24 °C [31]. *L. infantum* promastigotes were counted on Neubauer chambers and adjusted to $4x10^7$ parasites and sequentially washed in saline solution to remove culture medium. The pellet formed was used to perform DNA extraction together

with blood, splenic aspirate or skin from the control animals, using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

4. Duplex qPCR protocol

We designed a duplex qPCR in which we simultaneously amplify and detect *L. infantum* kDNA and a conserved region of the canine housekeeping gene 18S rRNA (gb|DQ287955.1) *Leishmania* kDNA amplification and detection used the following oligonucleotides [13]: LEISH-1, 5'-AACTTTTCTGGTCCTCCGGGTAG-3'; LEISH-2, 5'-ACCCCCAGTTTCCCGCC-3'; and LEISH-P, 5'-FAM-AAAAATGGGTGCAGAAAT-MGB/NFQ-3' (Life Technologies). For amplification and detection of the canine 18S rRNA sequence, we used the following new primers and probe: 18SCanis_F, 5'-TGCGAATGGCTCATTAAATC-3'; 18SCanis_R, 5'-CGTCGGCATGTATTAGCTCT-3'; and 18SCanis_P, 5'-HEX-TGGTTCCTTTGGTCGCTCGCT-BHQ1-3' (Biosearch Technologies, CA, USA).

qPCR reactions contained the Multiplex PCR Mastermix (IBMP/Fiocruz-PR, Curitiba, Brazil), 9 mM magnesium acetate ($Mg(OAc)_2$) and 2 mg/mL BSA (molecular biology grade, Roche) as final concentrations. The singleplex reaction for detection of *Leishmania* kDNA also contained 5 µL of extracted DNA (varying from 50 to 500 ng, as measured by UV absorbance at 260 nm), 200 nM of LEISH-P, 900 nM of each primer LEISH-1 and LEISH-2, and DNAse/RNAse-free H_2O to 25 µL. The duplex reactions contained the same reagents as the singleplex protocol, supplemented with 160 nM of each primer 18SCanis_F and 18SCanis_R, and 40 nM of the 18SCanis_P.

For clarity purposes, the terms “liquid” and “traditional” refer to reactions performed using reagents stored in freezer (-20 oC), which have to be manipulated in the traditional way (i.e., thawed before use). The terms “gelified” and “ready-to-use” refer to reactions performed using reagents that can be store above freezing temperatures, and do not need to be manipulated by the user since they are preloaded into the reaction vessels.

Gelified reactions were produced by mixing qPCR reagents (enzyme, buffer, salts, nucleotides, primers and probes), at final concentrations, with a gelification solution [28,29,33]. This mixture was then aliquoted onto the 96-well plates, and the plates were submitted to 3 cycles of vacuum (30 mBar) of 30 minutes each, at constant temperature (30 ^{o}C), as previously described [28,34]. Both liquid and gelified reactions were run in an ABI7500 Fast Real-Time PCR System (Life Technologies) with the following temperature profile: 1x 50 ^{o}C/2 minutes; 1x 95 ^{o}C/10 minutes; 45x [95 ^{o}C/15s, 60 ^{o}C/60s].

Seven-point standard curves for each tissue type (blood, spleen or skin) were prepared using DNA extracted from 2 x 10^5 *L. infantum* or *L. major* promastigotes per microliter as starting material. The extracted DNA was 10-fold serially diluted in *Leishmania*-negative tissue-specific canine DNA (10-100 ng/µl, as measured by UV absorbance at 260 nm) obtained from healthy animals. All reactions were performed in triplicate and data are expressed in terms of cycle threshold (Ct) mean values. For each tissue type, relevant Ct cut-off values were calculated by Receiver-Operator Characteristic (ROC) curve analysis to determine positivity for *L. infantum* or *L. major* DNA, as previously described [31]. Ct cut-off values presenting 100% of specificity and the higher sensitivity values were chosen for each tissue type. Quantification of

Leishmania kDNA was performed using an absolute method by comparing the mean Ct values obtained from each tissue type to the Ct values on the corresponding seven-point standard curve.

5. Statistical Analysis

To prevent any bias, results of the molecular diagnostic techniques were read without any knowledge of the other serological or parasitological results. ROC analysis, used to establish the Ct cut-off value, was performed using GraphPad Prism software v.5.0 (GraphPad Prism Inc., San Diego, CA). For each tissue type analyzed, corresponding sensitivity and 95% confidence intervals (CI) were calculated using splenic culturing as the reference standard. Comparisons among singleplex and duplex results for each individual sample were evaluated by the Wilcoxon signed rank sum test. The X^2 or Fisher tests were used to compare diagnostic methods results among all animals included in the study and between assyntomatic and symptomatics dogs. McNemar's test was used to assess results among the same samples tested in singleplex relative to the duplex protocol or in the regular format ("Liquid") and the ready-to-use format ("Gel"). A p-value below 0.05 was considered statistically significant.

Results

The existence of singleplex reactions is not a guarantee that the corresponding duplex qPCR will exhibit the same characteristics, such as efficiency and limit of detection. Losses of sensitivity and specificity may be due to competition of targets for the enzyme or unspecific interactions between the two sets of oligonucleotides or with the genomic targets. That is, the main issue

in the development of multiplex PCR assays is the difficulty of adjusting two or more different reactions occurring simultaneously without losing accuracy [35]. Therefore, we optimized the singleplex reaction described by Francino *et al.* [13] into a duplex reaction using qPCR reagents produced at our own production facility. Figure 1A shows the detection of 10-fold dilutions of *L. infantum* DNA performed in the singleplex (blue lines) or duplex format (red lines). There is virtually no difference between the detection of each dilution point. Singleplex and duplex reactions exhibited efficiency of 101.84 and 105.47 and an equal limit of detection of 0.05 parasites/µl, respectively. For clarity purposes, the detection of the second target in the duplex reaction depicted in Figure 1A is shown only in Figure 1B. Figure 1B depicts a typical duplex reaction which concomitantly detects the *L. infantum* DNA dilutions (red lines) and the constitutive 18S gene (blue lines). Detection of the 18S gene in these samples was possible because the *L. infantum* promastigotes DNA was diluted in DNA extracted from *Leishmania*-negative canine splenic aspirate, as described in Materials and Methods. Mean Ct intervals for the detection of the conserved canine region for each tissue were established as follows: 15.9-19.9 for blood, 16.6-19.0 for skin, and 17.5-19.8 for splenic aspirate samples. All samples used in this study presented mean Ct values within these ranges, thus considered suitable for *Leishmania* diagnosis assessment.

Figure 2 shows a direct comparison between the detection cycle of *L. infantum* DNA in canine blood samples analyzed with both the singleplex and the duplex reaction. Figure 2A shows that some samples were detected in a higher Ct in the duplex format, suggesting loss of sensitivity. Figure 2B shows that the difference observed between the detection cycles with the singleplex and the

duplex format ranged from -4.1 to +0.6, meaning that some samples were detected 4.1 Cts higher in the duplex relative to the singleplex format. On the other hand, some samples did not change their detection level, or were detected earlier. However, the overall difference between reactions is negligible, averaging a mean Ct value slightly higher than 1 (1.22) for the singleplex relative to the duplex protocol (p=0.0248) (Figure 2B). Furthermore, it is important to highlight that the observed difference did not affect qPCR positivity in our study, irrespective which protocol was used (p=1.0, McNemar's test). That is, all samples considered positive by the singleplex reaction were also considered positive by the duplex reaction.

Figure 3 shows the reportable dynamic range for *L. infantum* DNA quantification by qPCR when the parasite's DNA was diluted in DNA extracted from Leishmania-negative blood, skin or splenic aspirates. Reactions performed in all three DNA matrices exhibited a successful linear detection of up to 0.1 parasite per reaction. It should be noted that our reaction was able to detect 0.05 parasite per reaction (Figure 3, stars), although not always in the linear range of detection.

DNA amplification efficiency can be affected by several parameters, such as presence of inhibitors in the sample or suboptimal concentration of oligonucleotides (primers and/or probe), and can be estimated by the slope of the dilution curve detection. As described in Figures 1-3, we optimized the qPCR so that the mean amplification efficiency calculated from at least five independent standard curves was 96.8% for blood or skin samples (slope = -3.4±0.1 for each), and 105.4% for splenic aspirate samples (slope = -3.2±0.2), with corresponding R^2 of 0.99 irrespective of the tissue.

We then used a convenience sample to test our duplex qPCR, so that we were able to ran a ROC analysis, determine Ct cut-off values and maximize the reaction's sensitivity and specificity. Cut-off values for blood samples was established at Ct 42.99, with prediction rates of 100% sensitivity (CI 73.4– 100) and 100% specificity (CI 71.5–100); for skin samples, a Ct cut-off value of 40.0 provided prediction rates of 100% sensitivity (CI 73.5–100) and 100% specificity (CI 69.2–100); finally, in splenic aspirate samples, the Ct cut-off value of 38.2 corresponded to prediction rates of 91.67% sensitivity (CI 61.5–99.8) and 100% specificity (CI 73.5–100). ROC analysis for the three tissues revealed an area under the curve of 1.0, indicating a high probability ($p < 0.001$) that any randomly chosen positive sample would be correctly classified.

Although the duplex qPCR described here was designed for detection of *L. infantum* DNA, we also analyzed it using DNA extracted from *L. major*, *L. amazonensis*, or *L. braziliensis* promastigotes. Our results suggest that only *L. major* DNA exhibits similar qPCR profile and limit of detection as *L. infantum* DNA, while detection of DNA from 10^5 *L. amazonensis* promastigotes is at a similar Ct than DNA from 10^0 *L. infantum* promastigotes. We also tested the analytical specificity of the newly designed canine 18S rRNA oligonucleotides and found no detectable qPCR amplification using DNA samples of *Leishmania* spp (data not shown).

Next, blood, skin and splenic aspirate samples from 82 dogs were used to evaluate our duplex qPCR protocol. All the dogs were previously characterized by the reference method of splenic aspirate culturing, by which 42 were considered positive and 40 negative. The results from each diagnostic technique employed using different tissues are compared in Table 1. The qPCR of splenic

aspirate samples was able to detect *L. infantum* DNA in 92.9% of the splenic culturing from positive animals, followed by 50% when using skin samples, and 35.7% when using blood samples. Concerning these diagnostic techniques results, no statistically significant differences were noticed. Regarding the clinical status, duplex qPCR of splenic aspirate detected more positive samples than the other detection methods, but not in a significant way, identifying 72% as positive among asymptomatic and 77.8% among symptomatic dogs (Table 2). Among dogs with negative splenic culturing tests, qPCR from splenic aspirate samples was able to detect *L. infantum* DNA in 45% of these animals, while qPCR from skin detected *L. infantum* DNA in 42.5% and qPCR from blood detected *L. infantum* DNA in 27.5%. Results presented in Table 2 provide convincing evidence of the high positivity rates derived from the qPCR technique in comparison to parasitological and serological diagnostic methods.

Since qPCR reagents are temperature-sensitive, extreme care must be taken to keep them below -20 ^{o}C during transportation and storage. To overcome this hurdle, we applied the gelification technology to our reagents to store them in a ready-to-use format, pre-loading them onto the plates inside the production facility. Figure 4 shows that the reportable range for linear *L. infantum* DNA detection is not significantly affected by the gelification process (Fig 4A). Although the observed limit of detection for the gelified reaction was found to be 1 parasite/reaction, we were able to detect 0.1 parasite/reaction outside the linear range of detection.

Interestingly, parameters such as amplification efficiency and linearity (R^2) obtained by the gelified duplex qPCR were comparable to those obtained with the regular, liquid reaction format (Fig 3 and Fig 4A). Amplification efficiency for

the detection of *L. infantum* DNA by the gelified qPCR in blood, skin or splenic aspirate samples was 84.7%, 81.3%, and 82.4%, respectively. R^2 was 0.96 for all three reactions.

A direct comparison between the same samples tested with the regular format ("Liquid") and the ready-to-use format ("Gel") shows that there is no significant difference between them using McNemar's test (Fig 4B). Although some samples clearly had their detection affected by the gelification process, on average the delay was less than 1 Ct in the detection by the gelified reaction versus the liquid format. Figure 4C summarizes all the differences in Ct observed for the detections in Figure 4B.

Discussion

The successful output of a qPCR is directly correlated to the quality of the sample and/or its extracted DNA. One straightforward strategy to control the quality of the sample and, at the same time, the overall quality of the system (reagents, instrument and analysis software) is to amplify a housekeeping gene [22–26]. We developed a new reaction for the detection of the canine 18S rRNA gene and combined it with a published reaction for detection of *L. infantum* [13] into a new duplex qPCR that concomitantly detects the target and the internal control gene. Our results demonstrate that the duplex qPCR format did not affect the analytical sensitivity of *L. infantum* DNA amplification relative to the single reaction format (Figures 1 and 2). The detection of less than one parasite per reaction is possible because the target gene is present in multiple copies in the genome, thus being dissolved into solution when the parasites are lysed.

For *Leishmania* spp, the target gene is present at approximately 50 copies per parasite genome [17].

The duplex protocol was successfully used for concomitant detection of leishmania and canine DNA targets in blood, skin or splenic aspirate samples (Figure 3). It is important to highlight that the detection of the highly abundant 18S gene did not affect the detection of leishmania DNA, even when the latter was present in much lower amounts (Figure 1). Blood and skin are known to contain qPCR inhibitors, such as hemoglobin, HSA (human serum albumin), melanin and collagen [36], which were most likely removed during nucleic acid extraction. Our results corroborate that hypothesis, since we did not observe any meaningful difference between the qPCR, liquid or gelified, performed with samples originated from spleen, blood or skin. Therefore, when we consider the efficiency of the qPCR and the limit of detection, blood, skin or splenic aspirates are equally good sources for detection of *Leishmania* DNA.

The present study did not attempt to extensively test the analytical specificity of the new qPCR with respect to *Leishmania* DNA detection. However, our preliminary assessments show that our reaction detects *L. major* with the same sensitivity as *L. infantum*. Interestingly, our reaction would also be able to detect the presence of *L. amazonensis* in a sample with high parasitemia, because the detection of 10^5 *L. amazonensis* promastigotes is similar to the detection of 10^0 *L. infantum* promastigotes.

Although qPCR is a powerful and sensitive technique, the absolute requirement for maintaining freezing temperatures is a critical point and a major hurdle for its adoption as a routine diagnostic tool. This is specially true for *Leishmania* and other neglected tropical diseases detection because they

usually are endemic to countries with deficient lab infrastructure, where a point of care test would be most necessary. Therefore, almost as a *sine qua non* condition, a point of care test should not be influenced by the transportation/storage temperature [27]. Several technologies have been used in the last years to overcome this obstacle, of which the gelification technology stands out for being less harsh on the sensitive qPCR reagents [28,29]. Indeed, pre-storage of gelified qPCR reagents on the reaction vessel has been previously done, aiming the detection of *Plasmodium* spp [30, 31], *Campylobacter* spp [28] or *T. cruzi* [31]. Our results show that the gelified qPCR for detection of *Leishmania* DNA exhibits similar clinically-relevant linear detection limit as the traditional liquid format, irrespective of the tissue used as sample matrix (Figure 4). This format has previously been shown to allow qPCR reagents to be stored at 4 ºC for up to 12 months or at 22 ºC for 1-3 months without significant loss of sensitivity [28,29,34]. Furthermore, pre-loading the reaction in a ready-to-use format directly into the reaction vessel decreases the manipulation of reagents and minimizes the risk of operator mistakes.

We used our duplex qPCR to analyze 82 naturally-infected dogs and observed that, among the positive animals, splenic aspirates are the most effective tissue for detecting *L. infantum* infection, and should be preferred over blood or skin if sanitary conditions allows proper collection. We believe that the known spleen tropism of the parasite is the explanation for the higher positivity in splenic samples [37]. However, we suggest that skin should be considered as a viable alternative tissue for sample collection in environments with little-to-none infrastructure or skilled personnel, mainly because the collection procedure is less invasive and require less technical skills than splenic aspirate.

In addition, since skin lesions are known to contain live parasites [38], skin samples with active infection may have increased probability of *Leishmania* spp detection, and possibly increased diagnostic positivity.

Although parasitology and serology are advantageous due to their simplicity vis-à-vis molecular techniques, they are nonetheless limited and can lead to misleading results, thereby neglecting the presence of *Leishmania* infection in dogs [15,31]. In light of this consideration, we suggest that less specific testing techniques should be used for screening and more specific techniques such qPCR should be subsequently applied as a confirmatory test. This protocol would greatly enhance the accuracy of LV diagnosis and lead to an important improvement in control efforts.

Conclusion

We present a duplex qPCR able to detect *Leishmania infantum* or *L. major* and canine genomic DNA in a single reaction. Detection of host DNA in the same reaction strengthen CVL diagnosis by acting as an internal reaction control. The duplex and singleplex qPCR have similar performance in detecting *L. infantum* infection, with a clinically relevant detection limit, irrespective of the tissue tested (blood, skin or splenic aspirate) or the reagents storage format (liquid or gelified). Moreover, the duplex qPCR described herein offered greater sensitivity in comparison to the methods routinely used for CVL diagnosis.

List of abbreviations

VL – visceral leishmaniasis

CVL – canine visceral leishmaniasis

DPP® CVL – Dual Path Platform test for CVL diagnosis

EIE® CVL – enzyme-linked immunosorbent assay (ELISA) test for CVL diagnosis

cPCR – conventional PCR

qPCR – quantitative PCR

kDNA – kinetopast DNA

Ct – cycle threshold

ROC – Receiver-Operator Characteristic

CI – confidence interval

Declarations

Ethics Statement

All canine tissue samples used in this study and their specific collection procedures were approved by the CPqGM - FIOCRUZ Institutional Review Board for Animal Experimentation under Permit Number 007/2013.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors's contributions

RP and MS performed the qPCR experiments, analyzed the results, performed the statistical analyses, and drafted the manuscript. LS, LP and JJ extracted the DNA from the different tissues and performed the parasitological (splenic culturing) and serological experiments (EIE and DPP). PV, DF, and MK conceived and designed the study. AC analyzed the results, participated in the

conception and design of the study, and in the writing of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Reportable range for *L. infantum* DNA quantification by qPCR. *Panel A* shows the amplification plot of 10-fold serial dilutions of parasite DNA (ranging from 0.1 to 10^5 parasites) detected using singleplex (blue lines) or duplex (red lines) qPCR protocols. The thick horizontal red line represents the detection threshold. The duplex qPCR was carried out using *Leishmania infantum* DNA serially diluted into DNA extracted from leishmania-negative splenic aspirate. *Panel B* shows the amplification plot the duplex reaction alone, depicting the detection of *L. infantum* DNA (red lines) as well as the canine 18S gene (blue lines). The thick horizontal red line represents the detection threshold for *L. infantum* qPCR while the thick horizontal blue line represents the detection threshold for the 18S qPCR. Each concentration of DNA was tested in triplicates. Curves are representative of at least three independent experiments.

Figure 2. Comparison of threshold cycles between the singleplex and the duplex qPCR for detection of *Leishmania* DNA. *Panel A* shows the corresponding difference in Ct values obtained from the qPCR analysis of individual samples tested by singleplex or duplex protocols. The same sample was tested independently by both protocols, and the threshold cycles were plotted and connected by dotted lines. *Panel B* plots the differences between Ct values from the same sample using singleplex or duplex reactions, showing that the mean difference is less than 1 Ct (thick horizontal line). Comparison among singleplex and duplex results for each individual was evaluated by Wilcoxon signed rank sum test ($p<0.05$).

Figure 3. Comparison of dynamic ranges between *L. infantum* DNA detection in blood, skin or splenic aspirates. Ct values obtained for duplex qPCR reactions are plotted against known concentrations of parasite DNA (ranging from 0.1 to 10^5 parasites) diluted in leishmania-negative DNA from blood, skin or splenic aspirate. DNA representing 0.05 parasite's genome were stochastically detected, thus being shown as stars outside the linear regression. Mean reaction efficiencies from at least three independent experiments were respectively 96.8% for blood and skin samples,and 105.9% for splenic aspirate samples, all three with a R^2 of 0.99.

