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PCR MULTIPLEX EM TEMPO REAL PARA DETECÇÃO DE FONTES ALIMENTARES E DE *LEISHMANIA* SPP. EM FLEBOTOMÍNEOS

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KAMILA GAUDÊNCIO DA SILVA SALES

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Tese apresentada ao Doutorado em Biociências e Biotecnologia em Saúde do Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, como requisito parcial para obtenção do título de Doutor em Ciências.

Orientador: Dr. Filipe Dantas Torres

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RESUMO

No presente trabalho, objetivou-se desenvolver um ensaio de PCR em tempo real multiplex para detectar simutaneamente DNA de cão, humano e *Leishmania* spp. em flebotomíneos. Primers e sondas TaqMan visando os genes mitocondriais: citocromo c oxidase subunidade I e citocromo b de cão e humano, respectivamente, foram combinados em um ensaio multiplex, no qual também foram incluídos primers e uma sonda TaqMan visando o DNA do cinetoplasto de *Leishmania*. O ensaio multiplex foi 100% específico, com sensibilidade analítica de 10^3 fg por reação para cão e humano e 1 fg para *Leishmania*. Ao testar fêmeas de flebotomíneos ingurgitadas coletadas em campo (95 *Migonemyia migonei* e dois *Nyssomyia intermedia*), 50 *M. migonei* foram positivas para um ou dois alvos (taxas de positividade: 45,4% para humanos, 4,1% para cães e 12,4% para *Leishmania* spp.). Este ensaio multiplex de PCR em tempo real representa um novo e rápido teste para detectar DNA de cão, humano e *Leishmania* spp. em fêmeas de flebotomíneos e, portanto, uma ferramenta para avaliar o risco de transmissão de *Leishmania* em áreas onde as leishmanioses são endêmicas.

Palavras-chave: Flebotomíneos. Alimentação sanguínea. PCR em tempo real. Multiplex.

SALES, Kamila Gaudêncio da Silva. **Multiplex real-time PCR for detection blood meals and *Leishmania* spp. in sand flies**. 2019. Thesis (Doctorate in Biosciences and Biotechnology in Health) - Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, 2019.

ABSTRACT

The goal of the present study was to develop a multiplex real-time PCR assay for simultaneous detection of DNA from dog and human and *Leishmania* spp. in sand flies. Primers and TaqMan probes targeting the mitochondrially encoded cytochrome *c* oxidase I and cytochrome b genes of dog and human, respectively, were combined in a multiplex assay, which also includes primers and a TaqMan probe targeting the *Leishmania* kinetoplast DNA. The multiplex assay was 100% specific, with analytical sensitivities of 10^3 fg/reaction for dog and human and 1 fg for *Leishmania*. By testing field-collected engorged female sand flies (95 *Migonemyia migonei* and two *Nyssomyia intermedia*), 50 *M. migonei* were positive for one or two targets (positivity rates: 45.4% for human, 4.1% for dog and 12.4% for *Leishmania* spp.). This multiplex real-time PCR assay represents a novel fast assay for detecting DNA from dog, human and *Leishmania* spp. in female sand flies and therefore a tool for assessing the risk of *Leishmania* transmission to these hosts in areas where leishmaniases are endemic.

Keywords: Phlebotomine sand flies. Blood meal. Real-time PCR. Multiplex.

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1 INTRODUÇÃO

A presente tese, apresentada na forma de artigos, teve como objetivo principal o desenvolvimento de um ensaio de PCR multiplex em tempo detecção de DNA de *Leishmania* spp., cão e humano em flebotomíneos. Contudo, durante os quatro anos de doutorado, realizaram-se vários estudos de campo e laboratório correlacionados com o objetivo principal da tese.

Em 2017 publicamos na revista *Plos Neglected Tropical Diseases* o artigo "Sand fly population dynamics and cutaneous leishmaniasis among soldiers in na Atlantic forest remnant in northeastern Brazil" (Artigo 1), o qual descreve um estudo sobre a dinâmica populacional de flebotomíneos em um campo de treinamento militar em uma área de remanescente da Mata Atlântica no nordeste do Brasil, onde vários surtos de leishmaniose cutânea têm sido esporadicamente descritos. Os resultados deste trabalho sugeriram que o risco de leishmaniose cutânea por *Leishmania braziliensis* na área estudada é permanente, aparentemente não correlacionados aos picos populacionais dos flebotomíneos. No mesmo estudo, detectamos por PCR em tempo real o DNA de *Leishmania* spp. em fêmeas de flebotomíneos, utilizando um protocolo padrão descrito na literatura.

Ainda em 2017, otimizamos o ensaio de PCR em tempo real utilizado no estudo supracitado e publicamos na revista *Molecular and Cellular Probes* o artigo "*Leishmania*-FAST15: A rapid, sensitive and low-cost real-time PCR assay for the detection of *Leishmania infantum* and *Leishmania braziliensis* kinetoplast DNA in canine blood samples" (Artigo 2). Nesse artigo descrevemos um ensaio de PCR em tempo real (chamado de "*Leishmania*-FAST15") para a detecção e quantificação do DNA de *L. infantum* e *L. braziliensis* em diferentes tipos de amostras. As principais vantagens desse ensaio é que ele utiliza um pequeno volume de reação e é de rápida execução, com os resultados disponíveis em menos de 34 minutos. Desta forma, minimizando os custos dos ensaios e tempo necessário para obter os resultados. Além disso, esse ensaio pode acelerar as decisões de tratamento para pacientes caninos e humanos.

Em 2019 publicamos na revista *Parasites and Vectors* o artigo "Home sweet home: sand flies find a refuge in remote indigenous villages in north-eastern Brazil, where leishmaniasis is endemic" (Artigo 3), cuja a proposta foi de reunir dados epidemiológicos que pudessem indicar a ocorrência de um ciclo de transmissão peri-doméstica/doméstica de *L. braziliensis* em aldeias indígenas localizadas em uma área rural remota do estado de Pernambuco. Os resultados obtidos nesse trabalho sugerem que diferentes flebotomíneos

vetores podem ser adaptados às habitações humanas, aumentando assim o risco de transmissão de *Leishmania* nos ambientes doméstico e peridoméstico.

Por fim, em 2020, aprovamos para publicação na revista *Parasites and Vectors* o artigo "Fast multiplex real-time PCR assay for simultaneous detection of dog and human blood and *Leishmania* parasites in sand flies" (Artigo 4) no qual descrevemos um ensaio rápido de PCR multiplex em tempo real para detecção simultânea de DNA de *Leishmania* spp., cão e humano em flebotomíneos, com alta sensibilidade e especificidade analíticas, além de custo relativamente baixo. Os testes com amostras de campo do Artigo 3 confirmaram a boa performance do novo ensaio. Sabe-se que os cães são considerados os principais reservatórios de *L. infantum* no ambiente doméstico. Portanto, a detecção de *Leishmania*, sangue de cães e humanos em flebotomíneos, poderia indicar o risco eminente de transmissão de *Leishmania* spp. no ambiente doméstico e peridoméstico. Desta forma, o novo ensaio de PCR multiplex em tempo real desenvolvido no presente trabalho poderá representar uma ferramenta para avaliar a infecção por *Leishmania* spp. em fêmeas de flebotomíneos e para investigar se e com que freqüência essas fêmeas se alimentam de cães e humanos, permitindo assim estimar o risco de infecção nesses hospedeiros.

1.1 Revisão de literatura

1.1.1 Sistemática e identificação de flebotomíneos

Os flebotomíneos são pequenos insetos e possuem diferentes nomes vulgares, tais como "mosquito-palha" (devido à sua coloração amarelada semelhante à palha vegetal), "asa-dura" (por possuírem asas que se mantêm eretas durante o pouso), "tatuquira" (devido ao hábito de se abrigarem em buracos de tatu), "arrepiado" (por possuírem o corpo "piloso"), "pula-pula" (devido ao voo do tipo saltitante), "mosquitos" (por ser um inseto pequeno e que voa) e "cangalhinha" (por apresentarem a posição do tórax semelhante a uma "cangalha") (DIAS, 2011; MARTINS; WILLIAMIS; FALCÃO, 1978). Embora o termo mosquito seja utilizado para esses insetos, essa denominação está incorreta, pois o mesmo foi cunhado para insetos da família Culicidae e, sabidamente, flebotomíneos exibem características morfológicas, biológicas e genéticas distintas.