Figure 4. Detection of *L. infantum* DNA using a gelified, ready-to-use duplex qPCR. *Panel A* shows 10-fold serial dilutions of *L. infantum* DNA (ranging from 0.5 to 10^5 parasites) diluted in leishmania-negative DNA from blood, skin or splenic aspirates using the duplex qPCR in a gelified, ready-to-use format. Mean reaction efficiencies from at least three independent experiments using blood, skin or splenic aspirate samples were respectively 84.7 ± 2.5; 81.3 ± 1.1; and 82.4 ± 1.2, all three with a R^2 of 0.96 or higher. *Panel B* shows a direct comparison between the detection of a given sample by both reaction formats (liquid versus gelified, "gel"). *Panel C* is a plot of the differences between the threshold cycles of each individual detection performed in Panel B, showing that the mean difference is less than 2 Ct (1.68).

Tables

Table 1. Positive results of CVL diagnostic method (shown as %) performed on canine samples from 82 animals, which were divided into two groups according to splenic culturing result.

Diagnostic method	Positivity n (%)		
	Splenic culturing positive (n=42)	Splenic culturing negative (n=40)	Total (n=82)
qPCR Splenic aspirate	39 (92.9)	18 (45)	57 (69.5)
qPCR Skin	21 (50)	17 (42.5)	38 (46.3)
qPCR Blood	15 (35.7)	11 (27.5)	26 (31.7)
DPP® CVL	30 (71.4)	20 (50)	50 (61)
EIE® CVL	23 (54.8)	10 (25)	33 (40.2)

Table 2. Positive results of CVL diagnostic method (shown in %) performed on canine samples from 76 animals that tested positive in at least one method employed. Animals were divided into asymptomatic or symptomatic groups based on clinical score.

Diagnostic method	Positivity n (%)		
	Asymptomatic dogs (n = 40)	**Symptomatic dogs (n=36)**	**Total (n= 76)**
qPCR Splenic aspirate	29 (72.5)	28 (77.8)	57 (75)
qPCR Skin	19 (47.5)	19 (52.8)	38 (50)
qPCR Blood	12 (30)	14 (38.9)	26 (34.2)
DPP® CVL	28 (70)	22 (61.1)	50 (65.8)
EIE® CVL	16 (40)	17 (47.2)	33 (43.4)
Splenic culturing	22 (55)	20 (55.6)	42 (55.3)

Figure1

Click here to download Figure Figure 1 - qPCR curve.tiff

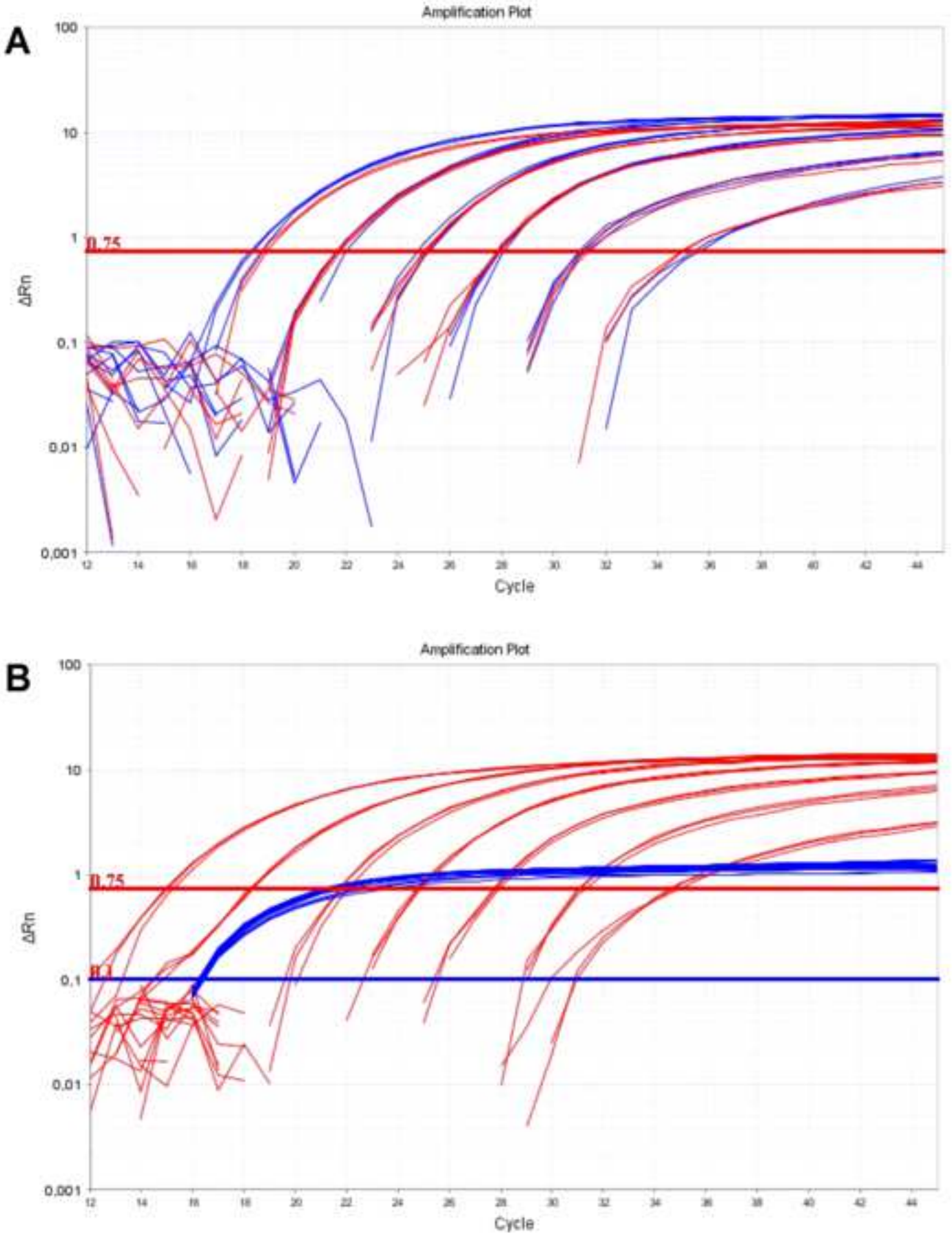

Figure2

Click here to download Figure Figure 2 - Singleplex multiplex.tiff

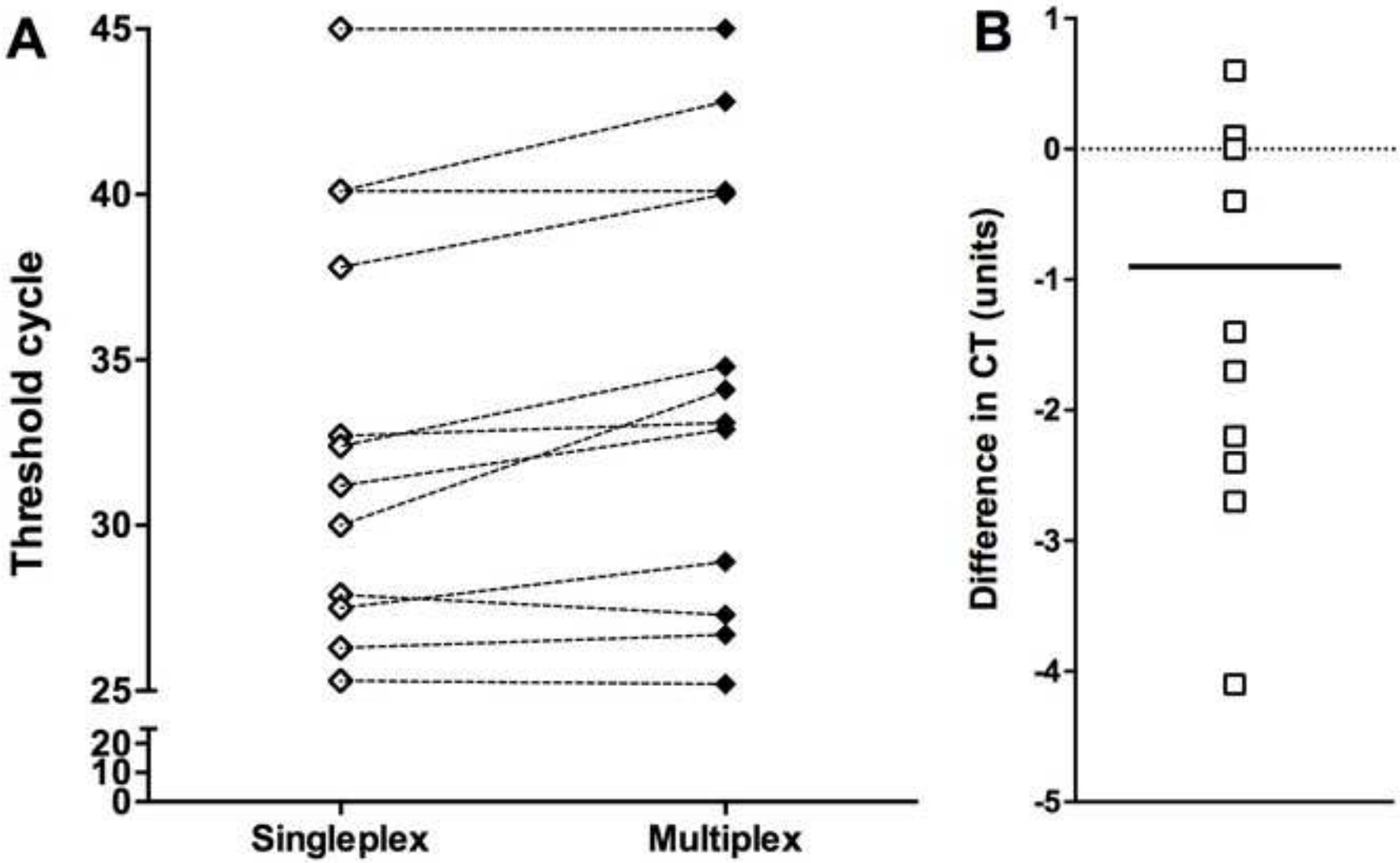

Figure3

Click here to download Figure Figure 3 - Blood Spleen and Skin LIQUID.tiff

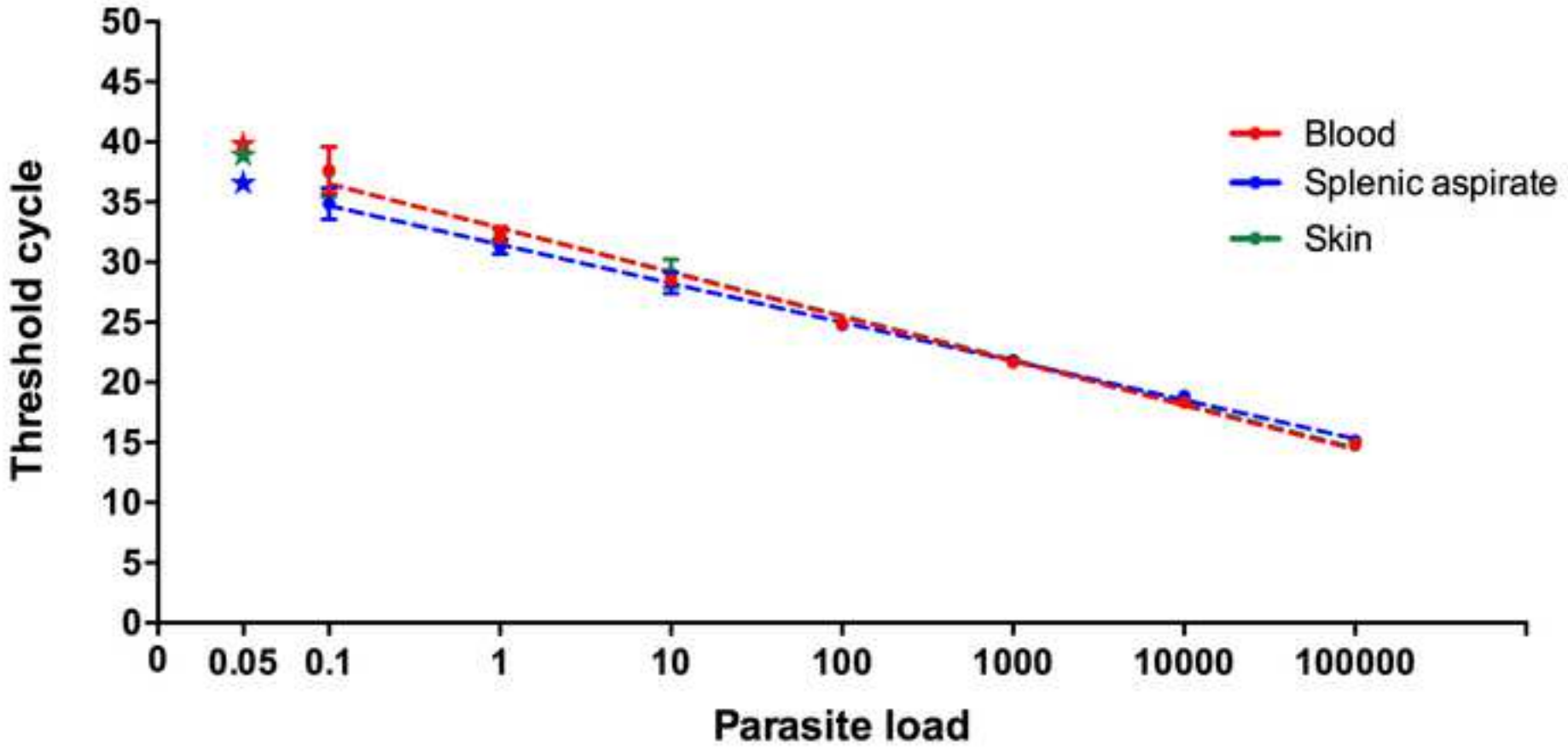

Figure4

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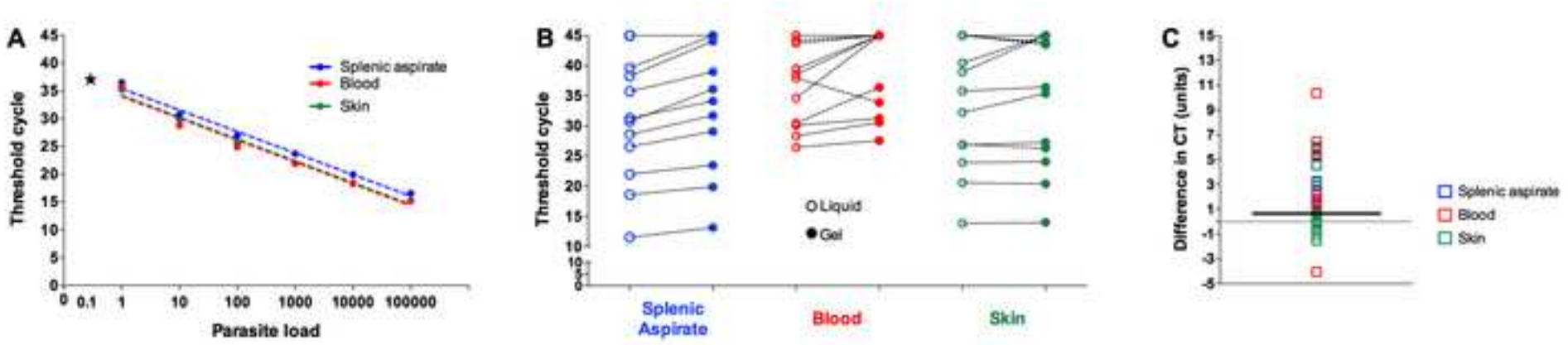


CAPÍTULO 2 – Estudo exploratório para identificação de biomarcadores de gravidade de uma amostra de cães coletada durante um estudo de corte transversal em Camaçari-BA

Nesta segunda etapa, foi realizado um estudo exploratório para identificação de biomarcadores de gravidade de amostras coletadas durante um estudo de corte transversal da população canina de Camaçari-BA, uma área endêmica para LVC. A escolha de Camaçari para este estudo deve-se ao fato de que casos caninos e humanos de LV vem sendo diagnosticados nessa região nos últimos anos, tendo sido relatados por diferentes estudos (CUNHA *et al.*, 1995; JULIÃO, 2004; BARBOZA *et al.*, 2006; GOMES NETO, 2007; SILVA *et al.*, 2010). Adicionalmente, o presente grupo de pesquisa realizou um estudo de corte transversal, nos anos de 2011 e 2012, identificando alta prevalência de LVC em diferentes bairros do município. Baseado nesses resultados prévios, para a realização desse estudo foram escolhidas regiões que apresentaram as maiores prevalências para LVC no período de 2011-2012, as localidades de Jauá, Pé de Areia, Barra do Jacuípe e Machadinho com 46%, 51%, 51,3% e 21,1% de prevalência de LVC, respectivamente (BORDONI, 2014).

O principal objetivo do presente estudo foi identificar diferentes possíveis biomarcadores, e correlacioná-los com a gravidade da infecção por *L. infantum* apresentada pelos animais examinados. A identificação de biomarcadores relacionados com a gravidade da LVC pode ser uma ferramenta interessante tanto para definir o prognóstico para o animal doente e direcionar seu tratamento, nos países onde este é permitido; assim como também para avaliar sua participação ativa no ciclo de transmissão da LV, uma vez que já é bem descrito na literatura que cães com a forma ativa e mais grave de LVC tendem a transmitir um maior número de parasitos para o flebotomíneo (DA COSTA-VAL *et al.*, 2007; MICHALSKY *et al.*, 2007; VERCOSA *et al.*, 2008).

Desta forma, neste estudo de corte transversal, foram avaliadas as hipóteses de associações entre a presença de biomarcadores de exposição à saliva do vetor e biomarcadores inflamatórios com a gravidade da infecção por *L. infantum*, que

podem revelar associações de interesse e poderão ser validadas no futuro em um estudo longitudinal que está sendo desenvolvido paralelamente ao presente estudo.

2.1 OBJETIVO GERAL

- Avaliar biomarcadores de exposição à saliva do vetor e identificar biomarcadores inflamatórios, correlacionando-os com a gravidade da LVC e carga parasitária.

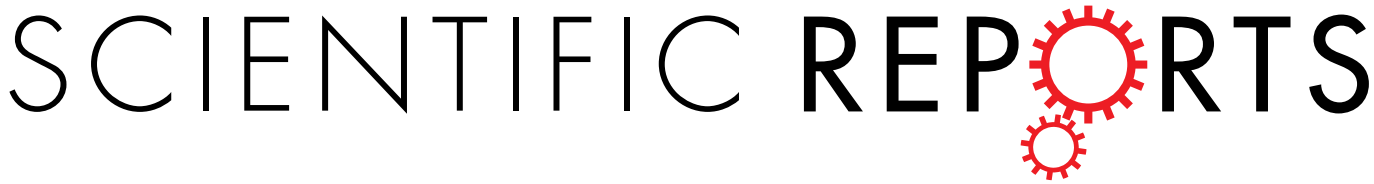
2.2 OBJETIVOS ESPECÍFICOS

- Avaliar LJM 11/LJM 17 como biomarcadores imunológicos de exposição à saliva do vetor;
- Realizar análise exploratória de diferentes biomarcadores inflamatórios, de ativação imune e de estresse oxidativo de LVC;
- Avaliar a presença de correlação entre os biomarcadores imunológicos de exposição à saliva do vetor, inflamatórios, de ativação imune e de estresse oxidativo com a gravidade de LVC e carga parasitária.

2.3 Artigo científico publicado na revista *Scientific Reports*

Manuela S. Solcà, Bruno B. Andrade, Melissa Abbehusen, Clarissa Teixeira, Jesus G. Valenzuela, Shaden Kamhawi, Ricardo Khouri, Patrícia Torres Bozza, Deborah Bittencourt Mothé Fraga, Valeria Matos Borges, Patrícia Sampaio Tavares Veras, Claudia Ida Brodskyn. **Circulating Biomarkers of Immune Activation, Oxidative Stress and Inflammation Characterize Severe Canine Visceral Leishmaniasis**. 2016.

No presente estudo foram avaliados simultaneamente biomarcadores imunológicos de exposição à saliva do vetor, biomarcadores de inflamação, ativação imune e estresse oxidativo em cães com diferentes quadros clínicos de LVC, visando identificar biomarcadores associados com a gravidade da doença clínica. Foi realizado um estudo exploratório de amostras de cães coletadas durante um estudo de corte transversal, em Camaçari-BA. A análise identificou uma bioassinatura distinta em cães com diferentes manifestações clínicas, caracterizada por uma diminuição dos níveis de LTB-4 e de PGE-2 de acordo com o agravamento da doença, e um aumento de CXCL1 e CCL2. Além disso, utilizando uma combinação de 3 parâmetros diferentes (LTB-4, PGE-2 e CXCL1) fomos capazes de discriminar entre escores clínicos diferentes pela construção de uma curva ROC. Foi detectado também, que cães com escores clínicos elevados apresentaram-se, mais frequentemente, com negatividade para IgG anti-saliva e elevadas cargas parasitárias. Este estudo permitiu a avaliação e identificação de vários biomarcadores em cães, que podem ser importantes para auxiliar, na avaliação do curso da doença e prognóstico pelo médico veterinário que estiver atendendo um cão com LVC, além de futuramente poder ajudar na distinção entre cães resistentes ou susceptíveis direcionando estratégias de controle da LVC em áreas endêmicas.



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Circulating Biomarkers of Immune Activation, Oxidative Stress and Inflammation Characterize Severe Canine Visceral Leishmaniasis

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Clinical manifestations in canine visceral leishmaniasis (CVL) have not been clearly associated with immunological status or disease progression. We simultaneously assessed biomarkers of inflammation, immune activation, oxidative stress, and anti-sand fly saliva IgG concentrations in dog sera with different clinical manifestations to characterize a biosignature associated with CVL severity. In a cross-sectional exploratory study, a random population of 70 dogs from an endemic area in Brazil was classified according to CVL clinical severity and parasitological evaluation. A panel of biomarkers and anti–sand fly saliva IgG were measured in canine sera. Assessment of protein expression of profile biomarkers identified a distinct biosignature that could cluster separately animal groups with different clinical scores. Increasing severity scores were associated with a gradual decrease of LTB4 and PGE2, and a gradual increase in CXCL1 and CCL2. Discriminant analyses revealed that combined assessment of LTB4, PGE2 and CXCL1 was able to distinguish dogs with different clinical scores. Dogs with the highest clinical score values also exhibited high parasite loads and higher concentrations of anti-saliva antibodies. Our findings suggest CVL clinical severity is tightly associated with a distinct inflammatory profile hallmarked by a differential expression of circulating eicosanoids and chemokines.

Visceral leishmaniasis (VL) is a widespread disease caused by the protozoan *Leishmania infantum*. This parasite is transmitted to humans and animals through the bite of the infected sand fly *Lutzomyia longipalpis*[1,2]. Dogs are considered the main urban reservoir of the parasite and its presence in the endemic area is known as a risk factor for the occurrence of human VL[3,4].

Clinical signs of canine VL (CVL) are non-specific, there is a widespread range of clinical manifestations varying from visceral to cutaneous presentation of the disease[5,6], on the other hand some animals do not display any clinical signs during the course of infection[7]. The resistance and susceptibility to CVL is directly correlated with the development of Th1 (IFN- γ, IL-2 and TNF-α), or Th2 (IL-4, IL-5, IL-10, IL-13 and TGF-β) immune

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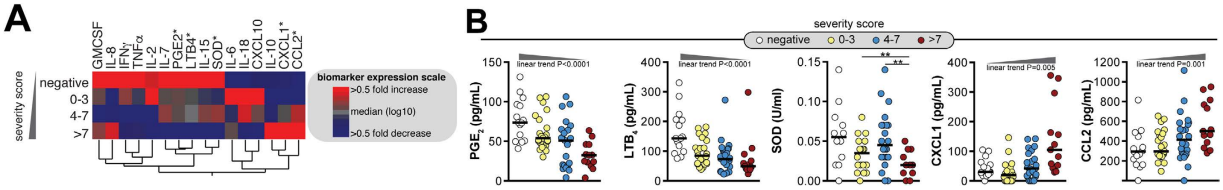

Figure 1. Distinct expression of immune and inflammatory markers in serum from dogs presenting with different VL clinical severity scores. (**A**) Hierarchical cluster analysis (Ward's method) with bootstrap was performed to depict the overall expression profile of the indicated serum biomarkers in the different study groups. (**B**) Scatter plots of biomarkers displaying significant statistical differences ($P < 0.005$) between the study groups using Kruskal-Wallis test. Non-parametric linear trend *ad hoc* tests were employed to examine the variation of the biomarker levels following the clinical severity score. SOD levels did not exhibit linear trend, thus data were compared using the Dunn's multiple comparisons test (**$P < 0.01$).

responses respectively, and the degree of immune activation is thought to directly impact the severity of disease[8,9]. Studies addressing the importance of other mediators, such as eicosanoids, Super Oxide Dismutase (SOD) and chemokines are few, especially in dogs[10,11]. Recently, our group has found the role played by LTB4 and PGE2 in modulating *Leishmania* infection in humans[11,12]. Experiments *in vitro* with human macrophages demonstrated that SOD increased the parasite burden in these cells due to the inhibition of reactive oxygen species (ROS)[10].

Knowledge of their role in CVL will further our understanding of the complex pathogenicity of the disease. Moreover, dogs constitute a model to study VL, since clinical signs in this specie have some similarities to those developed in humans[13,14], allowing its use in the study of new targets for prophylactic and therapeutic strategies.

It has been demonstrated that the production of anti-saliva antibodies in humans naturally exposed to *Lu. longipalpis* sand flies positively correlated with the development of delayed type hypersensitivity against *Leishmania*, and that in this setting, these antibodies are reported as a marker of protection against infection[15]. Notably, although dogs from endemic areas produce anti-saliva antibodies, there are no studies investigating whether these antibody levels correlate with protection, risk of disease development or transmission to sand flies.

Nonetheless, due to the complexity of CVL, there are difficulties in establishing a clear association between clinical manifestations and immunologic status. Herein, we hypothesize that only a single parameter of cellular or humoral immune responses cannot clearly define disease severity. Integrated studies of multiple biomarkers are needed to better understand their role in the outcome of *L. infantum* infection. In this cross sectional exploratory study, we identified a distinct biosignature in dogs with different clinical scores where an increase in the severity of disease was characterized by a continuous decrease in levels of LTB4 and PGE2 and an increase in levels of CXCL1 and CCL2. Additionally, using 3 different parameters (LTB4, PGE2 and CXCL1) we were able to discriminate between different clinical score ranges through the construction of ROC curves. Moreover, there is an augment in the frequency of dogs displaying anti-saliva IgG and high parasite load along with the increase of the clinical score. This study allows the evaluation of multiple biomarkers in dogs, which could be important for CVL surveillance in endemic areas.

Results

Expression of immune and inflammatory markers. After diagnosing CVL in the canine random sample 21.4% (15/70) were found to be negative for CVL whereas 78.6% (55/70) animals were infected. Clinical score evaluation on the infected dogs classified 40% (22/55) dogs with subclinical disease, 38.2% (21/55) with mild disease, and 21.8% (12/55) with severe disease.

All the biomarkers were analysed independently using univariate statistical analyses corrected for multiple observation, and only those ones that displayed significant differences among the different clinical groups were considered for the further analysis. A hierarchical clustering analysis of immune and inflammatory profiles in serum from dogs with different CVL clinical scores underlined a distinct biosignature associated with increased disease severity (Fig. 1). Remarkably, animals with higher severity scores (4–7 and >7) exhibited heightened serum concentrations of IL-10, CXCL1 and CCL2, whereas those with lower clinical scores (0–3) displayed increased levels of IL-6, IL-18 and CXCL10 relative to the average values of the entire study population (Fig. 1A). Infected dogs displayed reduced levels of several other biomarkers of inflammation and oxidative stress (Fig. 1A) when compared to uninfected ones. Amongst all the biomarkers, PGE2 and LTB4 values displayed a linear trend that decreased with disease severity (Fig. 1B). Conversely, we observed an upward linear trend in the amounts of CXCL1 and CCL2 with increasing clinical scores (Fig. 1B). In addition, dogs with a clinical score range from 4 to 7 displayed the highest serum levels of SOD, while those with the highest severity scores (>7) exhibited the lowest concentrations of this enzyme (Fig. 1B).

Network analysis of the circulating biomarkers in dogs. We next examined the relationships between the biomarkers within each clinical group using network analysis based on statistically significant Spearman correlations ($P < 0.05$). We observed that the correlations profile exhibited distinct characteristics in each study group (Fig. 2A and Supplemental File 1). In addition, in all groups, most of the observed statistically significant correlations were positive (Fig. 2A and Supplemental File 1). Increased frequency of significant negative correlations was detected in the group of uninfected animals as well as in dogs with the highest severity scores (Fig. 2A). Of note, in these two groups of dogs, the majority of statistically significant correlations in the networks involving

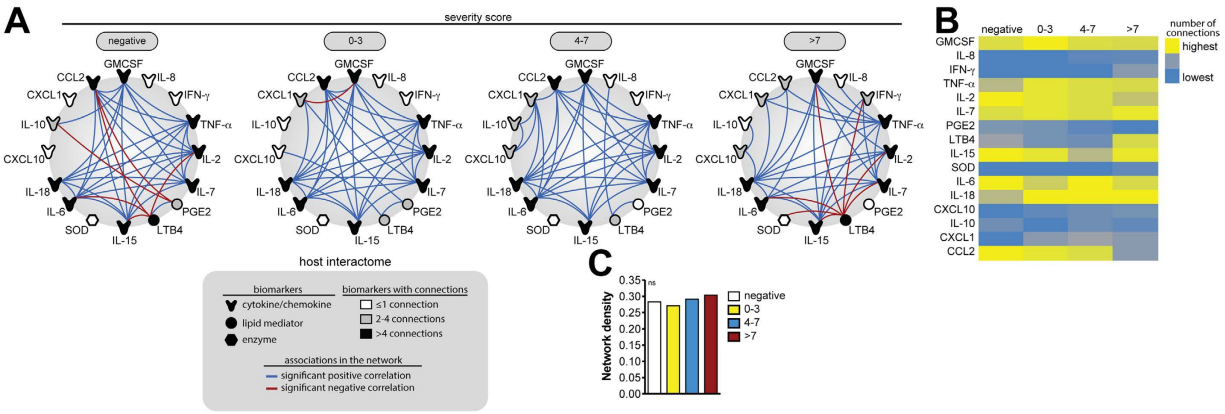

Figure 2. Network analysis of the circulating biomarkers in dogs with VL reveals a distinct biosignature of VL clinical severity. (A) The network analysis (host interactome) shows statistically significant Spearman correlations ($P < 0.05$) between of the biomarkers measured. See Supplemental File 1 for additional details on the strength (r value) and level of significance (P-value) for each individual correlation. **(B)** Heatmap of the number of statistically significant correlations involving each biomarker measured in dogs with different VL disease severity is shown. Details on the numbers of correlations are shown is Supplemental File 1. **(C)** Comparisons of the network densities is shown (density calculations are described in Methods). Data was compared using permutation test. ns, non significant.

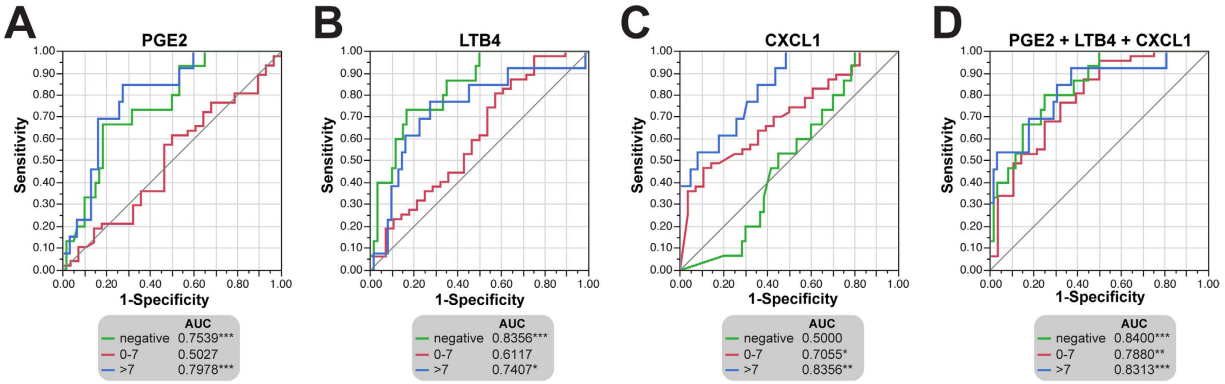

Figure 3. Using inflammatory markers to predict VL disease severity. (A–D) ROC curve analyses were performed to estimate in a quantitative way the performance of the different combinations of biomarkers used in the cluster analysis in segregating dogs diverging in VL clinical severity. AUC: area under the curve. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

LTB4 were negative associations (Fig. 2A). Moreover, following quantification of the number of significant correlations observed in each marker (node analysis), we found that the overall number of connections involving most of the markers was similar between the clinical groups (Fig. 2B and Supplemental File1). Interestingly, animals with the highest severity scores (>7) displayed increased participation of LTB4 while showing a decrease in the involvement of CCL2 in the network (Fig. 2B). Additional analyses revealed that the network densities were not significantly different between the groups (Fig. 2C).

ROC curve analyses using inflammatory markers to predict VL disease severity. ROC curve analyses were performed to estimate in a quantitative way the efficacy of different combinations of biomarkers in segregating dogs diverging in CVL clinical severity (Fig. 3). To simplify the analyses, the study groups were rearranged into: negative, score of 0–7 and score >7. We attempted to create a ROC curve combining the biomarkers that displayed a linear trend with disease severity score ranges (PGE2, LTB4, SOD, CXCL1 and CCL2, depicted in Fig. 1B). However, after testing different combinations (data not shown), the use of three biomarkers (PGE2, LTB4, and CXCL1) was shown to be the most efficient in distinguishing the clinical groups and therefore kept in the final ROC curve model. Figure 3A–C shows ROC curves for each marker PGE2, LTB4 and CXCL1, demonstrating that these three markers, when considered individually, have potential to distinguish the clinical groups. Notably, the combination of these biomarkers resulted in a better overall performance to discriminate the three clinical score categories (Fig. 3D).

Associations between antibodies production against salivary recombinant proteins LJM11 and LJM17 and parasite load of infected dogs. The associations between antibody production against

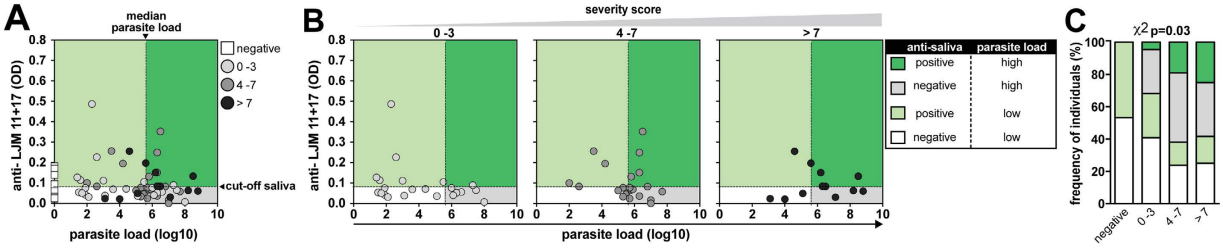

Figure 4. Associations between antibodies production against salivary recombinant proteins LJM11 and LJM17 and parasite load of infected dogs. (**A**) Correlations between parasite load and antibodies production against salivary recombinant proteins LJM11 and LJM17 in negative and infected dogs showing different clinical signs (n = 70). Dotted lines on the X-axis represent the median value of parasite load within the group of infected dogs, while dotted lines on Y-axis indicate the cut-off value for antibodies production against LJM11 and LJM17. In (**B**), the correlations were stratified according to clinical score presented by the dogs. White areas designate the quadrants that include the dogs displaying values of parasite load below the median and negative antibodies production; light green area designate the quadrants that include the dogs displaying values of parasite load below the median and positive antibodies production; dark green areas designate the quadrants that include the dogs displaying values of parasite load above the median and positive antibodies production; and grey areas designate the quadrants that include the dogs displaying values of parasite load above the median and negative antibodies production. In (**C**) the percentage of individuals within each area were compared between the groups with different clinical score using a chi-square analysis.

recombinant sand fly salivary proteins LJM11 and LJM17 and parasite load of infected dogs is presented in Fig. 4A–C. There was no correlation between antibody concentrations against these recombinant salivary proteins and parasite loads in dogs (Fig. 4A). We observed an increase in the frequency of dogs showing high parasite load and anti-saliva antibodies with the increase of severity disease (Fig. 4B,C dark green bars). Although it seems to be an increase in the frequency of dogs showing negative saliva serology and high parasite loads, the results were not statistically significant among the groups (Fig. 4B,C grey bars).

Discussion

In the present study, we evaluated different parameters associated to CVL severity, considering distinct groups of naturally infected dogs from a highly endemic area, in Camaçari, Bahia, Brazil. Our data revealed a distinct pattern of biomarkers correlated with the different clinical manifestations of CVL. We have also demonstrated that ROC curve analyses using combined biomarkers are able to distinguish dogs exhibiting different degrees of CVL severity. Furthermore, we identified that higher titers of antibodies to sand fly saliva is more frequent in highly parasitized dogs.

Cross sectional analysis of serum levels of cytokines and chemokines as well as LTB4 and PGE2 in dogs suggest that the relationships between the biomarkers in the study groups, and especially those related to LTB4 and CCL2, could be directly involved in the pathogenesis of CVL. Levels of LTB4 and PGE2 displayed a significant decrease following a gradual increase in disease severity. Recently, our group has shown the importance of eicosanoids in modulating immune responses in *Leishmania* infection[12]. In addition, PGE2 has been shown to contribute to parasite proliferation[11,16], whereas LTB4 increases macrophage activation and intracellular destruction[17–19]. Therefore, the balance between these two mediators seems to contribute to the control of *Leishmania* infection and inflammation. The decrease in the serum levels of both LTB4 and PGE2 found in our work indicate that dogs displaying severe disease may loose the ability to mount an efficient response to control the infection.

SOD-1 has been found to play a deleterious role in *Leishmania* infection, increasing parasite burden in *Leishmania*-infected human macrophages[10]. In our samples, an increase in SOD was observed in the group of dogs with clinical score between 4 and 7, indicating that this enzyme could be related to the pathogenesis of disease. However, dogs with the highest clinical score displayed low levels of this enzyme, possibly reflecting exhaustion of the immune system.

In the context of experimental or natural CVL infection, an up-regulation of chemokines expression in the spleen has been already described, although only CXCL10 and CCL5 were shown to be markedly elevated in oligosymptomatic dogs[20]. Concerning these chemokines, no significant differences in study groups were noticed. However, our findings showed a significant increase in CXCL1 and CCL2 serum levels with the increase of disease severity. CXCL1 is responsible for the recruitment of neutrophils while CCL2 is responsible for the recruitment of monocytes[21,22]. Results from literature have shown that dogs with CVL display neutropenia[23,24] and that derangement in neutrophil numbers and function seems to be important features of CVL[25,26]. Moreover, Menezes-Souza *et al.*[20] reported a higher expression of CCL2 in the skin of dogs with CVL that positively associated with a higher parasite density. The authors also reported an increase in the number of macrophages in the skin of symptomatic dogs displaying high parasitism. Our results as well as others studies suggest that these chemokines are recruiting immature or unresponsive macrophages, since animals with higher serum level of CXCL1 and CCL2 presented high levels of parasite in the spleen.

We identified a signature of CVL characterized by the absence of inflammatory mediators such as IFN-γ, TNF-α, IL-2, IL-7, PGE2, LTB4, IL-15 and SOD in dogs with severe CVL. Although dogs with subclinical disease do not present many of the mediators described above, an observed increase in the levels of IL-6 and IL-18,

suggests a restricted ability to control the infection. On the other hand, symptomatic dogs with a score higher than 7 showed a near absence of inflammatory mediators and an increase in IL-10, CCL2 and CXCL1 levels. Cytokines such as IFN- γ and TNF- α are considered the hallmark of protection in different clinical manifestations of leishmaniasis[8]. However, interactomes analysis showed that these protective cytokines do not have any correlation with the clinical status of the dogs, although in our study their levels decrease along the clinical score progression, but not in a significant way.

ROC curve analysis ultimately compared the overall performance of the diverse combination of candidate biomarkers in distinguishing different clinical manifestations of CVL. The combination of PGE2, LTB4 and CXCL1 was the one that better discriminated among the groups of animals, pointing out the importance of these mediators in the follow up and prognostic of clinical manifestations in CVL.