Os flebotomíneos pertencem ao filo Arthropoda, classe Insecta, ordem Diptera, subordem Nematocera, família Psychodidae e subfamília Phlebotominae (FORATTINI, 1973). A subfamília Phlebotominae apresenta uma longa história evolutiva e acredita-se que

os seus primeiros representantes tenham aparecido durante o Cretáceo Inferior aproximadamente 120-135 milhões de anos atrás (LEWIS, 1982). A primeira espécie de flebotomíneo foi descrita por Scopoli em 1786 na Itália, que após anos sendo denominada como *Bibio papatasi* teve a sua nomenclatura modificada, sendo hoje conhecida como *Phlebotomus papatasi* (MAROLI *et al*., 2013). Os primeiros flebotomíneos americanos (*i.e*., *Flebotomus vexator* e *Flebotomus cruciatus*, que ainda hoje não ocorrem no Brasil) foram descritos por Coquillett (1907).

Conforme a classificação adotada, a subfamília Phlebotominae é composta por seis gêneros: *Phlebotomus*, *Sergentomyia* e *Chinius* no Velho Mundo e *Lutzomyia*, *Brumptomyia* e *Warileya* no Novo Mundo (YOUNG; DUNCAN, 1994). Entre os gêneros de flebotomíneos do Novo Mundo, *Lutzomyia* é o maior e de mais ampla distribuição geográfica, com representantes desde os Estados Unidos até o Norte da Argentina e Uruguai. De acordo com a classificação tradicional, o gênero *Lutzomyia* estaria formado por 15 subgêneros, 11 grupos de espécies e duas espécies com descrição deficiente (YOUNG; DUNCAN, 1994).

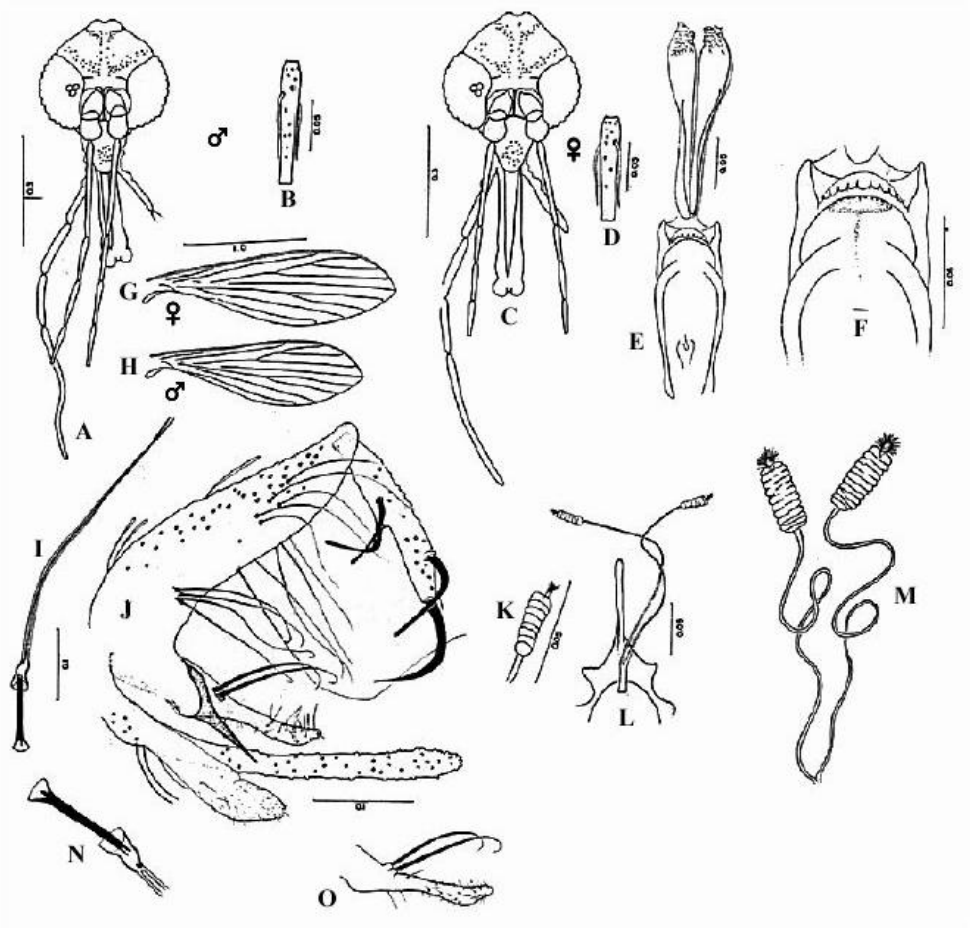
Recentemente, uma nova proposta de classificação (GALATI, 1995, 2003), que utilizou o método cladístico para estudar estes dípteros americanos, organizou os flebotomíneos do Novo Mundo em 23 gêneros: *Hertigia*, *Warileya*, *Brumptomyia*, *Oligodontomyia*, *Deanemyia*, *Micropygomyia*, *Sciopemyia*, *Lutzomyia*, *Migonemyia*, *Pintomyia*, *Dampfomyia*, *Expapillata*, *Pressatia*, *Trichopygomyia*, *Evandromyia*, *Psathyromyia*, *Viannamyia*, *Martinsmyia*, *Bichromomyia*, *Psychodopygus*, *Nyssomyia*, *Trichophoromyia* e *Edentomyia* (SHIMABUKURO *et al*., 2017).

Atualmente, são descritas aproximadamente 1.000 espécies de flebotomíneos válidas no mundo, das quais 530 são encontradas nas Américas, ocorrendo em 28 países (SHIMABUKURO, 2017). No Brasil, cerca de 279 espécies de flebotomíneos já foram registradas (AGUIAR; VIEIRA, 2018). Até este estudo, 42 espécies foram encontradas em Pernambuco (DANTAS-TORRES *et al.* 2010; AGUIAR; VIEIRA, 2018; SALES *et al*., 2019). A identificação dos flebotomíneos requer que o operador detenha habilidades e *expertise* taxonômica considerável a fim de diminuir ou excluir a possibilidade de equívocos na descrição das espécies (LATROFA *et al*., 2012).

Os febotomíneos adultos destinados à identificação toxonômica necessitam passar por uma delicada preparação dos espécimes (clarificação) e montagem em lâminas de microscopia na posição lateral e/ou dorso-ventral, para facilitar a visualização das características morfológicas: cibário, faringe, ascóides, número de segmentos antenais,

palpos, estruturas genitais (internas e externas), cerdas e nervuras das asas (AGUIAR; VIEIRA, 2018; YOUNG; DUNCAN, 1994) (Figura 1).

Figura 1 – Estruturas anatômicas de interesse taxonômico do macho e da fêmea de *Lutzomyia longipalpis*.



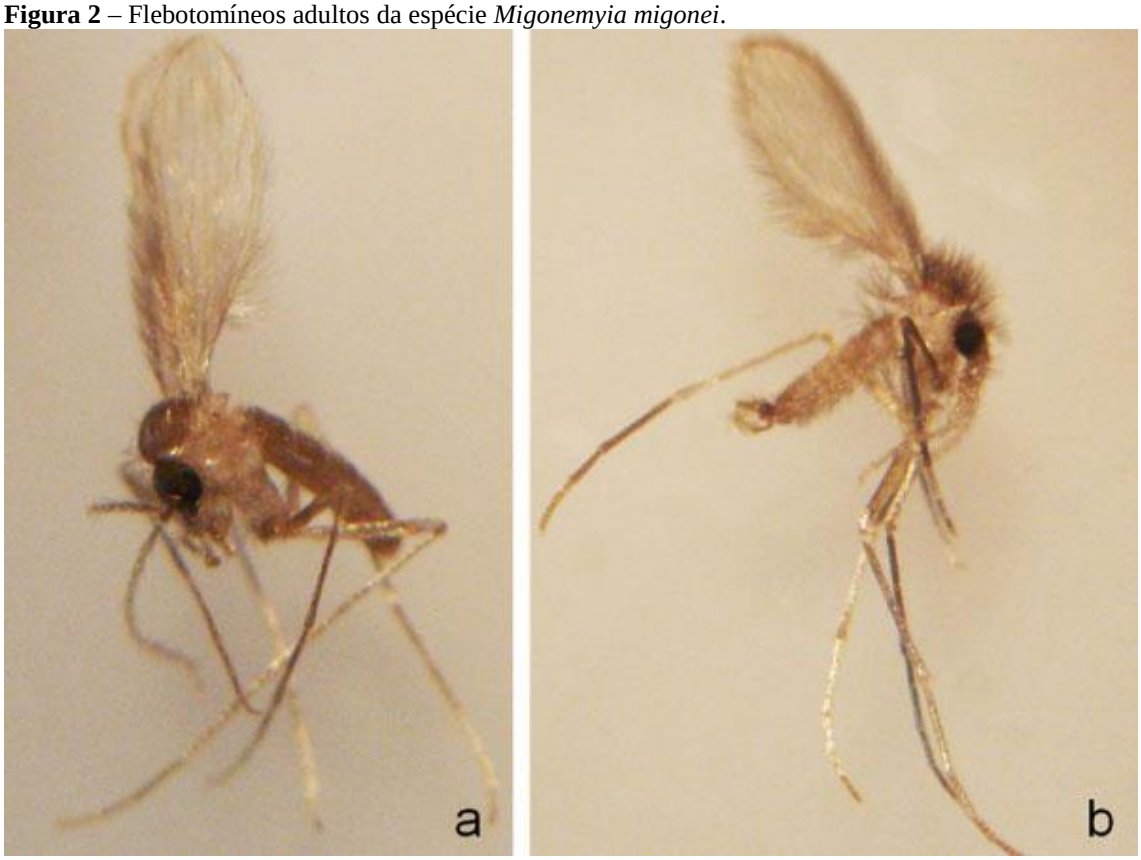
Fonte: Adaptado de Young e Duncan (1994).
Legenda: A) cabeça do macho; **B**) flagelômero II do macho; **C**) cabeça da fêmea; **D**) flagelômero II da fêmea; **E**) cibário e faringe da fêmea; **F**) cibário da fêmea; **G**) asa do macho; **H**) asa da fêmea; **I**) bomba e filamentos genitais; **J**) terminália do macho; **K**) corpo da espermateca; **L**) forquilha e espermateca; **M**) espermatecas; **N**) bomba genital; **O**) parâmero do macho.