Salivary recombinant proteins are of value as markers of vector exposure. In humans and dogs, LJM11 and LJM17 emerged as potential markers of specific exposure to *Lu. longipalpis*[27]. Additionally, Gomes *et al.*[15] positively correlates the appearance of an anti-saliva humoral response to an anti-*L. infantum* cell-mediated immunity raising the hypothesis that induction of immune response against sand fly saliva can facilitate the generation of a protective response against human VL. Thus, we can infer that anti-sand fly saliva antibodies can serve as an important epidemiological marker of vector exposure in endemic areas and even as a surrogate marker of protection. However, we observed an increase in the frequency of dogs with high parasite loads and anti-saliva antibodies with the increased of clinical scores. The strength of this dog study is the parasite load comparison together with the results of saliva serology. These type of data allowed the observation of the increased percentage of the highly infected and highly saliva exposed group along with disease severity. We speculate that dogs are likely more exposed to sand-fly bites in the endemic areas than humans, leading to an increase in the antibody production and higher chances of infection and elevated parasite load.

This study evaluated multiple biomarkers in dogs and defined a biosignature related to different clinical manifestations of CVL. Characterization of disease severity in CVL is essential to prevent the spread of the infection, since symptomatic animals are better disseminators of parasites in endemic areas. Further studies are in progress to define a particular biosignature for dogs that transmit parasites to sand flies allowing for better surveillance in areas of CVL transmission.

Methods

Study design. A random sample of 70 dogs was selected from a cross-sectional study conducted in the municipality of Camaçari, located in the State of Bahia, North-eastern Brazil (latitude: 12° 41′ 51″S; longitude: 38° 19′ 27″W). This area is endemic for both VL and CVL, with a seroprevalence of CVL ranging from 20 to 40% in the canine population (unpublished data).

Ethical aspects. This study was approved by the IGM - FIOCRUZ Institutional Review Board for Animal Experimentation under Permit Number 007/2013, within Brazilian Federal Law on Animal Experimentation (Law no. 11794), and following the guidelines for animal research established by the Oswaldo Cruz Foundation. All the dog owners signed an informed consent form, allowing the examination of the animals. Dogs were grouped according to CVL diagnosis and clinical score, as explained below.

CVL diagnosis. Serum and splenic aspirate samples were collected as described before[28] and used to perform anti–sand fly saliva serology and biomarkers analyses.

Parasitological evaluation of splenic aspirates was performed as previously described[29], as well as the use of qPCR technique to assess positivity in the splenic aspirate[28]. The splenic parasite load was measured by qPCR technique as described elsewhere[28]. Anti-*Leishmania* antibodies were detected using the double pathway platform-screening test (DPP® CVL, Bio-Manguinhos Unit, Rio de Janeiro, Brazil) followed by the confirmatory immunoassay (EIE® CVL, Bio-Manguinhos Unit, Rio de Janeiro, Brazil).

Dogs were considered infected if they present positive results in splenic culture or qPCR. Animals were considered uninfected if the above-mentioned tests were negative.

Composite clinical severity score for CVL. All animals were clinically examined and classified according to the parameters described in Table 1. Each clinical sign was given a grade of 0, 1 or 2 depending on the intensity of the clinical manifestation. The clinical score was calculated as the sum of grades for each clinical sign with same weights. The composite score could then range from 0 to 24 points. Infected animals with a clinical score of ≤ 3 were classified as without clinical disease (subclinical); a clinical score of $\geq 4 < 7$ were categorized as with mild disease and a clinical score of ≥ 7 was used to define severe disease.

Immunoassays. Cytokine and chemokine levels in serum were measured using a pre-defined luminex-based multiparametric kit (Milliplex Map Kit - canine cytokine magnetic bead panel, Life Technologies, Carlsbad, CA, USA). The markers examined were IFN-γ, IL-10, TNF-α, IL-2, IL-6, IL-7, IL-15, IL-8, CCL2, CXCL10, GM-CSF and CXCL1. Concentrations of PGE2, LTB4 and superoxide dismutase (SOD) were measured in serum samples from all the dogs using an enzyme-linked immunoassay (Cayman Chemical, Ann Harbor, MI, USA) as described previously[10,12].

Anti–sand fly saliva serologic testing. Recombinant *Lu. longipalpis* salivary proteins were expressed in HEK cells and HPLC purified as previously described[27]. Anti–sand fly saliva serologic test ELISA was performed as described elsewhere[27,30] with some adaptations for use in dogs. Antibody production against recombinant salivary proteins LJM11 and LJM17 was measured using the optical density values divided by the cut-off value of each experiment.

Clinical signs	Score based on intensity		
	0	1	2
Nutritional status	Normal or obese	Emaciate	Cachectic
Mucosa color	Normal	Anemic	—
Periocular dermatitis	Absent	Around one eye	Present in two eyes
Crust on ears	Absent	Present in one ear	Present in two ears
Ear Ulcers	Absent	Present in one ear	Present in two ears
Muzzle Depigmentation	Absent	In less than 1/3 of the muzzle	In more than 1/3 of the muzzle
Muzzle Hyperkeratosis	Absent	In less than 1/3 of the muzzle	In more than 1/3 of the muzzle
Muzzle Lesions	Absent	Initial mucous lesion	Larger ulcerated lesion
Spleen size	Not palpable	Enlarged	—
Onychogryphosis	Absent	Slight enlargement	Excessive enlargement
Alopecia	Absent	Focal	In more than 1/3 of the body
Seborrheic dermatitis	Absent	Focal	In more than 1/3 of the body
Lymphadenomegaly	Absent	One or two enlarged lymph nodes of the same pair	Enlarged lymph nodes of different pairs

Table 1. VL clinical parameters employed to calculate the clinical score of each animal included in the study.

Statistical Analysis. Median values with interquartile ranges (IQR) were used as measures of central tendency. The chi-square test was employed to compare frequencies between the study groups. Hierarchical cluster analysis (Ward's method) with bootstrap was performed to depict the overall expression profile of serum biomarkers in the negative and the different severity score groups. Significant statistical differences between groups of varying severity scores were evaluated using the Kruskal-Wallis test. Non-parametric linear trend *ad hoc* tests were employed to examine the variation of all biomarker levels following the clinical severity score. Biomarkers that did not exhibit linear trend were compared using the Dunn's multiple comparisons test.

Network analysis (host interactome) were generated from Spearman correlation matrices containing values of each biomarker measured in the serum samples, as described by Mendonça *et al.*[31]. The values were input in JMP 10.0 software (SAS, Cary, NC, USA). A heat map of the number of statistically significant correlations involving each biomarker was constructed for different severity score groups. Aiming to analyse the structure of the biomarker networks, the network density was calculated as described by Mendonça *et al.*[32].

Receiver-Operator Characteristic (ROC) curve analyses were used to test the power of some biomarkers (PGE2, LTB4, SOD, CLCX1 and CCL2) and their combination to distinguish dogs presenting with different stages of CVL clinical severity. For this analysis, the severity score groups 0–3 and 4–7 were combined. ROC curve analyses were performed using JMP 10.0 software. A p-value below 0.05 was considered statistically significant.

Correlations between parasite load and antibodies production against salivary recombinant proteins LJM11 and LJM17 in negative and infected dogs showing different clinical signs were evaluated. The median values of parasite load and the cut-off value for antibodies production against LJM11 and LJM17 were used as measures of central tendency, differentiating high and low parameter levels. The X^2 or Fisher tests were used to compare variables displayed as percentage.

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Author Contributions

Conceived and designed the experiments: M.S.S., C.R.T., R.K., P.T.B., D.B.M.F., V.M.B., P.S.T.V. and C.I.B. Performed the experiments: M.S.S., M.M.C.A., C.R.T. and R.K. Analysed the data: M.S.S., B.B.A., R.K., J.G.V., S.K., D.B.M.F., V.M.B., P.S.T.V. and C.I.B. Contributed reagents/materials/analysis tools: B.B.A., R.K., J.G.V., S.K., P.T.B., D.B.M.F., V.M.B., P.S.T.V. and C.I.B. Wrote the paper: M.S.S., B.B.A., C.R.T., R.K., J.G.V., S.K., P.T.B., D.B.M.F., V.M.B., P.S.T.V. and C.I.B.

Additional Information

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Supplementary information Solcà et al., 2016

Spearman correlations and *p*-values of the host interactome

NEGATIVE

Variable 1	Variable 2	R value	P value
IL2	CCL2	0,8528	<.0001
TNFa	CCL2	0,743	0,0015
CCL2	GM-CSF	0,8969	<.0001
IL 10	GM-CSF	0,5591	0,0303
IL 15	GM-CSF	0,8458	<.0001
IL 18	GM-CSF	0,7523	0,0012
IL2	GM-CSF	0,9069	<.0001
IL6	GM-CSF	0,7265	0,0022
IL7	GM-CSF	0,8572	<.0001
TNFa	GM-CSF	0,7794	0,0006
CCL2	IL 15	0,849	<.0001
IL 18	IL 15	0,7512	0,0012
IL2	IL 15	0,8459	<.0001
TNFa	IL 15	0,9091	<.0001
CCL2	IL 18	0,8981	<.0001
IL2	IL 18	0,7397	0,0016
TNFa	IL 18	0,6427	0,0098
CCL2	IL6	0,8014	0,0003
IL 15	IL6	0,8909	<.0001
IL 18	IL6	0,7331	0,0019
IL2	IL6	0,8768	<.0001
IL7	IL6	0,8791	<.0001
TNFa	IL6	0,8395	<.0001
CCL2	IL7	0,8832	<.0001
IL 15	IL7	0,8768	<.0001
IL 18	IL7	0,8212	0,0002
IL2	IL7	0,903	<.0001
TNFa	IL7	0,8904	<.0001
CCL2	LTB4 pg/ml	-0,5576	0,0308
IL 15	LTB4 pg/ml	-0,5624	0,0291
IL2	LTB4 pg/ml	-0,5293	0,0425
IL6	LTB4 pg/ml	-0,5916	0,0202
CCL2	PGE2 pg/ml	-0,5827	0,0226
IL 10	PGE2 pg/ml	-0,5252	0,0444
LTB4 pg/ml	PGE2 pg/ml	0,5893	0,0208
IL2	TNFa	0,79	0,0005

Subclinical 0-3

Variable 1	Variable 2	R value	P value
CCL2	IL6	0,9496	<.0001
IL7	IL6	0,9431	<.0001
IL2	IL6	0,9361	<.0001
IL2	IL7	0,9289	<.0001
TNFa	IL7	0,9245	<.0001
CCL2	IL7	0,9228	<.0001
TNFa	CCL2	0,8935	<.0001
TNFa	IL6	0,8914	<.0001
IL 18	IL 15	0,8828	<.0001
CCL2	IL 15	0,8616	<.0001
IL 15	GM-CSF	0,8601	<.0001
IL 18	IL7	0,8583	<.0001
IL2	TNFa	0,854	<.0001
TNFa	IL 18	0,8483	<.0001
IL 18	GM-CSF	0,8439	<.0001
IL2	CCL2	0,8422	<.0001
IL 15	IL7	0,8343	<.0001
TNFa	GM-CSF	0,8256	<.0001
IL7	GM-CSF	0,8191	<.0001
TNFa	IL 15	0,8173	<.0001
CCL2	IL 18	0,8144	<.0001
IL 15	IL6	0,7995	<.0001
IL2	IL 18	0,7968	<.0001
IL 18	IL6	0,7811	<.0001
CCL2	GM-CSF	0,7536	<.0001
IL6	GM-CSF	0,7526	<.0001
IL2	GM-CSF	0,7449	<.0001
IL2	IL 15	0,7178	0,0001
LTB4 pg/ml	PGE2 pg/ml	0,6311	0,0012
CXCL1	IL8	0,5809	0,0037
CXCL10	LTB4 pg/ml	0,4697	0,0237
CXCL1	PGE2 pg/ml	0,4688	0,024
CXCL1	GM-CSF	-0,4428	0,0344

CVL Moderate 4-7

Variable 1	Variable 2	R value	P value
IL2	GM-CSF	0,9199	<.0001
IL2	IL7	0,9012	<.0001
IL 18	IL 15	0,8767	<.0001
IL7	IL6	0,8532	<.0001
IL7	GM-CSF	0,8402	<.0001
IL 15	IL7	0,839	<.0001
IL 18	IL6	0,8377	<.0001
IL 18	IL7	0,8362	<.0001
TNFa	IL7	0,8321	<.0001
TNFa	GM-CSF	0,83	<.0001
IL 15	IL6	0,8277	<.0001
IL2	IL6	0,8277	<.0001
IL2	TNFa	0,8178	<.0001
TNFa	IL6	0,8001	<.0001
TNFa	IL 18	0,791	<.0001
IL2	IL 15	0,7791	<.0001
IL2	IL 18	0,7705	<.0001
IL6	GM-CSF	0,7471	<.0001
LTB4 pg/ml	PGE2 pg/ml	0,7409	<.0001
IL 18	GM-CSF	0,7221	<.0001
IL 15	GM-CSF	0,7126	<.0001
TNFa	IL 15	0,6603	0,0004
CCL2	IL6	0,588	0,0025
CXCL1	CXCL10	0,5673	0,0038
TNFa	CCL2	0,5646	0,004
CCL2	CXCL1	0,5396	0,0065
CXCL1	IL 10	0,5254	0,0084
IL8	LTB4 pg/ml	0,5163	0,0098
CXCL1	IL6	0,5148	0,01
CCL2	IL 18	0,5099	0,0109
CXCL1	IL 18	0,4575	0,0246
CCL2	GM-CSF	0,4449	0,0294
IL2	CCL2	0,4347	0,0338
CCL2	IL7	0,4213	0,0403
CXCL10	IL 10	0,411	0,046

CVL Severe >7

Variable 1	Variable 2	R value	P value
CCL2	CXCL1	0,7088	0,0067
CCL2	IL8	0,6923	0,0087
CXCL1	IL8	0,7692	0,0021
CXCL10	IL 18	0,5746	0,04
CXCL10	IL 10	0,5654	0,044
GM-CSF	LTB4 pg/ml	-0,5691	0,0424
IL 15	GM-CSF	0,9692	<.0001
IL 15	IL7	0,923	<.0001
IL 15	IL6	0,7721	0,002
IL 15	INFg	0,6328	0,0203
IL 15	LTB4 pg/ml	-0,5989	0,0305
IL 18	GM-CSF	0,8852	<.0001
IL 18	IL 15	0,809	0,0008
IL 18	IL7	0,6829	0,0101
IL 18	IL6	0,6424	0,0179
IL2	IL6	0,9487	<.0001
IL2	IL7	0,8796	<.0001
IL2	IL 15	0,8359	0,0004
IL2	GM-CSF	0,8349	0,0004
IL2	IL 18	0,6968	0,0081
IL2	LTB4 pg/ml	-0,6093	0,0271
IL6	GM-CSF	0,7351	0,0042
IL6	LTB4 pg/ml	-0,6155	0,0251
IL7	GM-CSF	0,8717	0,0001
IL7	IL6	0,8374	0,0004
IL7	LTB4 pg/ml	-0,6357	0,0195
IL7	INFg	0,5553	0,0488
INFg	LTB4 pg/ml	-0,663	0,0135
SOD U/ml	LTB4 pg/ml	-0,6929	0,0181
TNFa	IL 15	0,6769	0,011
TNFa	CCL2	0,6648	0,0132
TNFa	CXCL1	0,6593	0,0142
TNFa	GM-CSF	0,6354	0,0196
TNFa	IL8	0,6209	0,0235
TNFa	IL7	0,5635	0,0449

Node Analysis

marker	negative	0-3	4-7	>7
GMCSF	7	8	7	7
IL-8	0	0	1	1
IFNg	0	0	0	3
TNFa	6	7	7	7
IL-2	8	7	7	6
IL-7	7	7	7	8
PGE2	3	2	1	0
LTB4	5	2	2	7
IL-15	8	7	6	8
SOD	0	0	0	1
IL-6	8	6	8	7
IL-18	6	8	8	9
CXCL10	0	1	2	2
IL-10	2	0	2	1
CXCL1	0	3	5	3
CCL2	8	7	7	3

DISCUSSÃO GERAL

No presente trabalho foram avaliados biomarcadores de exposição à saliva do vetor, biomarcadores parasitológicos e identificados biomarcadores inflamatórios que se correlacionaram com a gravidade da infecção por *L. infantum* em cães. A carga parasitária se mostrou um bom marcador parasitológico, havendo correlação desta com o estado clínico dos animais infectados. Da mesma forma, a produção de anticorpos contra as proteínas da saliva do vetor LJM11/LJM17 se correlacionou com a gravidade do quadro clínico dos animais. Dentre os biomarcadores inflamatórios avaliados, foi identificado um perfil caracterizado pelo aumento de CXCL1 e CCL2 e redução de LTB-4 e PGE-2 apresentado pelos animais com quadro clínico mais grave de LVC.

É importante ressaltar que a classificação dos animais utilizando como base os sinais clínicos não é a melhor forma para determinar o estadiamento da LVC (SOLANO-GALLEGO *et al.*, 2009). Diversos autores apontam que as alterações hematológicas e bioquímicas provocadas pela infecção do parasito são também importantes para determinar a gravidade da infecção no animal (TROPIA DE ABREU *et al.*, 2011; FREITAS *et al.*, 2012). Um parâmetro importante a ser avaliado é a função renal, uma vez que a LVC está associada a uma alta prevalência de doença renal crônica (COSTA *et al.*, 2003). Solano-Gallego *et al*., (2011) em conjunto com outros participantes do grupo LeishVet, criaram um método de estadiamento da LVC baseado em 4 graus, desde doença leve até muito grave, baseado nas manifestações clínicas do animal, em conjunto com os achados hematológicos, bioquímicos e de urinálise. Em nosso estudo optamos por estadiar os cães baseado em presença e intensidade de sinais clínicos e da carga parasitária, e não uma avaliação mais completa dos animais, incluindo avaliações bioquímicas e hematológicas, principalmente, por razões de custo.

A quantificação do número de parasitas nos tecidos dos animais infectados auxilia no monitoramento da progressão da doença clínica, ajudando a avaliar a gravidade da infecção nos animais (MANNA *et al.*, 2008a), uma vez que os sinais clínicos apresentados pelos animais acometidos por *L. infantum* podem ser inespecíficos e podem não corresponder diretamente com a disseminação do parasita no organismo, como já foi demonstrado por Paiva Cavalcanti *et al.,* (2015).

No presente trabalho (Capítulo 1), foi observada associação positiva significativa entre a intensidade da carga parasitária no baço e o número de sinais clínicos presentes nos cães. Os animais sem sinais clínicos ou os que apresentaram de 1-3 sinais clínicos tiveram cargas parasitárias menores no tecido esplênico, quando comparados com animais com quadro clínico de LVC mais grave (SOLCA *et al.*, 2014). Esta correlação evidencia que a carga parasitária pode ser um bom biomarcador parasitológico para correlação com o quadro clínico dos animais. Esse resultado foi reforçado na segunda parte do estudo (Capítulo 2), no qual a carga parasitária também mostrou associação positiva, estatisticamente significativa, com a gravidade do quadro clínico da LVC (dado não apresentado no *paper* publicado de Solcà *et al.,* 2016).

O tecido esplênico apresentou maior taxa de positividade e maior carga parasitária nos cães infectados por *Leishmania*, quando comparado aos outros tecidos avaliados (Capítulo 1). Este achado pode ser explicado, devido ao baço ser um órgão importante de interação entre o sistema imune e os parasitos durante o curso da infecção (SANTANA *et al.*, 2008). Além disso, já foi demonstrado que o baço mantém a infecção do parasito durante todo o curso da LVC (REIS *et al.*, 2006a).

Um avanço na forma de quantificar a carga parasitária foi obtido na presente tese, que consistiu no desenvolvimento e validação do protocolo de qPCR em configuração *duplex* em formato líquido e no formato em gel (*ready-to-use*), inicialmente estabelecido em protocolo *singleplex* (Capítulo 1) (FRANCINO et al., 2006; SOLCA et al., 2014). Esse avanço, abre a possibilidade de, não somente, podermos quantificar a carga parasitária dos animais infectados, como utilizá-lo como uma ferramenta diagnóstica de mais fácil utilização, que poderia ser útil para clínica e estudos científicos (ROURA *et al.*, 2013). A detecção precisa da carga parasitária presente no cão infectado pela *Leishmania* é adicionalmente importante no monitoramento das infecções naturais, para acompanhamento de animais em tratamento e em acompanhamentos de cães avaliados em estudos experimentais (ROLÃO *et al.*, 2004; PENNISI *et al.*, 2005; MANNA *et al.*, 2006; MANNA *et al.*, 2008a; MANNA *et al.*, 2008b).

A detecção simultânea do gene 18S canino, no protocolo de qPCR desenvolvido, reforça a validade dos resultados desse método, principalmente para os resultados negativos. Naranjo *et al*., 2011 também empregaram como alvo para o

controle interno da reação o 18S rRNA, avaliando cães infectados por *L. infantum*, já outros autores empregaram outros alvos como beta actina (MANNA *et al.*, 2008a; MANNA *et al.*, 2009; MAIA *et al.*, 2010) e beta globulina (QUARESMA *et al.*, 2009). A confirmação da presença do DNA genômico nas amostras caninas, exclui a possibilidade de resultados falso negativos, pois é possível atestar que quando ocorre amplificação do DNA do hospedeiro, a ausência de amplificação do DNA de *Leishmania* não é devida a ausência de DNA ou presença de inibidores na amostra. O formato *duplex* da qPCR desenvolvida reforça ainda mais esse resultado, pois diminui o tempo de execução, combinando as duas reações de qPCR em uma, permitindo dessa forma a avaliação de amostras com pouco rendimento de DNA genômico após sua extração.

Contudo, a qPCR continua um método complexo para ser inserido na rotina diagnóstica da LVC, principalmente como teste de triagem na saúde pública. Ademais, o tecido esplênico, apesar de sua elevada sensibilidade na detecção do parasito, apresenta uma coleta que demanda conhecimento e habilidade para execução, equipamentos específicos assim como medicamentos, sendo desta forma inviável de ser realizada em estudos epidemiológicos de saúde pública (GONTIJO e MELO, 2004; BARROUIN-MELO *et al.*, 2006; CARVALHO *et al.*, 2009; WATSON *et al.*, 2011; SOLCA *et al.*, 2014). No Brasil, a triagem para LVC é realizada por um teste rápido que apresenta uma série de vantagens como o fato de dispensar equipamentos laboratoriais, necessidade de uma amostra de coleta pouco invasiva e rapidez que fazem com que este seja o teste ideal a ser empregado na triagem dos cães em área endêmica em programas de saúde pública (BOARINO *et al.*, 2005; GRIMALDI *et al.*, 2012a). Todavia, a qPCR poderia ser empregada como teste confirmatório, principalmente no seu formato duplex *ready to use*, para uso em clínicas veterinárias, mas principalmente tem uma aplicabilidade para avaliação diagnóstica de LV em seres humanos, uma vez que alguns indivíduos infectados são também HIV positivos, não produzindo anticorpos específicos contra o parasito e sendo dificilmente detectados em testes sorológicos (ALVAR *et al.*, 1997; ALVAR *et al.*, 2008).

Neste trabalho foi mostrado como a carga parasitária é um importante biomarcador para o monitoramento da infecção (Capítulo 1), desta forma a qPCR é uma ferramenta muito importante para pesquisa em LVC. A avaliação precisa da carga parasitária em infecções experimentais pode permitir avanços em estudos de

avaliação da patogênese da enfermidade, assim como, comparações confiáveis em estudos de avaliação da eficácia de drogas anti-*Leishmania* (ROURA *et al.*, 2013). Pennisi *et al.*, (2005) empregaram a qPCR para monitorar os efeitos do tratamento por allopurinol em cães experimentalmente infectados por *L. infantum*, demonstrando como essa abordagem pode mostrar claramente a eficácia da droga, ao longo do tratamento, uma vez que foi possível por essa técnica demonstrar que o tratamento levou à diminuição do número de parasitas em diferentes tecidos dos animais.

No Brasil, o tratamento da LVC é fiscalizado pelo MS, que não recomenda esta prática através da emissão de uma instrução normativa, sendo proibido o uso de medicamentos humanos para o tratamento dos animais (Portaria Interministerial n^{o} 1426, de 11 de julho de 2008). Contudo, existe uma série de outros medicamentos, como allopurinol e miltefosina, que são empregados para tratar animais infectados (KOUTINAS *et al.*, 2001; PENNISI *et al.*, 2005; SARIDOMICHELAKIS *et al.*, 2005; TORRES *et al.*, 2011) associado ao uso de métodos repelentes. Ribeiro et al. (2013), pelas recomendações do grupo Brasileish sugerem que quando a opção for o tratamento, devem ser instituídos os protocolos que produzam cura clínica e redução da carga parasitária, que deve ser sustentada por meio de investigações clínicas e laboratoriais. Assim a qPCR pode ser importante também para clínicos veterinários que tenham interesse em tratar os animais acometidos por *L. infantum* e monitorar a progressão da doença de forma ambulatorial com uma ferramenta acurada e relevante.

Uma perspectiva do presente estudo é a possibilidade de podermos acompanhar prospectivamente a infecção em animais subclínicos, para avaliar o desfecho clínico destes, validar os biomarcadores de gravidade encontrados e possivelmente encontrar outros biomarcadores indicativos de resistência ou susceptibilidade à infecção. Em nosso estudo, a utilização da qPCR *duplex* foi usada como uma ferramenta indispensável para detecção da infecção por *L. infantum* e quantificação precisa da carga parasitária dos animais e, com isso, avaliação da disseminação do parasito e realização de estudos de correlação da carga com os biomarcadores identificados (Capítulo 2).

Em nosso estudo (Capítulo 2), foi observada associação entre maior frequência de cães com alta carga parasitária e presença de anticorpos anti-saliva

de flebotomíneos com maior gravidade da doença nos cães. Previamente, foi demonstrado em humanos, que a produção de anticorpos anti-saliva apresenta correlação positiva com o desenvolvimento de reação de hipersensibilidade tardia contra a *Leishmania* e constitui um potencial marcador de proteção contra a infecção parasitária (GOMES *et al.*, 2002). No presente trabalho, mostramos que essa tendência parece ser oposta uma vez que os cães mais acometidos apresentaram maior frequência de positividade para anticorpos anti-saliva no soro. Anteriormente, foi demonstrado que cães da área endêmica produzem anticorpos anti-saliva (TEIXEIRA *et al.*, 2010), contudo não existem estudos que investiguem se a frequência e os níveis desses anticorpos se correlacionam com o risco de desenvolvimento de doença ou transmissibilidade para os flebotomíneos em cães. Adicionalmente, foi identificada uma tendência dos animais com produção de anticorpos anti-saliva apresentarem maior carga parasitária, o que pode indicar uma maior exposição destes animais ao inseto vetor. Vale ressaltar que este é um estudo transversal, onde poucas inferências podem ser assumidas, sendo, portanto, necessário um estudo de acompanhamento para determinar se a exposição prévia à saliva do vetor pode influenciar no curso da infecção por *L. infantum*. A identificação de um biomarcador que permita avaliar a exposição dos animais ao vetor e que se correlacione com o desfecho clínico poderá auxiliar no entendimento do ciclo da LV e LVC nas áreas endêmicas.

Nesta segunda etapa foi identificada uma bioassinatura distinta em cães com diferentes manifestações clínicas, caracterizada por menores níveis de biomarcadores como LTB-4 e PGE-2 (Capítulo 2) em animais com quadro grave de LVC. Já foi demonstrado que a diminuição dos níveis de PGE-2 contribui para a proliferação parasitária (ARAUJO-SANTOS *et al.*, 2014; SAHA *et al.*, 2014) enquanto que LTB-4 aumenta a ativação de macrófagos e a destruição intracelular do parasita (CHAVES *et al.*, 2014; TAVARES *et al.*, 2014). Portanto, o equilíbrio entre esses dois mediadores parece contribuir para o controle da infecção por *Leishmania*. Araujo-Santos *et al*., (2014) demonstraram que o equilíbrio na relação PGE-2 / LTB-4 pode ser importante para a modulação da resposta imune provocada pela saliva do vetor, permitindo o aumento da viabilidade do parasita, bem como a carga parasitária dentro dos leucócitos durante os primeiros momentos de exposição a *L. infantum*.

Ademais, no presente trabalho, foi encontrada uma bioassinatura caracterizada também por maiores níveis de CXCL1 e CCL2 nos cães apresentando doença mais grave (Capítulo 2). CXCL1 é uma quimiocina expressa por macrófagos, neutrófilos e células epiteliais, e tem atividade quimioatratora de neutrófilos (ABBAS, LICHTMAN e PILLAI, 2012). Estudos sugeriram que CXCL1 pode ser específica para a inflamação de origem infecciosa, já tendo sido detectada significativamente aumentada no sobrenadante de células mononucleares caninas estimuladas com lipopolissacárideos (KARLSSON *et al.*, 2016). CCL2 por sua vez, é referida como proteína quimioatratora de monócitos, recruta monócitos, células T de memória e células dendríticas para os locais de inflamação produzidos por lesão tecidual ou infecção (ABBAS, LICHTMAN e PILLAI, 2012). Até o presente momento, nenhum estudo procurou avaliar a associação destas quimiocinas com a gravidade da infecção por *L. infantum* em cães, sendo no futuro interessante realizar uma correlação com os níveis dessas quimiocinas e com os achados laboratoriais de leucograma para averiguar sua influência na quantidade de monócitos e neutrófilos circulantes.

É bem descrito na literatura a importância de outras citocinas como IFN-y e TNF-α para ativar macrófagos e matar os parasitas (REIS *et al.*, 2010). Estas citocinas são consideradas como marcadores de proteção em diferentes manifestações clínicas de leishmaniose. A IL-10, uma citocina anti-inflamatória, é considerada uma das citocinas responsáveis pela gravidade da doença, uma vez (SOLANO-GALLEGO *et al.*, 2006; YABSLEY *et al.*, 2008)que seus níveis já foram encontrados aumentados nos sobrenadantes de cultura celular de pacientes com LV, contribuindo para a imunossupressão observada nesses indivíduos (SAHA *et al.*, 2007). Entretanto, nossos resultados não demonstram nenhuma correlação entre essas citocinas e o estádio clínico dos cães, embora os seus níveis sejam mais baixos em cães com menor gravidade do quadro clínico, mas não de forma significativa.

Vale salientar que não pesquisamos a presença de coinfecção nos cães utilizados em nosso estudo. Os agentes causadores de hemoparasitoses mais comuns em cães são *Ehrlichia canis*, *Babesia canis* e *Anaplasma platys* (GUILLEN LLERA *et al.*, 2002; BEUGNET e MARIE, 2009; MEKUZAS *et al.*, 2009; VASCELLARI *et al.*, 2016). Estes são patógenos intracelulares que utilizam monócitos-macrófagos, hemácias e plaquetas como células hospedeiras (GREENE,

2006). A epidemiologia destas hemoparasitoses caninas e da LVC sobrepõe-se em muitas áreas do mundo, tropicais e não tropicais, uma vez que a atividade dos vetores (carrapatos e flebotomíneos) e os períodos de transmissão desses patógenos são semelhantes (BEUGNET, CHALVET-MONFRAY e LOUKOS, 2009; MENN, LORENTZ e NAUCKE, 2010; OTRANTO e DANTAS-TORRES, 2010).

O aspecto mais importante de avaliar a presença de outros patógenos em estudos de patogênese e resposta imune é que a maioria destes também se dissemina da pele para o baço, fígado e medula óssea, da mesma forma que *L. infantum* e ativam mecanismos imunológicos do hospedeiro que induzem uma gama de respostas imunopatológicas semelhantes (MEKUZAS *et al.*, 2009; VASCELLARI *et al.*, 2016). Já foi relatada a produção de citocinas pró-inflamatórias, como o TNF-α por parte dos linfócitos T CD8+ em animais infectados por *E. canis* (ISMAIL *et al.*, 2004), adicionalmente já foi demonstrado que a imunopatogenia da erliquiose canina pode estar relacionada à produção descontrolada de TNF-α pelos linfócitos T CD8+ na fase aguda da infecção e, em menor grau, durante a fase assintomática da infecção (HARRUS *et al.*, 1997; HARA *et al.*, 2004). Apesar de não termos encontrado níveis expressivos aumentados dessas citocinas, no presente estudo, a presença de coinfecções pode levar a um sinergismo na resposta imune específica da LVC (MEKUZAS *et al.*, 2009; CARVALHO, 2015), assim no estudo longitudinal em progressão decidimos realizar essa pesquisa para poder discriminar realmente quais os biomarcadores específicos da infecção por *L. infantum.*

O estudo exploratório realizado foi muito importante uma vez que permitiu avaliar diferentes possíveis biomarcadores, e correlacioná-los com a gravidade da infecção por *L. infantum* apresentada pelos animais examinados. Estudos transversais como esse, permitem uma análise mais ampla de redes de causalidade na qual são elaboradas hipóteses que podem ser validadas em estudos longitudinais. Adicionalmente, estudos transversais são mais rápidos, mais baratos, mais fáceis em termos logísticos (FRONTEIRA, 2013). Contudo, vale salientar que em um estudo de corte transversal o animal está sendo avaliado pontualmente quanto a sua situação clínica, não sendo possível distinguir por exemplo, um animal subclínico resistente de um animal subclínico em fase de latência. Desta forma, é importante que os biomarcadores identificados no estudo transversal sejam validados sucessivamente em um estudo longitudinal, o que está sendo desenvolvido. Neste estudo longitudinal será possível confirmar os dados deste

trabalho e avaliar quais biomarcadores estão relacionados com a progressão da enfermidade e seus diferentes desfechos clínicos, uma vez que o estudo de coorte permite distinguir um cão resistente de um animal em fase de latência, que irá desenvolver a forma clínica da LVC, pois avaliamos a progressão da infecção e da doença nos animais acompanhados.

Os cães assintomáticos, em fase de latência, são importantes alvos de estudo, pois quando estes são mantidos na área endêmica podem participar da dinâmica de transmissão da LV, uma vez que os cães sintomáticos geralmente são removidos e os animais latentes permanecerão e poderão desenvolver LVC (BURATTINI *et al.*, 1998; NUNES *et al.*, 2008). Assim, a identificação precoce de marcadores de progressão de cães assintomáticos que estão em fase de latência, nos quais ocorrerá o aparecimento de sinais clínicos, permitirá intervenções mais rápidas e eficazes no controle da LV no Brasil. Assim como, a identificação de um conjunto de biomarcadores dentre os animais que não desenvolvem o quadro clínico da doença e que poderão ser relacionados com a resistência a LVC, podendo ajudar a identificar esses animais no futuro, em áreas endêmicas, e indicar a eutanásia quando o cão tiver um papel na transmissão da LV.

A identificação dos biomarcadores de progressão da doença pode ser importante para uma melhor compreensão da patogênese da doença, e, futuramente, direcionar o prognóstico e manejo terapêutico dos animais acometidos, bem como novos alvos para estratégias profiláticas e terapêuticas. Assim, neste trabalho foi possível concluir que existem biomarcadores parasitológicos como a carga parasitária, biomarcadores imunológicos e inflamatórios como CXCL1, CCL2, LTB-4, PGE-2, além da produção de anticorpos específicos contra as proteínas LJM11 e LJM17 da saliva do vetor, que são capazes de diferenciar animais infectados por *L. infantum* de acordo com seus diferentes quadros clínicos.

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Colaborações em artigos científicos

- **Anexo 1 – Colaboração em artigo científico publicado**
    - Fraga, D. M. B. *et al*., 2016 - The Rapid Test Based on *Leishmania infantum* Chimeric rK28 Protein Improves the Diagnosis of Canine Visceral Leishmaniasis by Reducing the Detection of False-Positive Dogs. PLoS Neglected Tropical Diseases.
- **Anexo 2 – Colaboração em artigo científico publicado**
    - Borja, L. S. *et al*., 2016 - Parasite load in the blood and skin of dogs naturally infected by *Leishmania infantum* is correlated with their capacity to infect sand fly vectors. Veterinary Parasitology.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

The Rapid Test Based on *Leishmania infantum* Chimeric rK28 Protein Improves the Diagnosis of Canine Visceral Leishmaniasis by Reducing the Detection of False-Positive Dogs

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Abstract

Visceral Leishmaniasis (VL) has spread to many urban centers worldwide. Dogs are considered the main reservoir of VL, because canine cases often precede the occurrence of human cases. Detection and euthanasia of serologically positive dogs is one of the primary VL control measures utilized in some countries, including Brazil. Using accurate diagnostic tests can minimize one undesirable consequence of this measure, culling false-positive dogs, and reduce the maintenance of false-negative dogs in endemic areas. In December 2011, the Brazilian Ministry of Health replaced the ELISA (EIE CVL) screening method and Indirect Immunofluorescence Test (IFI CVL) confirmatory method with a new protocol using the rapid DPP CVL screening test and EIE CVL confirmatory test. A study of diagnostic accuracy of these two protocols was done by comparing their performance using serum samples collected from a random sample of 780 dogs in an endemic area of VL. All samples were evaluated by culture and real time PCR; 766 out of the 780 dogs were tested using the previous protocol (IFI CVL + EIE CVL) and all 780 were tested using the current protocol (DPP CVL + EIE CVL). Performances of both diagnostic protocols were evaluated using a latent class variable as the gold standard. The current protocol had a higher specificity (0.98 vs. 0.95) and PPV (0.83 vs. 0.70) than the previous protocol, although sensitivity of these two protocols was similar (0.73). When tested using sera from asymptomatic animals, the current protocol had a much higher PPV (0.63 vs. 0.40) than the previous protocol (although the sensitivity of either protocol was the same, 0.71). Considering a range of theoretical CVL prevalences, the projected PPVs were higher for the current protocol than for the previous protocol for each theoretical prevalence value. The findings presented herein show that

the current protocol performed better than previous protocol primarily by reducing false-positive results.

Author Summary

Visceral Leishmaniasis (VL) is a major public health problem. Its control is based on detection and culling of positive dogs, treatment of human cases and vector control. Canine cases often precede the occurrence of human cases; hence, disease control in dogs is important. Use of accurate diagnostic tests is required to avoid culling false-positive dogs and to minimize the number of false-negative dogs that are maintained in endemic areas. In December 2011, the Brazilian Ministry of Health changed the diagnostic protocol for canine VL (CVL). In the present study, the accuracy of this current protocol was compared to the previous one using serum samples of 780 dogs from an endemic area of VL. The findings revealed that the current protocol performed better than the previous protocol primarily by reducing false-positive results. Considering different theoretical prevalence values, the current protocol misdiagnosed fewer dogs than the previous one.

Introduction

Visceral leishmaniasis (VL) is a major public health problem worldwide. This disease in Brazil and Europe is caused by the protozoan parasite *Leishmania infantum*, which is transmitted to humans by the bite of sandflies from the genus *Lutzomyia* [1]. Dogs are considered the main reservoir of urban VL since: i) these animals harbor high parasitism in skin that offers a high capacity of parasite transmission to sandflies, ii) humans and dogs coexist in close proximity and iii) canine cases generally precede the occurrence of VL in humans [1–4].

The identification and euthanasia of serologically positive dogs is one of the primary VL control strategies recommended by the governments of some countries, such as Brazil. The use of accurate diagnostic tests for canine VL (CVL) can reduce failures on VL control program by minimizing maintenance of false-negative animals and culling of false-positive dogs that impact on euthanasia controversial measure, subsequently, decreasing dog owners' compliance and society disagreement. More accurate tests could also reduce the number of false-negative dogs that are maintained in endemic areas [4].

CVL is typically diagnosed by parasitological, serological and molecular tests. In December 2011, the program of the Brazilian Ministry of Health for monitoring and control of leishmaniasis replaced the enzyme-linked immunosorbent assay (EIE CVL) screening method and the indirect immunofluorescence assay (IFI CVL) confirmatory test with a new serodiagnostic protocol for CVL composed of the Dual Path Platform (DPP CVL) screening test and the EIE CVL confirmatory test [5]. The evaluation of sensitivity and specificity revealed low values for previous protocol that detects infection by determining seropositivity in dogs. This low performance is probably due to undesirable preservation of blood samples normally collected onto filter papers. This simple procedure for sample collection is performed easily and facilitates sample storage and transportation. However, it often submits the biological specimens to stress conditions that might damage samples and lead to unreliable test results [2,6–8]. Additionally, the low sensitivity and specificity offered by the old protocol can be explained by further reasons: i) both screening EIE CVL and confirmatory IFI CVL tests have been performed using blood samples that were collected in endemic areas and then sent to reference laboratories, where the tests were performed,

ii) EIE CVL and IFI CVL tests are time-consuming techniques, whereas IFI CVL has an additional difficulty to be standardized and interpreted depending on the ability of the observer to detect the antigen-antibody reaction by fluorescence microscope. This may lead to misinterpretation of the results and may compromise IFI CVL reproducibility in different laboratories.