A taxonomia clássica (morfológica) é inquestionável e sua utilização resolve a maioria das dificuldades de identificação. Contudo, em situações em que existem espécies crípticas (morfologicamente indistinguíveis), a taxonomia morfológica ainda pode apresentar limitações. Atualmente, com a introdução de novos métodos, como análise cromossômica, morfometria multivariada, isoenzima, análise molecular, filogenética e a espectrometria de massa, a classificação desse grupo de insetos tem gerado resultados mais robustos e elucidativos (AKHOUNDI *et al*., 2016).

1.1.2 Biologia e ecologia

Os flebotomíneos são insetos de pequeno porte medindo geralmente de 3 a 4 mm de comprimento (KILLICK-KENDRICK, 1999) e são holometábolos, isto é, apresentam desenvolvimento com metamorfose completa, com estágios de ovo, larva (quatro estádios ou ínstares), pupa e adulto. Quando adultos eles apresentam características peculiares, incluindo corpo "piloso", pernas longas e delgadas (Figura 2), voo do tipo saltitante e baixo, asas lanceoladas que se mantêm eretas em forma de "V" durante o repouso. Nesta fase possuem hábitos crepusculares e noturnos (BRAZIL; BRAZIL, 2003).

Figura 2 – Flebotomíneos adultos da espécie *Migonemyia migonei*.



Fonte: Dantas-Torres (2009).
Legenda: Fêmea (**a**) e macho (**b**).

As formas imaturas podem ser encontradas em diferentes ambientes domésticos (*e.g*., porões de casas, construções abandonadas, fendas no chão e paredes), peridomésticos (*e.g*., tocas de animais, aterros, latrinas e sob pedras) e silvestres (*e.g*., cavernas, toca de roedores, formigueiros e raízes tubulares) (FELICIANGELI, 2004; FORATTINI, 1973; SANGIORGI *et al*., 2012).

Os ovos são alongados de forma oval ou elipsoide, pálidos após postura, ao passo que em seguida a exposição ao ar eles escurecem (Figura 3). Medem de 300 a 500 mm de comprimento, por 70 a 150 mm de largura e exibem nervuras ou protuberâncias em seu exocório, as quais são peculiares de cada espécie ou complexo de espécies (BRAZIL; BRAZIL, 2003). A postura é feita isoladamente ou em pequenos grupos. E os ovos permanecem aderidos ao substrato, devido a uma substância produzida pelas glândulas acessórias (BRAZIL; BRAZIL, 2003). Esta substância é composta principalmente de ácidos graxos e tem como função de impermeabilização à água, podendo também atuar como feromônio de oviposição (BRAZIL; BRAZIL, 2003; MAROLI, 2013; RANGEL; LAINSON, 2003; REY, 1992).

De um modo geral, em condições de laboratório, as fêmeas adultas de flebotomíneos depositam em média de 30 a 70 ovos durante um único ciclo gonotrófico, embora haja grande variação de acordo com a espécie e condição ambiental (VOLF; VOLFORA, 2011). A quantidade de ovos depende de vários fatores, inclusive da quantidade de sangue ingerido pela fêmea (RANGEL; LAINSON, 2003).

Figura 3 – Ovos de flebotomíneos após a exposição ao ar.

Fonte: A autora.

Entre sete e 17 dias após a postura acontece à eclosão das larvas. Para que isso ocorra é necessário que o ambiente apresente condições favoráveis em termos de temperatura (25-27°C) e umidade (70-80%). Ao contrário de outros dípteros nematóceros, como mosquitos (Culicidae) e simulídeos (Simuliidae), os flebotomíneos se desenvolvem em microhabitat terrestre em vez de aquáticos (BRAZIL; BRAZIL, 2003).

A fase larval passa por quatro estádios de desenvolvimento (L1-L4). Em geral, são pequenas, esbranquiçadas, de aspecto vermiforme (Figura 4), sendo constituídas por 12 segmentos, sendo três torácicos e nove abdominais (iguais, com exceção dos dois últimos que são modificados à função locomotora). Apresentam apêndices cefálicos (antenas), que mudam de acordo com o estádio larval e a espécie, sendo as peças bucais do tipo triturador. Alimentam-se de matéria orgânica, das cascas dos ovos eclodidos e dos corpos dos adultos mortos após a postura. Todo segmento abdominal é recoberto por cerdas, que podem variar de acordo com o estádio larval (BRAZIL; BRAZIL, 2003). O período de desenvolvimento larval geralmente dura cerca de três semanas (VOLF; VOLFORA, 2011)

Figura 4 – Larva de flebotomíneo (estádio L4).

Fonte: A autora.

As pupas possuem coloração branco-amarelada, permanecendo aderidas ao substrato até a emergência dos adultos (Figura 5). São compostas por 13 segmentos, sendo os quatro

primeiros mais ou menos fundidos, formando o cefalotórax, ao passo que os nove restantes são individualizados e constituem o abdômen (BRAZIL; BRAZIL, 2003; FERRO *et al*., 1997). No final do período de desenvolvimento pupal, a pupa já começa a assumir uma forma semelhante à do adulto. O período de desenvolvimento pupal dura cerca de 7 a 10 dias, ao final do qual emergem os flebotomíneos adultos (VOLF; VOLFORA, 2011).

Figura 5 – Pupa de flebotomíneo.

Fonte: A autora.

Os flebotomíneos adultos, logo após a eclosão, permanecem pouco ativos. Eles apresentam dimorfismo sexual evidente, o macho apresenta uma probóscide curta, enquanto que nas fêmeas a probóscide é mais longa e adaptada para a realização do repasto sanguíneo (BRAZIL; BRAZIL, 2003). Os últimos segmentos abdominais são modificados para constituir a genitália dos flebotomíneos, sendo essa externa nos machos e interna nas fêmeas. Os machos apresentam grandes modificações na porção final do abdômen, com a presença de apêndices (lobos epandriais, parâmeros, gonocoxito, gonóstilo, dutos ejaculadores e espinhos) que formam a genitália externa (estruturas com valor taxonômico). Já nas fêmeas, esses apêndices externos estão ausentes e a genitália interna é constituída basicamente de espermatecas e glândulas acessórias (BRAZIL; BRAZIL, 2003). Em geral, a longevidade do adulto em condições naturais é praticamente desconhecida, mas as observações em o

laboratório indicam que os flebotomíneos podem viver de 20 a 30 dias, embora esse possa não ser o tempo de vida real (BRAZIL; BRAZIL, 2018).

Os flebotomíneos adultos, tanto fêmeas quanto os machos, possuem o aparelho bucal do tipo picador-sugador, medindo de 0,2 a 0,4 mm o que permite efetuar alimentação de carboidratos como: glicose, frutose, sacarose, turamose, melezitose, rafinose, maltose e trealose, obtidos de seiva de planta, néctar e secreções de afídeos ou outros homópteros (ALEXANDER; USMA, 1994).