DPP CVL is a rapid test based on a multi-epitope, recombinant chimeric protein (rK28) resulted from fusion of *L. infantum* genes: k9, single repeat units of k39 and k26 [9] that has been adopted as the screening method in a new protocol established by the Brazilian government. DPP CVL rapid test is an immunochromatographic assay that offers several advantages: i) rK28 was proven to provide very high levels of sensitivity and specificity for canine VL [9], ii) DPP CVL has a great potential for facilitating faster decision, since it is a point-of-care screening test that gives result within 15 minutes, iii) DPP CVL in association with the confirmatory test EIE CVL give results within 15 days, in comparison to previous protocol (EIE CVL + IFI CVL) that results were only liberated after a lengthy time interval that varied from one to two months. Thus, the incorporation of this rapid test into the current protocol accelerates the implementation of the control measures in endemic areas. In addition, this procedure uses only small blood samples and does not require specialized equipment and supplies [10].

The use of tests presenting low accuracy has serious epidemiological consequences: false-negative dogs are undetected thereby maintaining the parasite life cycle in endemic areas, and detection of false-positive dogs results in excessive dog culling. The lack of a reliable gold standard test for CVL hinders the assessment of diagnostic protocol performance and can result in misinterpretation of diagnostic test accuracy [11–16]. Indeed, although the common used gold-standard, culturing of *L. infantum*, is highly specific, its low sensitivity [17] hampers the evaluation of other diagnostic techniques.

In light of this limitation, latent class analysis (LCA) has been shown to be a valuable alternative to the classical validation approach of using parasitological methods as gold standards [18,19]. LCA is based on the theory that the observed results of different imperfect tests for the same disease are influenced by a latent common variable that cannot be directly measured, but can reflect accurately the true disease status. Previous studies employing LCA have accurately assessed serological [20–24] and molecular [12,25] diagnostic methods.

Despite the advantages of DPP CVL [10,14,26,27] for CVL diagnosis, few studies have assessed its performance [28,29]. To the best of our knowledge, the present study is an initial attempt designed to compare the accuracy of the current (DPP CVL and EIE CVL) and previous protocol (EIE CVL and IFI CVL) for CVL diagnosis employing a latent class variable as the reference standard. Serum samples were obtained during a cross-sectional study performed in an endemic area for VL in Brazil.

Methods

Ethics Statement

All experimental procedures involving dogs were carried out according to the Brazilian Federal Law on Animal Experimentation (Law no. 11794), the guidelines for animal research established by the Oswaldo Cruz Foundation (FIOCRUZ) and the Brazilian Ministry of Health Manual for the Surveillance and Control of VL [4]. The Institutional Review Board approved the present study for Animal Experimentation (CEUA, protocol no. 015/2009). Dog owners who agreed to participate in the study signed a Free, Prior and Informed Consent (FPIC) form.

Study Area

A cross-sectional study was conducted in the municipality of Camaçari, located in the State of Bahia in Northeastern Brazil. Using district sketches of households throughout 36 districts in

Camaçari obtained from the Zoonosis Control Center, a sample of domiciled dogs was randomly selected, during the years of 2011 and 2012. The sample size was calculated using Epi Info 3.5.1 (The Centers for Disease Control and Prevention—CDC, USA) based on estimates of the canine population (15,820 dogs) derived from an anti-rabies vaccination campaign and an expected CVL prevalence of 20% (5% margin of error, 95% confidence interval).

Sampling

Dogs were classified as asymptomatic or symptomatic based on the presence or absence of the following clinical signs: emaciation, alopecia, anemia, conjunctivitis, dehydration, dermatitis, erosion, ulcerations, lymphadenopathy, and onychogryphosis. They were classified as asymptomatic when presented 0 until 3 signs or symptomatic when presented more than 3 signs. Blood and splenic aspirate samples were obtained for CVL diagnosis from each dog at the same time. Blood was collected by venipuncture in sterile tubes to obtain serum. All serum samples were stored at -20°C until serological testing. Splenic aspirate samples were obtained using a puncture technique previously described by Barrouin-Melo and collaborators (2006) [30], and modified by Solcà and collaborators (2014) for ultrasound-guided collection. All 780 splenic samples were evaluated by culture and real time PCR; 766 out of the 780 serum samples were tested using the previous protocol (IFI CVL + EIE CVL) and all 780 were tested using the current protocol (DPP CVL + EIE CVL) (S1 Fig).

Parasitological Testing

Splenic aspirate samples were cultivated in Novy-Mac Neal-Nicolle (NNN) medium supplemented with 20% FBS (Fetal Bovine Serum, Gibco BRL, New York, USA) and 100 μg/mL of gentamicin. The cultures were maintained at 24°C for four weeks and examined weekly for the presence of parasites [31].

Serological Tests

All serological diagnostic test kits for CVL (DPP CVL, EIE CVL and IFI CVL Bio-Manguinhos) were used in accordance with manufacturer's recommendations.

DNA Extraction

DNA was extracted from splenic aspirate samples using DNeasy Blood & Tissue kit from Qiagen (Hilden, Germany), in accordance with manufacturer's recommendations. DNA concentrations were determined using a digital spectrophotometer (Nanodrop—ND-1000 Thermo Scientific, Wilmington, USA), then aliquoted at a concentration of 30 ng/μL and stored at -20°C until real time PCR amplification.

Real Time PCR

DNA extracted from splenic aspirate samples was amplified using real time PCR technique, in accordance with the protocol established by Francino and collaborators (2006) [32] and modified by Solcà and collaborators (2014). Control samples were added in all of the real time PCR experiments. As positive controls were used splenic aspirate samples from two dogs that had previously been identified in an endemic area as positive for *Leishmania* infection and as negative controls were employed splenic aspirates of two healthy dogs from the municipality of Pelotas, Rio Grande do Sul, Brazil, an area non-endemic for CVL.

Statistical Analysis

All test readers executing and reading the index tests had prior training and great experience in CVL diagnosis. All diagnostic testing was carried out under blinded conditions, which means that test readers interpreted the results obtained from each diagnostic technique for a given sample without knowledge of the other tests' results. The interpretation of the results using the previous and current diagnostic protocols classified dogs as positive when both tests (screening and confirmatory) presented positive results. Epi Info 3.5.1 (The Centers for Disease Control and Prevention—CDC, Atlanta, USA) and STATA 12.0 (StataCorp LP, Texas, USA) software programs were used to analyze results.

LCA was performed to define a latent class variable to evaluate the accuracy of the diagnostic tests and employed as previously described in Solcà and collaborators (2014). Latent variable modeling used the results of the following diagnostic techniques as indictor variables: serological (EIE CVL, DPP CVL and IFI CVL Bio-Manguinhos), parasitological (culture of splenic samples), and molecular (real time PCR of splenic aspirate) tests. We chose a two-class latent class model based on goodness of fit criteria, such as the Akaike information criterion (AIC) and Bayes information criterion (BIC). We also used the Lo-Mendel-Rubin test and the entropy for model evaluation [33]. MPlus version 5 software was used to implement LCA [34].

The performance of the diagnostic tests and protocols was estimated using the latent class variable as the reference standard. Diagnostic performance was calculated in 2 x 2 contingency tables of positive and negative test results, using the command diagt in Stata. We determined specificity, positive predictive values (PPV), negative predictive values (NPV) and diagnostic accuracy with 95% exact binomial confidence intervals (CI). Diagnostic accuracy was calculated as the number of true positive + number of true negative/total number of tested serum samples. Differences among diagnostic protocols regarding their performance (sensitivity and specificity) were assessed using McNemar chi-square test (p-value < 0.05), for all dogs and for two categories of disease status based on symptomatology. The number of animals considered as false negative and false positive was also calculated for each of the diagnostic techniques evaluated, considering as true positive those dogs that were positive according to the latent class variable.

Results

From April 2011 until July 2012, 780 dogs pure and mixed-breed with estimated ages from 1 to 10 years old, were enrolled in the study. According to the presence of clinical signs of CVL, 47.8% dogs were asymptomatics and 54.2% symptomatics. Five diagnostic tests were used to determine the proportion that tested positive in this random population. The IFI CVL yielded the highest percentage of positivity (36%), whereas the splenic aspirate culture yielded the lowest percentage of positivity (13.1%). Among the remaining tests, the EIE CVL, real time PCR and DPP CVL tests were positive in 24.9%, 22.4% and 16.9% of the dogs, respectively (Fig 1 and Table 1).

Using LCA, 14.1% of the 780 dogs were classified as positive (Table 1). Evaluation of LCA entropy showed that a high accuracy in the classification of dogs by LCA was achieved, with value of 0.97. *A posteriori* average probabilities that dogs were properly classified in the latent classes "positive" and "negative" were, respectively, 95% and 99%. Moreover, the test of Lo-Mendel-Rubin indicated that the model with two classes produced better results than that with only one class ($p < 0.01$). These results are supported by the analysis of AIC and BIC (AIC = 3025.996, BIC = 3077.249).

The real time PCR and culture techniques yielded the highest sensitivities, 0.97 and 0.90, respectively, when the latent class variable served as the reference standard. Among the three

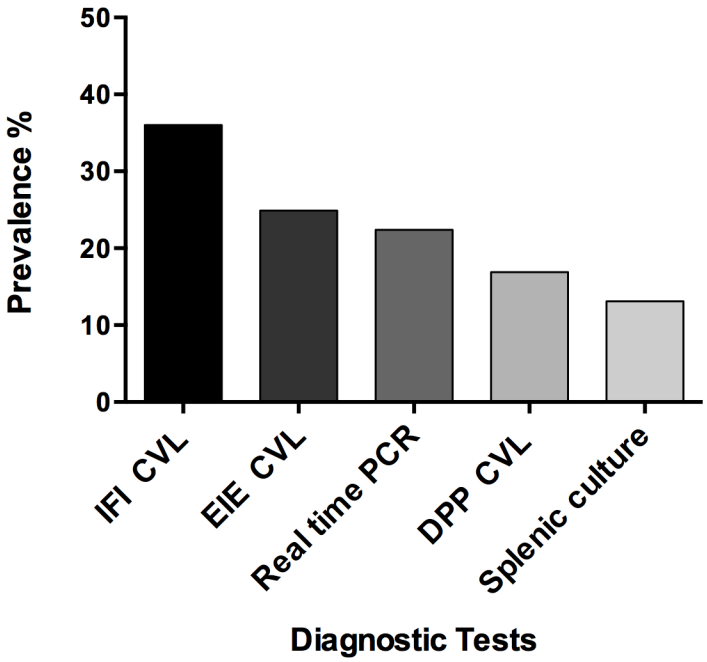

Fig 1. Percent of positive results of five CVL diagnostic tests performed on canine sera samples from an endemic area of Camaçari.

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serological tests, IFI CVL and DPP CVL had the highest sensitivity (0.86) and EIE CVL (0.79) (Table 2). Regarding specificity, culture was found to be the most specific (1.00), followed by DPP CVL (0.94), then real time PCR (0.90), EIE CVL (0.84) and IFI CVL (0.73).

When the latent class variable was considered as the reference test, the PPV of culture was 1.00. Among the other four techniques, DPP CVL had the highest PPV (0.71), followed by real time PCR (0.61), EIE CVL (0.45) and IFI CVL (0.37). Likewise, among serological tests, DPP CVL (0.98), followed by IFI CVL (0.97) and EIE CVL (0.96) showed the highest NPV (Table 2).

The measures of diagnostic accuracy of the current diagnostic protocol were then compared to those of the previous protocol (Table 3). Both protocols had equally high sensitivity (>0.72; McNemar's chi-square test, $p = 0.051600$) and NPV (0.96), whereas the new protocol consistently had a higher specificity (>0.97, $p = 0.0078$) and PPV (>0.83). The diagnostic accuracy was higher when current diagnostic protocol was compared to the previous protocol (0.94 vs. 0.92).

Table 1. Prevalence of latent classes and conditional probabilities according to the LCA model for CVL diagnoses.

Technique	Frequency Positive n = 780 (%)	Latent classes	
		Positive n = 110 (14.1%)	Negative n = 670 (85.9%)
		Conditional probabilities (%)	
IFI CVL	36.0	85.9	26.6
EIE CVL	24.9	79.2	15.8
Real time PCR	22.4	96.2	10.1
DPP CVL	16.9	84.1	5.6
Culture	13.1	88.1	0.0

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Table 2. Performance of diagnostic tests considering the latent class variable as the gold standard.

Diagnostic tests	Sensitivity	Specificity	PPV	NPV
DPP CVL	0.86 (0.78–0.92)	0.94 (0.92–0.97)	0.71 (0.63–0.79)	0.98 (0.96–0.99)
EIE CVL	0.79 (0.70–0.86)	0.84 (0.81–0.87)	0.45 (0.38–0.52)	0.96 (0.94–0.98)
IFI CVL	0.86 (0.78–0.92)	0.73 (0.69–0.77)	0.37 (0.31–0.43)	0.97 (0.95–0.98)
Real time PCR	0.97(0.92–0.99)	0.90 (0.87–0.92)	0.61 (0.54–0.68)	1.00(0.99–1.00)
Culture	0.90 (0.83–0.95)	1.00 (0.99–1.00)	1.00 (0.96–1.00)	0.98 (0.97–0.99)

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Table 3. Performance of current and previous protocols for CVL diagnosis, considering the latent class variable as the gold standard.

Diagnostic tests		Sensitivity	Specificity*	PPV	NPV	Accuracy
Previous protocol	**EIE CVL + IFI CVL (Asymptomatic dogs)**	0.71 (0.42–0.92)	0.96 (0.93–0.98)	0.40 (0.21–0.61)	0.99 (0.97–1.00)	0.95
	EIE CVL + IFI CVL (Symptomatic dogs)	0.74 (0.64–0.82)	0.94 (0.90–0.96)	0.79 (0.69–0.87)	0.92 (0.88–0.95)	0.89
	EIE CVL + IFI CVL (Total)	0.73 (0.64–0.81)	0.95 (0.93–0.96)	0.70 (0.61–0.78)	0.96 (0.94–0.97)	0.92
Current protocol	**DPP CVL + EIE CVL (Asymptomatic dogs)**	0.71 (0.42–0.92)	0.98 (0.96–0.99)	0.63 (0.35–0.85)	0.99 (0.97–1.00)	0.97
	DPP CVL + EIE CVL (Symptomatic dogs)	0.73 (0.63–0.82)	0.97 (0.94–0.98)	0.88 (0.78–0.94)	0.92 (0.89–0.95)	0.91
	DPP CVL + EIE CVL (Total)	0.73 (0.63–0.81)	0.98 (0.96–0.99)	0.83 (0.74–0.90)	0.96 (0.94–0.97)	0.94

*The specificity of previous and current protocol was statically different, based on McNemar test (p = 0.0078).

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Comparing the performance of current protocol (DPP CVL + EIE CVL) to that of DPP CVL alone revealed that sensitivity showed higher value for DPP CVL (0.86) than that for the current protocol (0.73), although PPV showed a slight lower value for DPP CVL (0.71) compared to PPV for the current protocol (0.83).

When the dogs were categorized according to the presence of clinical signs of CVL (Table 3), the sensitivity of both diagnostic protocols was similar in asymptomatic and symptomatic dogs. However, in symptomatic dogs, the new protocol had higher specificity and PPV (0.97 and 0.88, respectively) than the previous protocol (0.94 and 0.79, respectively). In addition, in asymptomatic dogs, the PPV of the current protocol was significantly higher, by 22.5%, than that of the previous protocol ($p = 0.0078$). Also, difference was observed in diagnostic accuracy of protocols when they were used in symptomatic dogs (0.91 vs. 0.89) and asymptomatic dogs (0.97 vs. 0.95). To generalize the better performance of current protocol to other settings, the PPV and NPV were calculated for the current and previous protocol accordingly to different theoretical values of CVL prevalence (Table 4). For each estimated prevalence value, the current protocol was estimated to yield higher PPVs, ranging from 0.23 to 0.99, whereas the projected PPVs for the previous protocol ranged from 0.13 to 0.98. Regarding NPV, both protocols yielded similar projected values, ranging from 0.47 to 1.00.

Discussion

The present study primarily demonstrated that the DPP CVL + EIE CVL protocol, in comparison with the EIE CVL + IFI CVL protocol, performed better for the serodiagnosis of CVL. The adoption of this new protocol offered several advantages, due to inclusion of the rapid DPP

Table 4. Estimates of PPV and NPV of current and previous protocols for CVL diagnosis, considering the latent class variable as the gold standard, by theoretical values of CVL prevalence.

	Previous protocol		Current protocol	
Prevalence (%)	PPV	NPV	PPV	NPV
1	0.13	1.00	0.23	1.00
5	0.43	0.99	0.62	0.99
10	0.61	0.97	0.77	0.97
15	0.71	0.95	0.84	0.95
20	0.78	0.93	0.88	0.94
30	0.86	0.89	0.93	0.89
50	0.93	0.78	0.97	0.78
80	0.98	0.47	0.99	0.47

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CVL screening test, which can be performed easily and quickly and does not require specialized equipment and personnel [27,28].

Several authors previously discussed that the lack of a perfect gold standard test for CVL hampers the evaluation of diagnostic tests for CVL [12,13]. Previous studies have proven that LCA is effective for evaluating diagnostic tests' performance [12,20,21,35–38]. Herein, using a latent class variable as the reference standard, we were able to comprehensively compare two protocols for serodiagnosis of CVL using serum samples collected from 780 randomly selected dogs from an endemic area of VL. The use of LCA had an additional advantage: we were able to evaluate the performance of both real time PCR and culture. Very few studies have evaluated the performance of real time PCR, and most studies that evaluated the performance of CVL diagnostic techniques used culture as the gold standard [10,14,26,28]. Using LCA, we found that real time PCR and culture were the most sensitive techniques. Among the serological tests evaluated, DPP CVL had the best performance. Although IFI CVL had the highest sensitivity, it was the least specific, as previously described by de Santis and collaborators (2013) and Laurenti and collaborators (2014). Regarding the performance measure that has epidemiological relevance, the PPV, the DPP CVL had the highest PPV among the serological tests evaluated as previously described by da Silva and collaborators (2013).

In addition to evaluating each individual test for CVL diagnosis, we compared the performance of previous and current protocols employed in Brazil. The usefulness of protocols was evaluated by determining PPVs and NPVs of each protocol. The better individual performance of the DPP CVL was reflected in the 13% higher PPV of the current protocol for CVL detection compared to the previous protocol. Both protocols yielded a NPV of 0.96, suggesting that when these protocols have negative results it is highly probable that serum are from dogs that are actually uninfected. By contrast, the current diagnostic protocol provides a greater PPV (0.83) than that of the previous protocol (0.70), indicating that the current protocol provides a greater level of assertiveness in diagnosing positive dogs. Although the new protocol showed a higher specificity and PPV than the previous one, the sensitivity is still limited (around 0.73) in both protocols, meaning that the maintenance of false-negative dogs in endemic areas still represents a public health concern and more efforts should be done to try to find out better protocols or new antigens to reduce the maintenance of infected dogs in areas of zoonotic transmission.

Considering questions rose about the wisdom to diagnose CVL using DPP CVL + EIE CVL instead of DPP CVL alone, the comparison of performances showed that a higher sensitivity value (0.86) and lower PPV (0.71) for DPP CVL compared to DPP CVL + EIE CVL (0.73 and

0.83, respectively) that might cause detection of false positive dogs. Mostly to avoid this, a confirmatory test, EIE CVL, has been associated to DPP CVL in the current protocol."

When current protocol is applied for diagnosing asymptomatic and symptomatic dogs, it showed similar performance for sensitivity (0.71, 0.73) and specificity (0.98, 0.97), respectively. While, the level of NPV (0.99) was greater, the level of PPV (0.63) was much lower for asymptomatic dogs in comparison to NPV (0.92) and PPV (0.88) for symptomatic animals. In accordance to this results, Otranto and collaborators (2009) showed that recently exposed or newly infected dogs might not be detected by serological tests, since these false-negative animals do not seroconvert soon after infection or they may develop a cellular type of immune response that are not detected using serological tests. In addition to this difficulty, no appropriate gold standard for *Leishmania* infection detection in asymptomatic dogs was established, highlighting the necessity for the development of new tests to improve diagnosis of asymptomatic dog.