Após os flebotomíneos ingerirem uma substância açucarada, esta é armazenada no divertículo, de onde é levada para o trato digestivo e em seguida é absorvida. Esses carboidratos são utilizados principalmente como fonte de energia para exercerem as suas principais atividades de voo, acasalamento, postura e garantir a sobrevida (BRAZIL; BRAZIL, 2003). Além disso, os açúcares desempenham um papel importante no desenvolvimento e infectividade de *Leishmania* spp., não só no controle da flora intestinal e atuando como bacteriostáticos, mas também como fonte de energia para os parasitos (BRAZIL; BRAZIL, 2018).

Apenas as fêmeas são hematófagas (ALEXANDER, 2000; BRAZIL; BRAZIL, 2003; FERRO *et al*., 1997). No período reprodutivo, as fêmeas (Figura 6) precisam de sangue, sendo este o alimento fundamental para a maturação dos ovos (FORATTINI, 1973). As fêmeas iniciam sua alimentação sanguínea entre 24 e 48 horas após a sua emergência da pupa (BRAZIL; BRAZIL, 2003, 2018). Normalmente realizam um único repasto, mas em algumas espécies foram observados mais de um repasto antes da oviposição, tal comportamento ocorreria devido às variações climáticas, altas temperaturas e baixa umidade, prejudicando a oviposição, sendo necessário mais de um repasto (BRAZIL; BRAZIL, 2003). A realização de mais de um repasto sanguíneo é um fenômeno de grande importância epidemiológica porque aumenta o poder de transmissão de patógenos por esses insetos (BRAZIL; BRAZIL, 2018).

O número de ovos produzidos está diretamente relacionado à quantidade de sangue ingerido (READY, 1979). De um modo geral, cada fêmea pode ingerir o volume de sangue igual ao próprio peso, logo a quantidade de sangue ingerido varia de acordo com a espécie, podendo variar de 0,1 a 0,6 mg (RANGEL; LAINSON, 2003).

Figura 6 – Fêmea adulta de flebotomíneo.

Fonte: Collins (2011).

Os flebotomíneos apresentam peças bucais relativamente curtas impedindo assim a penetração profunda na pele do hospedeiro (KAMHAWI, 2006). Em vista disso, suas peças bucais são adaptadas a dilacerar a pele do hospedeiro e romper pequenos vasos sanguíneos, formando assim uma pequena poça de sangue de onde se alimentam, processo esse chamado de telmofagia (ou "pool-feeding") (LEHANE; GUARNERI, 2009). Durante a hematofagia, as fêmeas secretam a saliva que contém diferentes substâncias, tais como anticoagulantes, antiagregantes plaquetários, imunossupressores e vasodilatadores que favorecem o fluxo sanguíneo durante a alimentação (KAMHAWI, 2006; RIBEIRO *et al*., 1995). Danos vasculares causados pela picada durante a hematofagia desencadeiam respostas fisiológicas de defesa no hospedeiro que são determinadas principalmente por dois eventos importantes: homeostase e processo inflamatório (RIBEIRO, 1987).

O tempo de sucção completa em espécies de flebotomíneos neotropicais, que se alimentam de animais de sangue quente, varia consideravelmente, de 1 a 5 minutos, enquanto o processo é muito mais lento em espécies que se alimentam em animais de sangue frio (BRAZIL; BRAZIl, 2018). Após a ingestão, o sangue é direcionado ao intestino médio e em seguida o bolo alimentar formado sofre a ação de enzimas secretadas pelo intestino médio (*e.g*., tripsinas e amino-carboxipeptidases). Assim, a digestão permite a conversão de nutrientes sanguíneos em proteínas necessárias para o desenvolvimento dos ovos e para o suprimento do gasto energético (PIMENTA *et al*., 2003). Além disso, sabe-se que compostos presentes na saliva de flebotomíneos exibem ação quimiotática para monócitos e

imunorreguladora, com capacidade de interagir com os macrófagos, aumentando sua propagação e dificultando a ação efetora destas células na destruição dos parasitos (MICHALICK, 2011).

A preferência dos flebotomíneos por determinados tipos de hospedeiros vertebrados para a obtenção do sangue é bastante variada. O comportamento pouco seletivo de algumas espécies leva esses insetos a se alimentarem em diversas fontes de vertebrados, inclusive humanos. Esse comportamento facilita a transmissão de patógenos (KILLICK-KENDRICK, 1999).

Cada espécie de flebotomíneo pode ter suas próprias preferências alimentares, embora a disponibilidade do hospedeiro seja um fator importante na determinação do repasto sanguíneo. Diversas espécies são oportunistas e se alimentam de animais, os quais têm acesso mais fácil, esse comportamento se confirma quando as mesmas espécies coletadas em diferentes áreas exibem padrões de alimentação diferentes (ABBASI; CUNIO; WARBURG, 2009).

A diversidade vegetal, animal e fatores microclimáticos que caracterizam as florestas tropicais fornecem recursos alimentícios, abrigos e condições ótimas de temperatura e umidade para o desenvolvimento dos flebotomíneos, influenciando a abundância e diversidade de espécies (SILVA *et al*., 2007). A degradação de habitats naturais pode forçar os flebotomíneos a se adaptarem ao ambiente modificado e desta forma são atraídos por habitações humanas, onde eles podem encontrar fontes de alimento (por exemplo, animais domésticos, humanos) (SALES *et al*., 2015).

1.1.3 Importância médica: leishmanioses, arboviroses e bartonelose

Os flebotomíneos apresentam grande importância médica e veterinária, pois são considerados vetores de protozoários, vírus e bactérias, além de causarem grande incômodo ao ser humano e outros vertebrados através de suas picadas dolorosas provocando muitas vezes reações alérgicas nos hospedeiros (MAROLI *et al*., 2013).

As leishmanioses são zoonoses causadas por várias espécies de protozoários flagelados que pertencem ao gênero *Leishmania,* família Trypanosomatidae e ordem Kinetoplastida. Essas doenças estão presentes em 98 países, distribuídos na Europa, África, Ásia e América, contudo, a sua notificação é compulsória em apenas 30 deles (ALVAR *et al*., 2012). As leishmanioses podem se apresentar em diferentes formas clínicas conforme a

espécie de *Leishmania* e a resposta imune do hospedeiro (BARRAL; COSTA, 2011). De modo geral, as leishmanioses são divididas em duas formas clínicas principais: leishmaniose cutânea (LC) e leishmaniose visceral (LV). As diferentes formas estão distribuídas em todo o mundo, com uma estimativa de 350 milhões de pessoas vivendo em regiões endêmicas (ALVAR *et al*., 2012).

Os 10 países com maior estimativa de casos de LC são: Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia, Sudão do Norte, Costa Rica e Peru, juntos representam entre 70 a 75% da incidência estimada global desta doença (ALVAR *et al.*, 2012). E ainda, mais de 90% dos casos de LV ocorrem em seis países: Índia, Bangladesh, Sudão, Sudão do Sul, Etiópia e Brasil. Estima-se que cerca de 1,6 milhões de novos casos dessas doenças surjam a cada ano, dos quais 500.000 são de LV e 1,1 milhão de LC (ALVAR *et al.*, 2012). As leishmanioses estão entre as seis doenças infecciosas mais importantes do mundo, o que pode ser explicado pelo coeficiente de detecção elevado, alto número de mortes e deformidades relacionadas (ALVAR *et al.*, 2012). Entre os patógenos que os flebotomíneos são capazes de transmitir, as leishmânias ocupam o primeiro lugar em importância em termos de morbidade e mortalidade no ser humano e outros animais (BARRAL; COSTA, 2011).

Atualmente, cerca 45 espécies do gênero *Leishmania* foram descritas, incluindo os quatro subgêneros (*i.e*., *Leishmania*, *Viannia*, *Sauroleishmania* e *Mundinia*). Entre estas espécies ~ 22 têm importância médica significativa (ESPINOSA *et al*., 2018). Nas Américas, entre as 15 espécies dermotrópicas de *Leishmania* circulantes, sete estão presentes no Brasil: *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *guyanensis*, *Leishmania* (*Leishmania*) *amazonensis*, *Leishmania* (*Viannia*) *lainsoni*, *Leishmania* (*Viannia*) *naiffi*, *Leishmania* (*Viannia*) *shawi* e *Leishmania* (*Viannia*) *lindenbergi*. Ao passo que, *Leishmania* (*Leishmania*) *infantum* é a espécie responsável por causar a LV, a forma mais grave da doença (MAIA-ELKHOURY; ALBUQUERQUE; SALOMÓN, 2018).