Across a range of plausible prevalence, the theoretical expectation for PPV varied among 0.13 to 0.98 for previous protocol, and 0.23 to 0.99 for current protocol. PPV and NPV of a diagnostic test are known to be influenced by the prevalence of a given disease in a population. Thus, as disease becomes more prevalent the probability of subjects to test positive in diagnostic tests will be higher among sick individuals. In the present study, the analysis using different theoretical prevalence revealed that the current protocol has high performance irrespective of disease prevalence. In accordance, higher PPVs provided by DPP CVL + EIE CVL for diagnosing CVL have additional advantages since in endemic countries, regardless of the prevalence of CVL, the current protocol compared to previous one would better discriminate truly uninfected dogs from those that have risky to be infected.

In summary, our findings show that the current protocol for diagnosis of CVL implemented in Brazil has an excellent accuracy (0.91 for symptomatic dogs and 0.97 for asymptomatic), due to its greater specificity values and PPV. Because of the simplicity of test procedures and rapidity of results, the data presented herein strongly support the idea that the introduction of DPP CVL into the diagnostic CVL protocol contribute to improve CVL diagnosis that can have consequent effects that impact positively on disease control.

Supporting Information

S1 Checklist. Standards for the Reporting of Diagnostic Accuracy Studies (STARD) checklist for reporting of studies of diagnostic accuracies.
(DOC)

S1 Fig. STARD flowchart. Standards for the Reporting of Diagnostic Accuracy Studies (STARD) description of the experimental design to calculate accuracy of CVL serodiagnostic protocols.
(TIFF)

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Author Contributions

Conceived and designed the experiments: DBMF LVP PSTV. Performed the experiments: DBMF LVP LSB LAB MdSS. Analyzed the data: DBMF LVP PGdSET LDAFA. Contributed reagents/materials/analysis tools: PGdSET LDAFA PSTV. Wrote the paper: DBMF LVP LDAFA PSTV.

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Research paper

Parasite load in the blood and skin of dogs naturally infected by *Leishmania infantum* is correlated with their capacity to infect sand fly vectors

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ABSTRACT

The sand fly *Lutzomyia longipalpis* is primarily responsible for the transmission of visceral leishmaniasis (VL) in the New World, and dogs are considered to be the main urban reservoir of this disease. In order to improve the efficacy of control measures, it is essential to assess the transmission capacity of *Leishmania infantum* to the sand fly vector by naturally infected dogs. The present study investigated the existence of correlations between canine clinical presentation and the intensity of parasite load in the blood, skin and spleen of naturally infected dogs. In addition, we also attempted to establish correlations between the intensity of parasite load in canine tissue and the parasite load detected in sandflies five days after feeding on naturally infected dogs. A total of 23 dogs were examined and classified according to clinical manifestation of canine VL. Blood samples, splenic aspirate and skin biopsies were collected and parasite DNA was quantified by qPCR. Canine capacity to infect *Lu. longipalpis* with parasites was evaluated by xenodiagnosis and parasite loads were measured five days after feeding. No significant differences were observed with respect to canine clinical manifestation and the parasite loads detected in the blood, skin and spleen samples obtained from naturally infected dogs. Regardless of clinical canine visceral leishmaniasis (CVL) presentation and the degree of parasite burden, almost half of the dogs successfully infected sandflies with parasites, albeit to a low number of sandflies with correspondingly low parasite loads. Parasite loads in both canine blood and skin were shown to be positively correlated with the canine infectiousness to the sand fly vector, and positive correlations were also observed with respect to these tissues and the sand fly infection rate, as well as the parasite load detected in sandflies following xenodiagnosis. In conclusion, this indicates that parasite loads in both blood and skin can function as potentially reliable markers of canine capacity to infect sand fly vector.

1. Introduction

Visceral leishmaniasis (VL) is a severe systemic disease that affects humans, dogs and other mammalian vertebrates (WHO, 2010). In the New World, VL generally results from infection by the protozoan parasite *Leishmania infantum* (syn. *L. chagasi*), which is transmitted to mammalian hosts mainly by the *Lu. longipalpis* sand fly vector (WHO, 2010). Abundant evidence indicate that domestic dogs are the main reservoir of VL in urban areas, which is supported by reports of canine epidemics preceding outbreaks in humans in endemic regions (Alvar et al., 2004; Bevilacqua et al., 2001).

Due to variability in the immune response to infection, dogs with visceral leishmaniasis (CVL) can present as either asymptomatic or with a progressively symptomatic form of the disease (Ciaramella et al., 1997; Rallis et al., 2005). In general, the capa-

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bility to transmit parasites to sandflies has been associated with disease severity. Several studies have reported that most symptomatic dogs are capable of transmitting parasites to sandflies in endemic areas, and that these animals are more infectious than asymptomatic dogs, confirming their importance in the transmission cycle of VL (Courtenay et al., 2002; da Costa-Val et al., 2007; Guarga et al., 2000). By contrast, other studies have demonstrated that a given dog's capacity to transmit parasites to sand fly vectors is independent of its clinical CVL presentation (asymptomatic, oligosymptomatic or polysymptomatic) (Guarga et al., 2000; Molina et al., 1994). Unfortunately, none of these studies has attempted to elucidate parameters for distinguishing dogs that are capable of transmitting parasites to sand fly vectors from those that are incapable of transmission. Since VL control measures in Brazil are based on serological diagnosis and the euthanasia of seropositive dogs (Ministério da Saúde et al., 2006), to increase the efficacy of such measures, it is essential to ascertain infectiousness of naturally infected dogs.

Parasite transmission occurs by the contact of sand fly proboscides with the skin and blood of an infected host vertebrate during blood-feeding (Bates, 2007). We hypothesized that a given infected dog would infect sandflies with *L. infantum* in accordance with the intensity of its tissue (skin or blood) parasite burden. To test this hypothesis, we quantified parasite load in the skin and blood of naturally infected dogs presenting a range of clinical manifestations and correlated the intensity of parasite load in these tissues with a given dog's capacity to infect sandflies with parasites.

2. Materials and methods

2.1. Ethical considerations

All experiments involving canine specimens were performed in compliance with Brazilian federal law for animal experimentation (Law 11794), in conformity with the Oswaldo Cruz Foundation (FIOCRUZ) animal experimentation guidelines, and according to the instructions outlined in the Brazilian Ministry of Health's manual for the surveillance and control of VL. The present study was approved by the Institutional Review Board (CEUA protocol no. 015/2009) of the Gonçalo Moniz Research Center in Bahia, Brazil (CPqGM–FIOCRUZ/BA). Dog owners who agreed to participate in the study signed a Free, Prior and Informed Consent (FPIC) form.

2.2. Canine specimens

A total of 23 dogs were recruited directly from their owners during the period of April 2011 and September 2012, some of whom reside in endemic regions within the State of Bahia, Brazil: (i) 15 animals were pets from the municipality of Dias D'Ávila, (ii) 6 from the municipality of Camaçari and (iii) 2 dogs domiciled in Salvador.

Dogs were included in the study if they tested positive for CVL by aspirate spleen culture or qPCR of blood, skin or spleen tissue. All dogs were clinically examined by a veterinarian and subsequently classified in accordance with the intensity of the most common clinical signs of CVL, including weight loss, alopecia, cutaneous lesions, conjunctivitis, onychogryphosis and lymphadenopathy. Thereafter, each detected clinical sign was categorized in grades of 0, 1, 2 or 3 in accordance with its intensity. Clinical scores were then calculated by summing the grades assigned for each clinical sign present in infected dogs. Animals were classified as without clinical manifestation (subclinical infection, clinical score 0–3) or with clinical disease (symptomatic infection, clinical score 3 +). The Municipal Center for Zoonotic Disease Control received notification regarding all positive dogs.

2.3. Sample collection

To perform parasitological diagnosis and determine parasite loads, blood collection, spleen aspiration and skin biopsies were performed. Samples of peripheral blood were collected by venipuncture (minimum of 5 mL) then aliquoted and stored at −80 °C. The dogs were anesthetized with acepromazine (0.1 mg/kg iv, Vetnil, Brazil) and splenic aspirate samples were obtained using a puncture technique previously described by Barrouin-Melo et al. (2006), which was modified to incorporate guidance by a portable ultrasound device (Solca Mda et al., 2014). Lidocaine solution (0.1%) was used as local anesthesia to perform cutaneous punch biopsies. Skin samples with a diameter of 4 mm were collected from the internal surface of a single lesion-free ear.

2.4. Parasitological testing and serological evaluation

Splenic aspirate samples were collected as explained above and culturing was performed as previously described (Barrouin-Melo et al., 2006; Solca Mda et al., 2014). Briefly, splenic aspirates were cultured for a period of four weeks in Novy–MacNeal–Nicolle (NNN) biphasic medium, supplemented with 20% FBS (Fetal Bovine Serum, Gibco BRL, New York, USA) and 50 μg/mL of gentamicin to avoid contamination (Sigma Chemical Co., St. Louis, MO) at 23 °C (Barrouin-Melo et al., 2006). Microscopy for parasite detection was performed at weekly intervals for no less than four weeks. Each splenic culture was prepared in duplicate and double-checked to avoid misidentification.

The serological evaluation was realized usind the immunochromatographic rapid test DPP® LVC (Bio-Manguinhos, Brazil). The diagnostic test procedures with DPP® LVC was realized in accordance with manufacturer recommendations.

2.5. Xenodiagnosis

All 23 animals were submitted to xenodiagnosis no later than 10 days following CVL diagnosis and tissue sample collection. Adult *Lu. longipalpis* were obtained from closed colonies at the Laboratory of Veterinary Infectious Diseases at the Federal University of Bahia and the other at the Gonçalo Moniz Research Center (FIOCRUZ-BA). Both sand fly colonies were originally formed by sandflies captured in the endemic municipality of Ipecaetá, located in northeastern Bahia. Insects from 8 to 12 generations were used in xenodiagnosis experimentation. To carry out this procedure, sandflies, including both 30–40 females and 10–15 males, were starved for 24 h, then placed in transparent PVC cylinders covered with fine mesh over one end. Each dog had a cylinder affixed to one of its ears without lesions or scabs, so as to maintain contact for 40 min, thus allowing the insects to feed on the dogs' blood. Xenodiagnosis was only considered successful when an estimated 70% or more of the sandflies were observed taking a blood meal. In only one xenodiagnosis procedure, the estimated percentage was lower than 70%, thus the procedure was considered invalid, and was repeated the next day. All sandflies were subsequently kept for a period of five days and allowed to feed on a saturated glucose solution. After five days, all surviving sandflies were frozen at −80 °C until DNA extraction. qPCR was then performed to determine the presence of parasite DNA and quantify parasite load. The capacity of naturally infected dogs to infect sand fly vectors with *L. infantum* was assessed by considering multiple factors: (1) canine infectiousness to sandflies, estimated as the percentage of dogs that successfully infected sandflies with *L. infantum* during xenodiagnosis; (2) sand fly infection rate, estimated as the number of sandflies that were successfully infected during xenodiagnosis; and (3) the average parasite load detected in successfully infected sandflies following xenodiagnosis.

2.6. DNA extraction from canine tissue and sandflies

DNA extraction from splenic aspirates, skin biopsies (4 mm), and whole blood (200 μL) samples was performed using the DNeasy Blood & Tissue kit in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The females *of Lu. longipalpis* used in xenodiagnosis were individually transferred to 1.5 mL microcentrifuge tubes and DNA extraction was performed as previously described (Michalsky et al., 2007). DNA concentration and quality were then assessed and each sample was aliquoted and stored at −20 °C.

2.7. Leishmania *kinetoplast DNA* (*kDNA) Quantification*

To determine parasite load in canine samples and sandflies, *Leishmania* DNA was quantified by qPCR assays employing an amplification procedure previously described (Francino et al., 2006) and modified by Solcà and collaborators (Solca Mda et al., 2014). The taqMan-MGB probe and PCR primers were designed to target conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum* to obtain a 120-bp amplicon. The qPCR amplification protocol (Solca Mda et al., 2014) employed the following primers: forward primer 5′-AACTTTTCTGGTCCTCCGGGTAG-3′ (Leish-1) and reverse primer 5′-ACCCCCAGTTTCCCGCC-3′ (Leish-2), both at a final concentration of 900 nM. A fluorogenic probe 5′-AAAAATGGGTGCAGAAAT-3′ was used for detection, synthesized using a FAM reporter molecule attached to the 5′ end, as well as a MGB-NFQ quencher linked to the 3′-end (Perkin-Elmer Applied Biosystems) at a final concentration of 200 nM. Parasite load was determined by comparing cycle threshold (CT) values derived from a standard curve, which was obtained from DNA amplification using 10-fold serial dilutions of *Leishmania* DNA performed in triplicate, ranging from 10^5 to 10^{-1} parasites. In order to minimize interplate variation, the averaged CT values for each sample were normalized based on a common fluorescence detection baseline value. A CT value was recorded at the point at which its fluorescence signal crossed the established detection baseline. In order to overcome the limitations caused by melanin present in skin samples submitted to PCR, all steps leading up to DNA amplification were performed in the presence of bovine serum albumin (2.5 μg/each reaction) (Sigma Chemical) to prevent the inhibition of PCR (Giambernardi et al., 1998).

Two separate receiver operating characteristic (ROC) curves were plotted to obtain the optimal CT cut-off point for considering either a canine or sand fly sample as positive. First, to determine the CT cut-off point for *Leishmania* DNA in canine samples, the amplification results from 20 *Leishmania*-negative samples and 20 *Leishmania*-positive samples were plotted on a ROC curve. Next, for *Leishmania* DNA detection in sand fly samples, a ROC curve was constructed using the amplification results from 40 sandflies used in the xenodiagnosis of two confirmed negative animals (non-infected dogs from a non-endemic area with negative results in serology by DPP® LVC, screening test recommended by Brazilian Health Ministry, and negative results in PCR and culture of spleen aspirate) and 12 male sandflies reared in a laboratory spiked with *Leishmania* DNA (positive controls). Finally, a range of CT cut-off points across each ROC curve were analyzed for sensitivity and specificity to determine its optimal CT cut-off value. Canine tissue and sand fly samples were considered positive when CT values were less than or equal to its corresponding ROC curve CT cut-off value.

To evaluate dog DNA integrity, amplification of the 18S rRNA gene (Perkin-Elmer Applied Biosystems) was used as an internal reference of genomic canine DNA. Only canine DNA samples that successfully amplified the 18S rRNA gene were included. Predeveloped TaqMan assay reagents were used in accordance with manufacturer's recommendations. The slope of the standard curve for the 18S rRNA gene corresponded to −3.406 (SD ± 0.415) and an average slope was determined from CT values obtained from five independent experiments with a corresponding correlation coefficient (r^2) of 0.993 (SD ± 0.002). The results of parasite loads in infected dogs are expressed as the number of parasites per 10 mg of DNA of canine host tissue.

2.8. Statistical analysis

Differences in parasite loads among dogs with different clinical manifestations, as well as parasite loads in infected sandflies used for xenodiagnosis, were evaluated using the Mann-Whitney test. Fisher's exact test was used to compare infectiousness of dogs with presenting different clinical manifestation. Spearman's rank correlation coefficient was used to conduct correlation analysis. All p values < 0.05 were considered to be statistically significant.

3. Results

3.1. Study group

Of the 23 dogs included in this study, 13 were classified as dogs with subclinical infection and 10 as with symptomatic infection (Table 1). Cultures of splenic aspirate were positive in nine animals (39%), 6 with subclinical infection (46%) and 3 with symptomatic infection (30%) while serology by DPP was positive in eight (35%), 3 with subclinical infection (23%) and 5 with symptomatic infection (50%). All dogs tested positive by qPCR with respect to at least one of the tissue types evaluated, including 18 positive skin samples (78.2%), 10 with subclinical infection, 77%; 8 with symptomatic infection, 80%, 18 splenic aspirate samples (78.2%), 11 with subclinical infection (75%) and 7 with symptomatic infection (70%), and eight blood samples (35%), 4 with subclinical infection (31%) and 4 with symptomatic infection (40%).

3.2. Parasite load in tissue samples from infected dogs with diverse clinical manifestations of CVL

The median values found for parasite load in each evaluated canine tissue type were: 316 parasites in skin and 1335 in splenic aspirate (Table 1). Since in 15 out of 23 dogs, parasite DNA was not detected in blood samples, the median parasite load resulted in 0. Considered only samples from dogs that qPCR tested positive (n = 8), the median parasite load resulted in 309. No significant differences were found when comparing detected parasite load values within a given tissue type with respect to clinical manifestations of CVL (dogs with subclinical or symptomatic infection) (Fig. 1).

With respect to correlation testing considering parasite burden among the three tissue types evaluated, positive correlations were observed between parasite load in the blood and spleen (Spearman's; r = 0.57, P < 0.001), blood and skin (Spearman's; r = 0.64, p < 0.0001), and spleen and skin (Spearman's; r = 0.57, p < 0.001).

3.3. Capacity of naturally infected dogs to infect sand fly vectors with parasites

An assessment of the capacity of naturally infected dogs to infect sand fly vectors with parasites revealed that among the 653 sandflies used in xenodiagnosis, only 74 (11.4%) tested positive for *Leishmania* DNA by qPCR (Table 1). Canine infectiousness to sandflies, calculated as the percentage of dogs that successfully transmitted parasites to sandflies during xenodiagnosis, was similar in dogs with subclinical infection (54%; 07/13) and symptomatic infection (40%; 4/10) (p = 0.39). Further analysis of these dogs' capacity to infect sandflies showed that 44% of the infected animals

Table 1
Evaluation of naturally infected dogs with different clinical manifestations according to positivity in diagnostic tests for Leishmania infection and the capacity to infect sand fly vector.
Clinical classification/Dog no.