O ciclo de vida das leishmânias se desenvolve parte no inseto vetor e parte no hospedeiro vertebrado (SACKS; PERKINS, 1984). Inicialmente a fêmea de flebotomíneo, ao realizar o repasto sanguíneo no hospedeiro infectado, ingere o parasito na forma amastigota (que apresentam morfologia esférica e sem flagelo, que se reproduzem por fissão binária) (RIBEIRO, 1987). Essa forma é conduzida ao intestino médio do inseto e envolta pela matriz peritrófica (MP) junto ao bolo alimentar (PIMENTA *et al*., 1997; SECUNDINO *et al*., 2005; WALTERS *et al*., 1993, 1995). Conseguinte, as amastigotas se diferenciam em promastigotas. Após várias mudanças e intensa multiplicação, as promastigotas se

diferenciam em promastigotas metacíclicas (forma infectante) (DESCOTEAUX, 1999; SACKS; PERKINS, 1984). Essa diferenciação ocorre nas primeiras 48 horas após a ingestão do sangue infectado e quando os parasitos alcançam o intestino anterior do vetor, estando assim, prontos para serem transmitidos a um hospedeiro vertebrado susceptível durante um repasto sanguíneo sucessivo (DA-CRUZ; PIRMEZ, 2005).

Killick-Kendrick (1990) sugeriu alguns parâmetros para a incriminação de uma espécie de flebotomíneo como vetor de *Leishmania* spp.: (a) as promastigotas precisam ser isoladas de fêmeas coletadas de campo não ingurgitadas recentemente; (b) as formas infectantes do parasito precisam ser visualizadas na porção anterior do intestino e da válvula estomodeal de fêmeas naturalmente infectadas ou derivadas de colônia após xenodiagnóstico; (c) o inseto precisa ter atração e picar o hospedeiro primário da leishmânia em questão; (d) é necessário haver associação ecológica, entre o flebotomíneo e os seres humanos e eventuais hospedeiros reservatórios; (e) o sucesso da transmissão experimental após infecção de uma espécie de hospedeiro natural ou modelo equivalente de laboratório. Aprimorando os critérios já existentes, Ready (2013) propôs ainda, a utilização de dados retrospectivos e modelagem matemática com base em um programa de controle planejado que demonstre a incidência da doença atenuando significativamente com uma diminuição da densidade do vetor.

No Brasil diversas espécies de flebotomíneos são consideradas vetoras primárias de leishmânias causadoras de LC: *Bichromomyia flaviscutellata*, *Lutzomyia gomezi*, *Ny. intermedia*, *Nyssomyia whitmani*, *Nyssomyia neivai*, *Nyssomyia umbratilis*, *Migonemyia migonei*, *Pintomyia fischeri*, *Psychodopygus wellcomei*, *Psychodopygus complexus* e *Psychodopygus ayrozai* (RANGEL *et al*., 2018). Contudo, existem outras espécies baseadas em evidências epidemiológicas ou parasitológicas, ou ambas, que podem estar envolvidas no ciclo de transmissão da LC (RANGEL; LAINSON, 2003; RANGEL *et al*., 2018).

Enquanto a LV tem *Lu. longipalpis* como principal espécie associada à sua transmissão, contudo, em determinadas áreas *Mg. migonei* e *Lutzomyia evansi* têm sido indicadas como potenciais vetores de *L*. (*L*.) *infantum* (CARVALHO *et al*., 2010; GUIMARÃES *et al*., 2016; LAINSON; RANGEL, 2005; SALOMÓN *et al*., 2010).

Em alguns biomas como a Mata Atlântica e a Caatinga, presentes no Estado de Pernambuco, a fauna flebotomínica é composta por cerca de 42 espécies (AGUIAR; VIEIRA, 2018; DANTAS-TORRES *et al*., 2010, 2017; MIRANDA *et al*., 2015; SALES *et al*., 2019). As espécies *Ny. whitmani* e *Ps. complexus* são consideradas as principais vetoras de *L*. (*V*.) *braziliensis* nas regiões da Zona da Mata Sul e Zona da Mata Norte de Pernambuco,

respectivamente (ANDRADE, 2005; BRANDÃO-FILHO *et al*., 1999). Em Pernambuco existe uma significativa riqueza de espécies de flebotomíneos quando comparado com outras áreas do Nordeste do país (*e.g.*, Alagoas, Ceará, Paraíba, Piauí, Rio Grande do Norte e Sergipe) (DANTAS-TORRES *et al*., 2010; GALATI, 2003).

Em várias regiões do Brasil a adaptação de flebotomíneos vetores ao ambiente modificado pelo homem vem sendo comprovada a cada dia em diferentes estudos, o que potencializa a procura desses insetos por diferentes fontes alimentares e consequentemente aumenta o risco de transmissão de *Leishmania* spp., inclusive em áreas urbanas ou suburbanas (DANTAS-TORRES *et al*., 2017; DONALISIO *et al*., 2012; LEONARDO; RABÊLO, 2004; MIRANDA *et al*., 2015; SALES *et al*., 2015; 2019; ZEILHOFER *et al*., 2008).

Os flebotomíneos também são potenciais transmissores de alguns vírus, dos quais os mais importantes fazem parte dos gêneros: *Phlebovirus* (família Bunyaviridae), *Vesiculovirus* (família Rhabdoviridae) e *Orbivirus* (família Reoviridae) (DEPAQUIT *et al*., 2010; MAROLI *et al*., 2013).

O gênero *Phlebovirus* abriga a maioria das espécies conhecidas (DEPAQUIT *et al*., 2010). Aproximadamente 39 sorotipos de *Phlebovirus* são relatados, entre estes 25 fazem parte do Novo Mundo (SHAW *et al*., 2003). No Velho Mundo o *Phlebovirus* tem sido associado à presença de flebotomíneos nas seguintes áreas: sul da Europa, África, Oriente Médio e Ásia Central e Ocidental (TESH *et al*., 1976). Entre os *Phlebovirus* temos os seguintes vírus: vírus da febre dos flebotomíneos de Nápoles, que inclui o vírus Nápoles, vírus Teerã, vírus Karimabad e vírus da Toscana; o vírus Salehabad, inclui os vírus Salehabad e vírus Arbia; vírus siciliano e o vírus Corfou (ANAGNOSTOU *et al*., 2011; COLLAO *et al*., 2010; MOUREAU *et al*., 2010).

Ademais, sugere-se que muitos desses vírus sejam mantidos em seus vetores artrópodes por meio da transmissão vertical (transovariana) (TESH, 1988). Nas Américas muitos sorotipos foram caracterizados a partir de flebotomíneos pertencentes ao gênero *Lutzomyia*, e na África, Europa e Ásia Central, principalmente de *Phlebotomus* e *Sergentomyia* (DEPAQUIT *et al*., 2010).

O vírus siciliano foi isolado de *Phlebotomus papatasi* capturado do Mediterrâneo para a Ásia Central e de espécimes de *Phlebotomus ariasi* na Argélia (IZRI *et al*., 2008). Na Itália *Phlebotomus* pertencente ao subgênero *Larroussius* (*i.e*., *Phlebotomus perniciosus*, *Phlebotomus perfiliewi* e *Phlebotomus neglectus*) são fortes candidatos à transmissão do vírus siciliano na ausência de *Ph. papatasi* (DEPAQUIT *et al*., 2010). A participação de diferentes

espécies de vertebrados na manutenção do ciclo de vida do vírus siciliano inclui: *Apodemus* spp., *Mus musculus, Rattus rattus, Clethrionomys glareolus, Meriones libycus, Gerbillus aureus* e *Mustela nivalis* (CHASTEL *et al*., 1983).

Na Grécia, o vírus Corfou foi isolado em *Ph. neglectus* (RODHAIN *et al*., 1985). O vírus Adria (semelhante ao vírus Arbia) foi detectado, porém não isolado, em flebotomíneos coletados na Albânia (PAPA; VELO; BINO, 2010; ANAGNOSTOU *et al*., 2011).

Segundo Tesh *et al*. (1992) o vírus Toscana foi isolado diversas vezes de *Ph. perniciosus* e *Ph. perfiliewi* e sua distribuição inclui: Espanha, França, Itália, Grécia, Chipre, Portugal e Turquia. Um novo *Phlebovirus* chamado de vírus Massilia foi isolado de *Ph. perniciosus* no sudeste da França (CHARREL *et al*., 2005).

Os flebotomíneos americanos identificados como vetores de *Phlebovirus* são: *Nyssomyia trapidoi*, *Nyssomyia ylephiletor*, *Bi. flaviscutellata*, *Ny. umbratilis*, *Psychodopygus panamensis* e *Lutzomyia sanguinaria* (ADLER; THEODOR, 1926). No Velho Mundo, as espécies vetoras compreendem: *Ph. papatasi*, *Ph. perniciosus* e *Ph. perfiliewi* (MAROLI *et al*., 2013).

Fazem parte do gênero *Vesiculovirus*: o vírus Chandipura e os vírus da estomatite vesicular VSV-Alagoas, VSV-Indiana e VSV-New Jersey (MAROLI *et al*., 2013). Aproximadamente 28 agentes virais do gênero *Vesiculovirus* infectam vertebrados e invertebrados (BHATT; RODRIGUES, 1967).

O vírus Chandipura foi identificado pela primeira vez em 1965 após o isolamento do sangue de dois pacientes da vila de Chandipura, no estado de Maharashtra, na Índia (BHATT; RODRIGUES, 1967). Este vírus afeta principalmente crianças e é caracterizada por doença gripal e disfunções neurológicas (MENGHANI *et al*., 2012). Surtos da infecção pelo vírus ocorreram em Andhra Pradesh e Maharashtra em 2003 onde tiveram 329 crianças afetadas e 183 mortes (RAO *et al*., 2004). O vírus Chandipura é atualmente endêmico apenas na Índia (DHANDA; RODRIGUES; GHOSH, 1970; MAVALE *et al*., 2007), contudo, a importação do vírus através de um indivíduo infectado com ou sem sintomas clínicos não podem ser excluídos (DEPAQUIT *et al*., 2010).

O vírus Chandipura já foi isolado de *Phlebotomus* spp. (DHANDA *et al*., 1972) e também foi detectado em espécime do gênero *Sergentomyia* (GEEVARGHESE *et al*., 2005), mostrando assim a capacidade do vírus em estar presente em dois diferentes gêneros de flebótomos. Não obstante, em áreas que o vírus está presente a espécie suspeita e predominante é *Ph. papatasi* (MAROLI *et al*., 2013).

O vírus estomatite vesicular causa estomatite em humanos e animais domésticos (gados, cavalos e porcos) estão presentes no Novo Mundo (MAROLI *et al*., 2013). Nos Estados Unidos, *Psathyromyia shannoni* é incriminada como um importante vetor do vírus por acometer rebanhos de bovinos e ovinos em diversas áreas (COMER *et al*., 1994). Na região amazônica do Brasil dois vírus do gênero *Vesiculovirus* (Vesiculovírus Carajás e Marabá) foram isolados em fêmeas de *Lutzomyia* spp. capturadas em Serra Norte, município de Marabá, Pará (COMER; TESH, 1991). As espécies *Ny. trapidoi*, *Ny. ylephiletor* e *Pa. shannoni* têm sido associadas com a transmissão de VSV (COMER; TESH, 1991).

Diferente dos outros grupos de vírus, o gênero *Orbivirus* representado pelo vírus Changuinola não exibe grande importância clínica e ainda é um grupo carente de informações (DEPAQUIT *et al*., 2010). Ademais, diferentes vírus como o Mayaro (*Alphavirus*) e o vírus do Oeste do Nilo (*Flavivirus*) são capazes de infectar, *in vitro*, uma linhagem celular de *Lu. longipalpis*, sugerindo assim a participação dessa espécie de flebotomíneo como potenciais vetores (TESH; MODI, 1983; PITALUGA *et al*., 2008).

Muitas dessas infecções são facilmente confundidas com malária, influenza e outras doenças virais respiratórias ou outras arboviroses (SHAW *et al*., 2003). É evidente o quão forte é a atuação dos arbovírus na saúde humana. Entretanto, o surgimento de novas doenças em todo o mundo vem crescendo, devido principalmente à deficiência das estratégias para limitar ou eliminar a transmissão desses patógenos (DEPAQUIT *et al*., 2010; MAROLI *et al*., 2013).

Outra doença que envolve a participação de flebotomíneos na transmissão é a doença de Carrión, febre de Oroya ou verruga peruana, causada pela bactéria *Bartonella bacilliformis* (MINNICK *et al*., 2014).

As *Bartonella* spp. são bactérias gram negativas, flageladas, intracelulares facultativas, aeróbias obrigatórias e oportunistas (MAROLI *et al*., 2013). A bartonelose por *B. bacilliformis* é uma zoonose encontrada apenas nos vales andinos do Peru e em algumas áreas do Equador e do sul da Colômbia (CLEMENTE *et al*., 2012).

Na fase aguda da doença ocorre anemia hemolítica severa, dores articulares, febre e icterícia (MAROLI *et al*., 2013; TICONA *et al*., 2010). Ainda, a ausência ou atraso do tratamento antibiótico pode levar a implicações fatais (PONS *et al*., 2016). Na fase crônica, considerando pessoas que foram anteriormente expostas, a bactéria *B. bacilliformis* induz a proliferação de células endoteliais, produzindo lesões cutâneas chamadas verrugas peruanas (MINNICK *et al*., 2014).

As espécies *Pintomyia verrucarum* e *Lutzomyia peruensis pescei* participam da transmissão dessa bactéria, ao passo que, no Peru, *Lutzomyia noguchii* e *Lu. peruensis* podem também estar envolvidas na transmissão (CLEMENTE *et al*., 2012; YOUNG; DUNCAN, 1994).

De fato, a transmissão vetorial é a via mais relevante da doença, contudo, outras vias possíveis devem ser levadas em consideração (*e.g*., transfusão sanguínea, contato acidental com sangue infectado, transmissão vertical) principalmente devido à natureza da doença, onde qualquer inoculação direta ou contato com sangue humano ou fluido infectado pode resultar na aquisição da enfermidade (PONS *et al*., 2016).

1.1.4 Relação vetor-parasito-hospedeiro: o caso das leishmânias

Há muito tempo tem sido observada a capacidade de parasitos (*e.g*., protozoários, nematóides, bactérias e vírus) em influenciarem a alimentação de seus vetores artrópodes (MOLYNEUX; JEFFERIES, 1986; HURD, 2003). No caso das leishâmanias, elas exibem inúmeras modificações genéticas e fenotípicas para garantir sua sobrevivência e transmissão de hospedeiro para hospedeiro (ROGERS; BATES, 2007). No intestino médio do flebotomíneo as leishmânias sofrem o efeito das enzimas digestivas, particularmente são proteases do tipo tripsina e quimotripsina. A produção destas proteases por células epiteliais do intestino médio inicia-se várias horas após a ingestão de sangue e as suas atividades atingem um pico entre 18-48 horas, dependendo da espécie de flebotomíneo (DILLON; LANE, 1993; TELLERIA *et al*., 2010).

As fêmeas de flebotomíneos durante repasto sanguíneo precisam contornar o sistema hemostático do hospedeiro, composto pela cascata de coagulação sanguínea, vasoconstrição, fibrinólise e agregação plaquetária (HURD, 2003). Ademais, a infecção provocará uma resposta comportamental específica do hospedeiro frente ao parasito e assim o vetor precisa superar as respostas imune, inata e adquirida, do hospedeiro (HURD, 2003). Para vencer esses obstáculos os flebotomíneos carregam, em sua secreção salivar, componentes fundamentais como anticoagulantes, anti-plaquetários, vasodilatadores imunomoduladores e moléculas anti-inflamatórias (KAMHAWI, 2000).

Além das barreiras fisiológicas, a alimentação dos flebotomíneos também pode ser influenciada por fatores físicos e biológicos dos microhabitats (FEITOSA; CASTELLÓN, 2006). A maioria das espécies se alimenta ao entardecer e durante a noite, quando a

temperatura cai e a umidade aumenta, ou seja, um ambiente favorável para ir à busca de recurso para suprir suas atividades energéticas (BRAZIL; BRAZIL, 2003).

Os flebotomíneos podem se alimentar preferencialmente em um determinado tipo de hospedeiro ou grupo de hospedeiros, ainda que a disponibilidade do hospedeiro seja um fator determinante do comportamento da alimentação sanguínea (ABBASI; CUNIO; WARBURG, 2009). As alterações antrópicas no ambiente em que os flebotomíneos estão presentes podem afastar a fonte de repasto, fazendo com que estes dípteros procurem fontes alternativas, inclusive o ser humano (FEITOSA; CASTELLÓN, 2006). É provável que muitas espécies de flebotomíneos sejam oportunistas e se alimentem em animais aos quais tenham acesso mais fácil, uma vez que as mesmas espécies coletadas em biótopos diferentes exibem padrões de alimentação distintos (ABBASI; CUNIO; WARBURG, 2009).