Clinical classification/Dog no.	Clinical Score	Diagnostic Test Type					Canine capacity of infect sandflies		
		DPP	Aspirate Spleen Culture	qPCR (*Leishmania* kDNA)			Infectiousness	Infection rate	Parasite load detected
				Skin	Spleen	Blood			
Subclinical infection									
01	0	–	+	89	274	1335	1	01/22 (4.5%)	3
02	0	–	–	0	0	113	0	0/31 (0%)	0
03	0	–	+	1238	0	660	0	0/42 (0%)	0
04	0	–	–	0	0	447	1	02/32 (6.25%)	3
05	2	+	+	18,020,075	6315	339,826	1	01/36 (2.7%)	45
06	1	–	–	0	0	18437	0	00/39 (0%)	0
07	2	–	–	24	0	0	0	0/14 (0%)	0
08	1	–	–	66	118	0	1	01/23 (4.3%)	4
09	1	–	–	645	0	628	1	1/41 (2.4%)	3
10	3	–	+	1553	0	966	1	25/31 (80.6%)	689
11	1	+	–	1014	0	26,274	0	0/25 (0%)	0
12	2	+	+	97	0	602	0	0/28 (0%)	0
13	2	–	+	59,187,691	509	17,105	1	01/28 (3.5%)	3
Symptomatic infection									
14	5	–	–	316	0	0	0	00/32 (0%)	0
15	7	–	–	67	0	0	0	0/23 (0%)	0
16	6	–	–	0	0	9382	0	00/25 (0%)	0
17	8	+	–	42,632	0	5640	0	0/25 (0%)	0
18	10	–	–	147	0	0	1	01/24 (4.1%)	8
19	12	+	–	737,615	58	17,602	0	0/13 (0%)	0
20	10	+	–	0	0	2198	0	0/23 (0%)	0
21	17	+	+	3,451,889,073	151194	17,863,788	1	28/36 (77.7%)	29774
22	8	–	+	30,625,119	344	19,058,029	1	11/29 (38%)	197
23	7	+	+	22,904,007	179	3440,041	1	02/28 (7.1%)	970
Total positivity rate		08/23 (35%)	09/23 (39.1%)	18/23 (78.2%)	08/23 (35%)	18/23 (78.2%)	11/23 (47.8%)	74/653 (11.3%)	1338

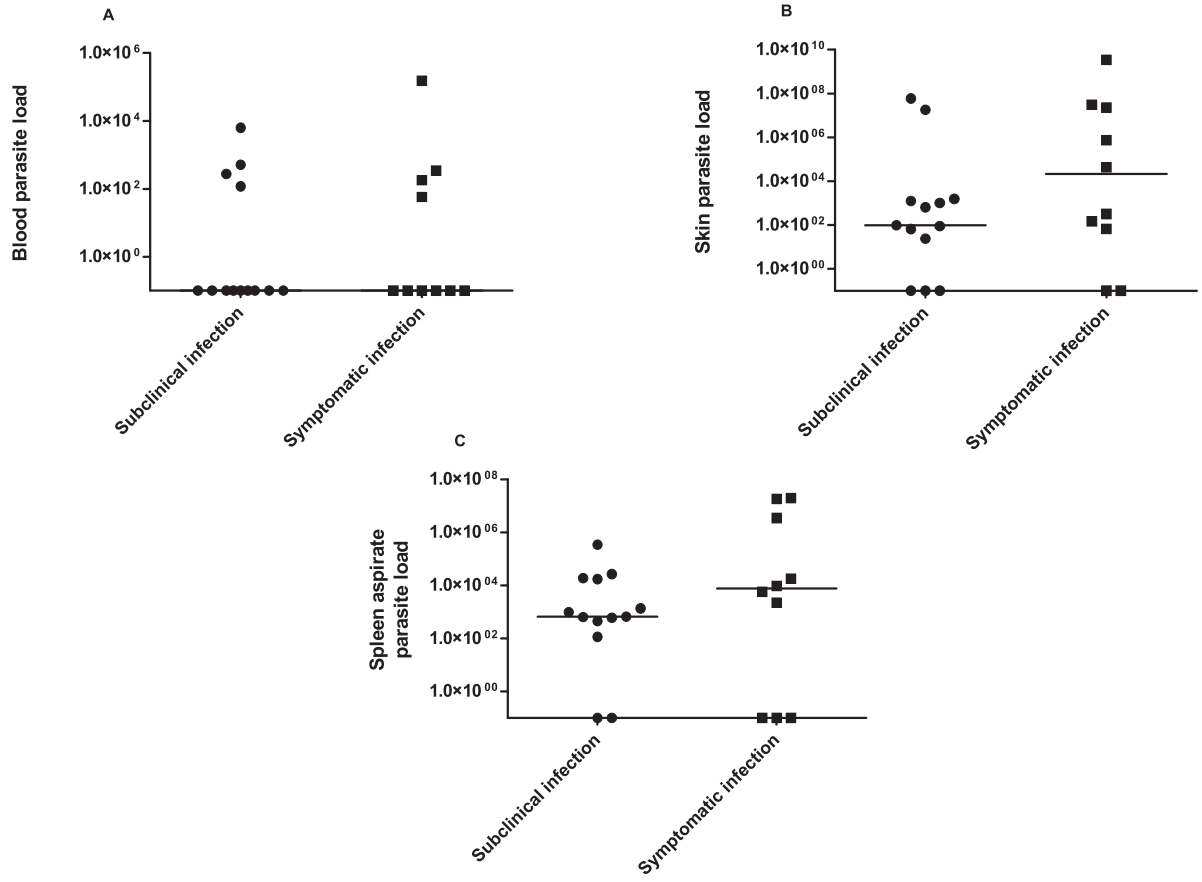

Fig. 1. Blood, skin and spleen samples parasite load of infected dogs according to clinical classification. Blood (A), skin (B), and spleen aspirate samples (C) were obtained from dogs classified with a different form of clinical manifestations of CVL: subclinical infection or symptomatic infection. Parasite load was detected using qPCR in all skin, blood, and splenic aspirate samples. Each plot represents the detected parasite load in an individual dog.

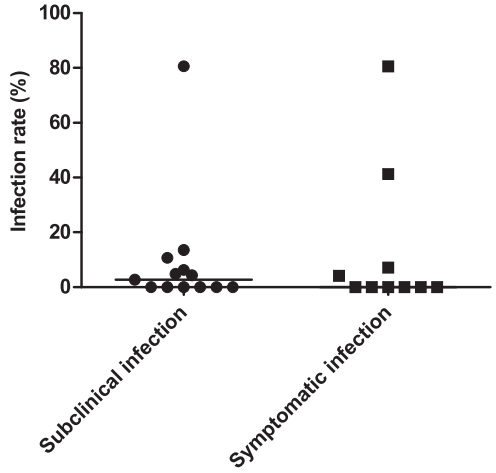

Fig. 2. Sand fly infection rate of naturally infected dogs according to clinical classification. Sand fly infection rates, calculated as the number of successfully infected sandflies following xenodiagnostic procedures, distributed according to clinical manifestation.

were capable of successfully infect sand fly vector with parasites, although 80% of these dogs infected to a relatively small number of sandflies (n = 1 to 2). Sand fly infection rate, which measures the number of sandflies that were positively infected following xenodiagnosis, remained similar between dogs with subclinical infection (9%) and with symptomatic infection (13%) dogs (Fig. 2).

Parasite load quantification performed in each sand fly submitted to xenodiagnosis revealed that parasite load ranged from 1 to 5971 parasites in the 74 infected sandflies, with a median value of 28 parasites, the 25% percentile was 4 and 75% percentile was 112.5 parasites. The median parasite loads among sandflies that became successfully infected was 10, after feeding on dogs with subclinical infection, and 84, after feeding on dogs with symptomatic infection. No differences were found when comparing the average parasite loads in infected sandflies, irrespective of the clinical classification of the animals on which the vectors fed (Fig. 3A). Fig. 3B illustrates individual sand fly parasite loads five days after xenodiagnosis. Most sandflies showed low parasite loads and, notably, a single highly symptomatic dog (score = 17, dog no 21) was found to be responsible for the highest parasite loads detected in sandflies (total: 29,774 parasites counted in 28 infected sandflies) (Fig. 3B).

Parasite load values in the dogs' blood and skin were shown to be positively correlated with canine infectiousness to sandflies (blood $r = 0.65$, $p < 0.001$; skin $r = 0.42$, $p = 0.05$) (Fig. 4A and B), yet no correlations were found with respect to spleen tissue ($r = 0.27$, $p = 0.21$). Interestingly, positive correlations were also found between parasite load in blood ($r = 0.59$ $P < 0.01$) or skin ($r = 0.43$, $P \leq 0.05$) and sand fly infection rate (Fig. 4C and D). Finally, a positive correlation was additionally found between parasite load in canine blood ($r = 0.66$, $P < 0.001$) and skin ($r = 0.54$, $P < 0.01$) and parasite burden in sandflies following xenodiagnosis (Fig. 5A and B). No correlations were found when comparing the parasite loads detected in splenic aspirate samples (Fig. 5C) to the parasite burden found in vectors five days after feeding.

4. Discussion

The present study represents an initial attempt to establish correlations between the intensity of parasite load in canine tissue and the parasite load detected in sandflies five days after feeding on naturally infected dogs with a range of clinical presentations. Almost half of the dogs were found to be capable of infect sand fly vectors with parasites, regardless of their clinical classification and corresponding clinical score. This finding supports the notion that a particular canine clinical manifestation of VL is not indicative of a dog's capacity to infect sand fly vectors with parasites. Remarkably, among these infective dogs, most (82%) infected to a relatively limited number of sandflies (between 1 and 2), which corresponds to approximately 10% of the total number of sandflies used in xenodiagnosis procedures. These results are consistent with those obtained by Courtenay et al. (2002), who similarly showed that most of the infective dogs studied infected no more than 10% of the sandflies employed. Taken together, these findings would seem to suggest that, in a given endemic area, one would expect a low number of infective dogs to successfully infect a proportionately low number of sandflies with parasites.

The present study utilized qPCR to detect parasite DNA in vectors five days following xenodiagnosis procedures, with low parasite burden found in the vast majority of sandflies. This may be explained by the choice to assess parasite load in the vectors at only five days post-xenodiagnosis. Undoubtedly, by quantifying the number of infective metacyclic forms of promastigotes under direct observation at seven days or later following blood-feeding, sensitivity would be enhanced. Additionally, this important information would provide a notion of how many infective parasites could be successfully transmitted to another mammalian host in a subsequent sand fly blood feeding. Another possibility to improve sensitivity with respect to this procedure is performing xenodiagnosis in animal lesion sites, as previously described by Aslan et al. (2016) in an experimental model of CVL. Future studies should strive to not only comprehensively address the proper site on the animal's skin to perform xenodiagnosis in naturally infected dogs, but also quantify parasite burden, in the sandflies that were successfully blood-fed, at time points seven days or later following blood-feeding.

The low infection rates and low parasite loads detected in sandflies in the present study support the notion that most vectors do not significantly impact the urban VL transmission cycle. Interestingly, we found a very high parasite load in the blood and skin of one of the dogs we evaluated, which was able to infect a high number of sandflies (78%) resulting in high parasite loads (5.9 to 10×10^3 parasites) at five days after feeding. We recognize that since our dog population represents a convenience sampling, the present findings are not indicative of the actual transmission cycle seen in a given dog population within an endemic area. Nonetheless, we speculate that a low number of highly infected dogs would likely have a more significant impact on parasite transmission to sandflies, while higher numbers of less-infected dogs should present less risk of transmissibility in the urban VL transmission cycle in endemic areas.

In the present report we also found that neither canine infectiousness nor sand fly infection rates have been found associated to CVL clinical manifestations similar to other authors (Guarga et al., 2000; Laurenti et al., 2013; Magalhaes-Junior et al., 2016; Molina et al., 1994). Different from us, other authors have demonstrated that both the capacity of dogs infect sandflies and sand fly infection rates are positively correlated with the presence of clinical signs in infected dogs (da Costa-Val et al., 2007; Vercosa et al., 2008). These discrepancies in the literature may be due to the lack of standardization regarding xenodiagnoses.

Xenodiagnosis is considered the only way to accurately identify which dogs are infective to sand fly vectors (Guarga et al., 2000; Michalsky et al., 2007; Molina et al., 1994), yet, due to its inherent limitations, this technique is unable to improve the effectiveness of programs designed to control leishmaniasis in endemic areas. Thus, the identification of tissues in which parasite load is positively correlated with canine infectiousness is paramount. Deane and Deane (1954) showed that the skin of asymptomatic dogs in an endemic area can harbor *Leishmania* parasites, indicating this tissue type could be a potential marker of infectiousness. Interest-

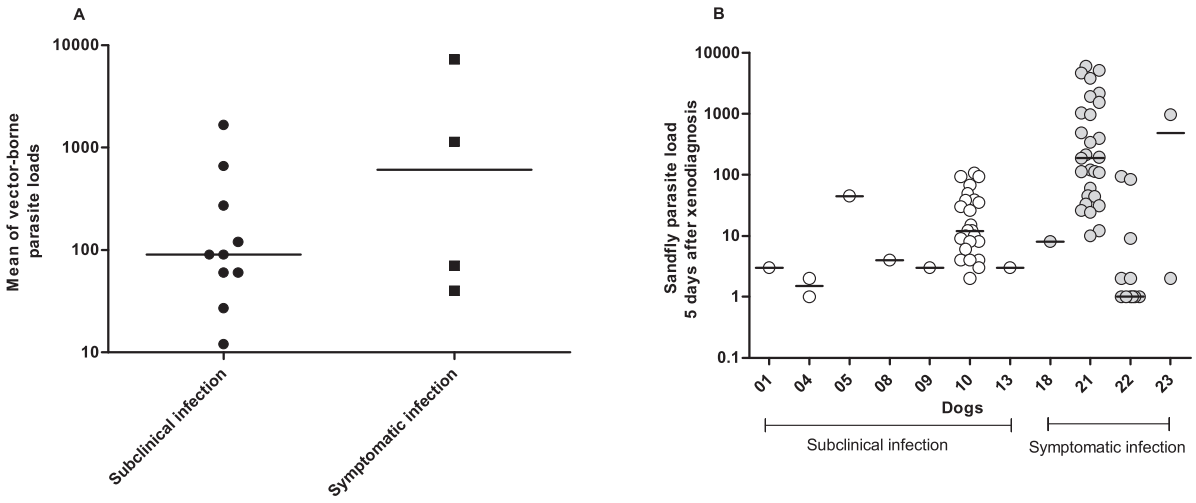

Fig. 3. Parasite load in infected sandflies fed on naturally infected dogs with a range of clinical classifications. (A) Mean parasite loads in sandflies measured 5 days after being fed on naturally infected dogs, which were classified according to clinical VL presentation. (B) Transmission capacity of infective dogs, measured as the parasite load in each sand fly 5 days following xenodiagnosis, according to clinical manifestation of CVL.

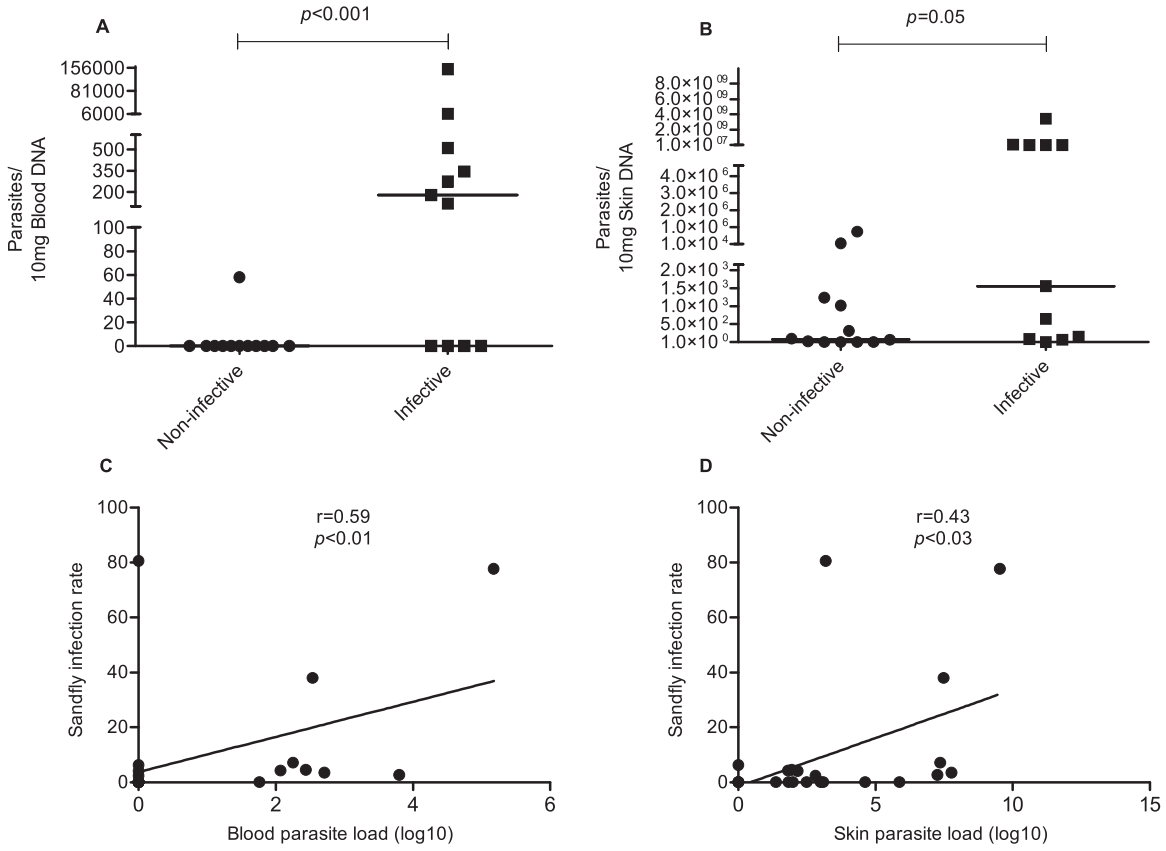

Fig. 4. Parasite load in the blood and skin of naturally infected dogs in association with canine capacity to infect sand fly: distribution of parasite load in blood (A) and skin (B) of non-infective and infective dogs. Correlation between parasite load in blood (C) and skin (D) of dogs with sand fly infection rate following xenodiagnosis. Parasite load was detected by qPCR in blood and skin samples and it is represented as the number of parasites measured in 10 mg of DNA. Each plot represents the detected parasite load in an individual dog. Relevant Spearman correlation coefficients (r) and *p* values are shown.

ingly, the present report we found positive correlations between parasite loads in both skin and blood with respect to canine infectiousness to the sand fly vector, as well as the sand fly infection rate and parasite load in sand fly vectors following xenodiagnosis. Furthermore, De Amorim et al. (2011) and Courtenay et al. (2002) also demonstrated an association between high parasite load in dog skin and canine infectiousness. These authors considered a high parasite load in dog skin to be a strong predictor of canine infectiousness to sandflies (Courtenay et al., 2014). Moreover, Vercosa et al. (2008) demonstrated a positive association between canine infectiousness to sandflies and an elevated degree of blood parasitism in dogs, which is in agreement with our finding of a positive correlation between parasite load in canine blood and dog capacity to infect sandflies. Taken together, these findings support the notion that

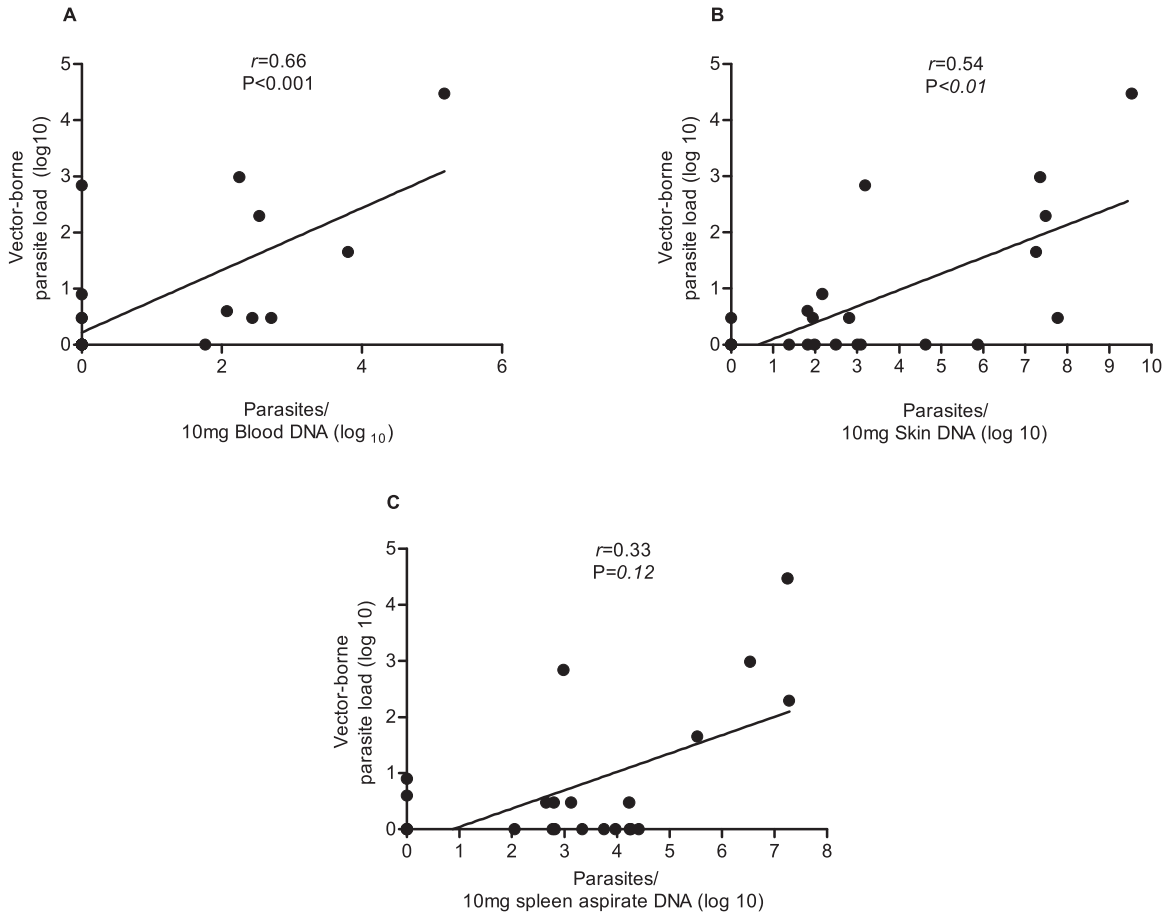

Fig. 5. Evaluation of parasite load in infected sandflies in relation to parasite load in different tissues of naturally infected dogs. Each plot represents the mean parasite load detected in sandflies 5 days following xenodiagnosis in accordance with the parasite load detected in canine tissue type: blood (A), skin (B) and splenic aspirate (C). Relevant Spearman correlation coefficients (r) and *p* values for each tissue type are shown in the respective plots.

parasite load in both canine skin and blood could potentially serve as reliable markers of the canine capacity to infect sand fly vector.

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