As fêmeas de flebotomíneos se alimentam de uma grande variedade de hospedeiros vertebrados, mamíferos (NERY; LOROSA; FRANCO, 2004; TIWANANTHAGORN *et al*., 2012; SALES *et al*., 2015; TANURE *et al*., 2015), répteis (BATES, 2007), anfíbios (BRAACK *et al*., 1981) e aves (SANT'ANNA *et al*., 2010). A atração que diferentes animais exercem sobre os flebotomíneos como fonte alimentar estabelece um importante fator para o entendimento das relações hospedeiro-vetor nos diversos ambientes, principalmente em áreas onde as leishmanioses são endêmicas (FONTELES *et al*., 2009). O odor, temperatura e dióxido de carbono produzidos pelos hospedeiros, são estímulos fundamentais para a orientação dos flebotomíneos a sugar o sangue (BRAZIL; BRAZIL, 2018). Curiosamente, os animais domésticos estão entre as fontes de sangue preferidas dos flebotomíneos no ambiente peri-doméstico, potencializando o risco de se tornarem vetores de *Leishmania* (RANGEL; VILELA, 2008).

1.1.5 Alimentação sanguínea

O sangue de vertebrados é um recurso rico em nutrientes, composto principalmente das seguintes proteínas: albumina, imunoglobulina, hemoglobina, sendo esta última a proteína em maior quantidade (WICHER; FRIES, 2006).

A maioria dos artrópodes hematófagos ingerem grandes quantidades de sangue em uma única refeição (GRAÇA-SOUZA *et al*., 2006), mosquitos e triatomíneos ingerem entre três e dez vezes seus pesos corporais em cada repasto (FRIEND; CHOY; CARTWRIGHT, 1965). Outro exemplo é o caso dos carrapatos, cujo volume de sangue ingerido por uma

fêmea pode ser até ser 100 vezes do peso inicial do ectoparasito (ROMOSER, 1996). Em flebotomíneos a quantidade de sangue ingerida varia de acordo com a espécie, mas, de um modo geral os flebotomíneos ingerem menos de 1µl de sangue, logo, comparado a outros artrópodes a quantidade é bastante pequena (RANGEL; LAINSON, 2003).

A evolução da hematofagia em insetos envolveu a modificação do aparelho bucal para promover o acesso ao sangue; as glândulas salivares tiveram que produzir moléculas específicas para inibir a hemostasia dos vertebrados; o intestino médio permitiu a neutralização de lesões imunológicas mediadas pelo sangue dos vertebrados e para ajudar na digestão e assimilação dos componentes do sangue (RIBEIRO, 1996; STARK; JAMES, 1996).

Para a ordem Diptera foram sugeridas duas vias evolutivas para que esse grupo chegasse à alimentação sanguínea, cada uma baseada em uma pré-adaptação. Na primeira proposta, as peças bucais especializadas para a perfuração de plantas ou presas se modificaram e muito facilmente suportaram a alimentação em um hospedeiro vertebrado. Na segunda, uma adaptação à proximidade do ninho, da toca, do pelo ou do cabelo do hospedeiro levou a uma a associação que resultou na alimentação de sangue em vertebrados (WAAGE, 1979).

A hematofagia foi um passo grande na evolução de patógenos transmitidos por vetores, pois a capacidade reprodutiva destes insetos aumentou com a possibilidade de novos alimentos. Estrategicamente ao conseguir este novo hábito alimentar, os artrópodes passaram a transmitir patógenos presentes no sangue dos hospedeiros (MEJIA *et al*., 2006).

De acordo com a classificação tradicional dentro da família Psychodidae a alimentação de sangue evoluiu em apenas três gêneros: *Phlebotomus*, *Lutzomyia* e *Sergentomyia* (YUVAL, 2006). O potencial reprodutivo das fêmeas de flebotomíneos depende em parte do tipo da fonte de sangue e da quantidade de nutrientes ingeridos (BENITO-DE MARTIN *et al*., 1994).

Nos flebotomíneos a digestão do sangue e a absorção dos nutrientes ocorrem no intestino médio, este compartimento é composto por células epiteliais colunares que secretam as enzimas digestivas. O lúmem do intestino médio é constituído por uma membrana quitino protéica chamada de Matriz Peritrófica (MP) que recobre o alimento ingerido, e que divide o conteúdo luminal em dois compartimentos, o espaço endoperitrófico e o espaço ectoperitrófico (RICHARDS; RICHARDS, 1977).

Todo o processo digestivo ocorre em três etapas primordiais: 1) Fase inicial: quando acontece uma diminuição da massa molecular dos polímeros pela ação de despolimerases, tais como amilases, celulases, hemicelulases e proteinases; 2) Fase intermediária: formação dos dímeros ou pequenos oligômeros, como maltose, celobiose e dipeptídeos, provindos da hidrólise de amido, celulose e proteínas; 3) Fase final: os dímeros são clivados em monômeros por dimerases (SILVA; LEMOS; SILVA, 2012). De um modo geral, em fêmeas de flebotomíneos o processo de digestão total do sangue dura em torno de cinco dias, podendo variar de acordo com a espécie de flebotomíneo e a origem do sangue do hospedeiro (HAOUAS *et al*., 2007; LEE *et al*., 2002).

1.1.6 Métodos de identificações de fontes alimentares

King e Bull (1923) realizaram os primeiros estudos sobre fonte alimentar em artrópodes, seguido por Rice e Barber (1935). Diversas metodologias já foram empregadas na identificação de sangue em flebotomíneos e atualmente o estudo da biologia desses insetos vem contribuindo significativamente para compreensão de suas interações desses insetos com potenciais hospedeiros de *Leishmania* (RODRIGUES *et al*., 2017).

Os métodos sorológicos tradicionais fazem parte dos ensaios mais utilizados na identificação de sangue em flebotomíneos, incluem: teste de precipitina (AFONSO *et al*., 2005; DIAS *et al*., 2003; FONTELES *et al*., 2009; JAVADIAN *et al*., 1977; MORRISON *et al*., 1993; NERY; LOROSA; FRANCO, 2004; OLIVEIRA-PEREIRA *et al*., 2008; TESH *et al*., 1971, 1972), ensaio de imunoabsorção enzimática (ELISA) (AGRELA *et al*., 2002; BONGIORNO *et al*., 2003; COLMENARES *et al*., 1995; GOMEZ; SANCHEZ; FELICIANGELI, 1998; MARASSÁ *et al*., 2004; ROSSI *et al*., 2008; SVOBODOVÁ *et al*., 2003), imunoeletroforese (MORSY *et al*., 1993) e difusão em gel de agarose (SRINIVASAN; PANICKER, 1992). Embora, todas as técnicas imunológicas tenham contribuído expressivamente na produção de informações importantes sobre a identidade dos hospedeiros vertebrados em diversos artrópodes hematófagos, elas ainda requerem mais tempo no processo dos ensaios e carecem de sensibilidade (MALEKI-RAVASAN *et al*., 2009); não detectam repastos sanguíneos mais antigos que 24 horas (TESH *et al*., 1971); apresentam possível reação cruzada e incapacidade de incriminar reservatórios não esperados (HAOUAS *et al*., 2007) e ainda podem até não distinguir espécies filogeneticamente próximas (SILVA *et al*., 2001).

A espectrometria de massa é uma ferramenta utilizada para investigar o conteúdo alimentar de flebotomíneos, entretanto, a amostra (conteúdo hemoglobínico) empregada nesta técnica não se mantém viável por mais de um ano (SILVA, 2006). Outras metodologias baseadas em observações visuais, capturas com isca humana, armadilhas contendo animais (*e.g.*, cão, coelho e galinha) também permite conhecer de forma subjetiva o comportamento alimentar dos flebotomíneos (MUNIZ *et al*., 2006). Porém, essas observações não permitem esclarecer o complexo hábito alimentar desses dípteros em condições naturais.

Alternativamente, métodos moleculares foram desenvolvidos para detecção de fonte alimentar em flebotomíneos, trazendo a possibilidade de aplicações metodológicas que permitam detectar quantidades pequenas de sangue (SANT'ANNA *et al*., 2008; HAOUAS *et al*., 2007). Estratégias para a amplificação do DNA utilizando a PCR (reação em cadeia da polimerase) (ABBASI; CUNIO; WARBURG, 2009; JIMÉNEZ *et al*., 2013, GARLAPATI *et al*., 2012); PCR em tempo real (qPCR) (SALES *et al*., 2015; RODRIGUES *et al*., 2017); PCR-RFLP (*Restriction Fragment Length Polymorphism*) (MALEKI-RAVASAN *et al*., 2009); ensaio de heteroduplex (ABBASI; CUNIO; WARBURG, 2009); hibridização reversa e sequenciamento de DNA (CHEN *et al*., 2016) permitem resultados mais acurados e com maior sensibilidade quando comparado com ensaios sorológicos.

Com a utilização da biologia molecular é possível analisar a tipagem do DNA em amostras que de outra maneira não poderiam ser analisadas, como amostras antigas e de baixa qualidade (DUARTE *et al*., 2001). A qPCR claramente indica a superioridade em relação à outras técnicas, em termos de sensibilidade e rapidez na obtenção dos resultados (YANG; ROTHMAN, 2004; RODRIGUES *et al*., 2017). As particularidades da qPCR permitem a eliminação da etapa laboriosa pós-amplificação (gel de agarose para eletroforese), necessária para visualização do produto amplificado na PCR convencional.

Diversos marcadores genéticos são explorados na identificação de fonte alimentar em artrópodes, entre eles o gene nuclear que codifica para Prepronociceptina (*PNOC*) (HAOUAS *et al*., 2007) (KENT, 2009); genes mitocondriais (citocromo b e citocromo c oxidase 1) (BOAKYE *et al*., 1999; SANT´ANNA *et al*., 2008) e genes do RNA ribossômico (*12S* e *16S*) (PICHON *et al*., 2003; HUMAIR *et al*., 2007; VALINSKY *et al*., 2014).

Dentre os marcadores disponíveis, o citocromo b que também pode ser simbolisado com: *COB, CYTB, MT-CYB* ou *UQCR3,* é o gene mais comum usado na identificação de repastos sanguíneos em artrópodes (KENT, 2009). Este gene corresponde a uma proteína caracterizada a partir de complexo III do sistema de fosforilação oxidativa mitocondrial, é a

única proteína neste complexo codificado pelo genoma mitocondrial (IRWIN *et al*., 1991). Ademais, o *CYTB* vem sendo utilizado como alvo para diagnóstico molecular e estudos evolutivos de vertebrados (IRWIN *et al*., 1991; NGO; KRAMER, 2003). O emprego do *CYTB* como marcador é bastante útil, pois ele detém uma localização no genoma mitocondrial (que não sofre recombinação) e existe uma grande disponibilidade de sequências completas no *Genbank* o que permite uma comparação extensa entre as espécies (PERKINS; SCHALL, 2002).

Outro gene mitocondrial também utilizado na identificação de sangue é o Citocromo c oxidase 1 conhecido como *COI, MT-CO1* ou *COX1* (KENT, 2009; CRABTREE *et al*., 2013). O *COI* é uma das três subunidades codificadas pelo DNA mitocondrial (mtDNA) do complexo respiratório IV, este complexo IV é a terceira e última enzima da cadeia de transporte de elétrons da fosforilação oxidativa mitocondrial (HEBERT; RATNASINGHAM; WAARD, 2003). O gene *COI* é frequentemente empregado para identificar espécies de animais, uma vez que sua sequência apresenta uma taxa de mutação frequentemente rápida o suficiente para diferenciar espécies estreitamente relacionadas e também porque a sua sequência é conservada entre membros da mesma espécie (HEBERT; RATNASINGHAM; WAARD, 2003).

O conhecimento sobre o padrão alimentar dos flebotomíneos juntamente com o entendimento da interação dos hospedeiros com o parasito é de grande importância ecológica e epidemiológica, pois fornece subsídios para indicação de potenciais reservatórios de *Leishmania* spp. Tal conhecimento poderá contribuir para adoção de medidas de prevenção e controle das leishmanioses. A introdução de ferramentas de biologia molecular como a PCR multiplex em tempo real para detecção de *Leishmania* spp., sangue de cão e humano, elementos chave no ciclo zoonótico da LV, poderia fornecer dados sobre a dinâmica de transmissão desse protozoário. Os cães são considerados os principais reservatórios de *L*. (*L*.) *infantum* no ambiente doméstico, logo a detecção de DNA de cães em flebotomíneos vetores pode servir como indicador de risco. Mormente, a detecção de sangue de humanos indicará que os flebotomíneos se encontram estabelecidos no ambiente peri-doméstico e, consequentemente, o risco eminente de transmissão.

A caracterização do sangue ingerido por fêmeas de flebotomíneos baseada na detecção do DNA do hospedeiro por PCR em tempo real é uma alternativa promissora em relação às técnicas tradicionais (*e.g*., teste de preciptina e ELISA), devido a sua alta sensibilidade e especificidade. Sales *et al*. (2015) desenvolveram protocolos de PCR em tempo real para

detecção de sangue de cinco hospedeiros (humano, galinha, cavalo, gato e cão) em flebotomíneos, os quais apresentaram resultados promissores. Todavia, a padronização desses protocolos para a detecção simultânea do DNA de diferentes hospedeiros e de *Leishmania* spp. em flebotomíneos seria de grande valia em termos de custo-benefício, uma vez que a detecção individual apresenta maior custo em comparação a um protocolo multiplex. Neste sentido, espera-se que essa ferramenta molecular auxilie nos estudos sobre a dinâmica de transmissão de *Leishmania* spp. no ambiente peri-doméstico, contribuindo assim para o desenvolvimento de novas medidas de prevenção e controle das leishmanioses.

2 OBJETIVO GERAL

Desenvolver um protocolo de PCR multiplex em tempo real para detecção do DNA de cão, humano e *Leishmania* spp. em flebotomíneos.

2.1 Objetivos específicos

a) Desenhar *primers* e sondas para detecção do DNA de cão e humano;

b) Avaliar os *primers* e sondas *in silico*;

c) Padronizar um protocolo PCR multiplex em tempo real para a detecção de DNA cão, humano e de *Leishmania* spp.;

d) Avaliar a aplicabilidade da PCR multiplex em tempo real para detecção de DNA de cão, humano e de *Leishmania* spp. em fêmeas de flebotomíneos coletados em áreas onde as leishmanioses são endêmicas.

3 ARTIGOS

Nessa seção, serão apresentados quatro artigos científicos na íntegra, produzidos durante o doutorado. Os artigos do 1 ao 3 são trabalhos correlacionados à pesquisa. O artigo 4 compõe todos os resultados dos objetivos propostos nesta tese.

3.1 Artigo 1

O artigo intitulado “Sand fly population dynamics and cutaneous leishmaniasis among soldiers in an Atlantic forest remnant in northeastern Brazil” foi publicado em 2017 na revista PLOS Neglected Tropical Diseases (doi.org/10.1371/journal.pntd.0005406), o qual relatamos um estudo sobre a dinâmica populacional de flebotomíneos em um campo de treinamento militar localizado em um remanescente de Mata Atlântica no nordeste do Brasil, onde surtos de leishmaniose cutânea por *L. braziliensis* tem sido esporadicamente descritos.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Sand fly population dynamics and cutaneous leishmaniasis among soldiers in an Atlantic forest remnant in northeastern Brazil

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Abstract

Outbreaks of cutaneous leishmaniasis are relatively common among soldiers involved in nocturnal activities in tropical forests. We investigated the population dynamics of sand flies in a military training camp located in a remnant of Atlantic rainforest in northeastern Brazil, where outbreaks of cutaneous leishmaniasis have sporadically been described. From July 2012 to July 2014, light traps were monthly placed in 10 collection sites, being nine sites located near the forest edge and one near a sheep and goat stable. Light traps operated from 5:00 pm to 6:00 am, during four consecutive nights. *Leishmania* infection in sand flies was assessed using a fast real-time PCR assay. Cases of cutaneous leishmaniasis among soldiers were also investigated. In total, 24,606 sand flies belonging to 25 species were identified. Males (n = 12,683) predominated over females (n = 11,923). Sand flies were present during all months, being more numerous in March (n = 1,691) and April 2013 (n = 3,324). *Lutzomyia choti* (72.9%) was the most abundant species, followed by *Lutzomyia longispina* (13.8%), *Lutzomyia complexa* (5.3%), representing together >90% of the sand flies collected. Forty cases of cutaneous leishmaniasis were recorded among soldiers from January 2012 to December 2014. *Leishmania* isolates were obtained from eight patients and were all characterized as *Leishmania braziliensis*. Soldiers and anyone overnighting in Atlantic rainforest remnants should adopt preventative measures such as the use of repellents on bare skin or clothes and insecticide-treated tents.

Author summary

Outbreaks of cutaneous leishmaniasis are relatively common among soldiers involved in nocturnal activities in tropical forests. However, there is limited information on the relationship between sand fly population dynamics and cases of cutaneous leishmaniasis in Atlantic forest remnants. In this study, we investigated the population dynamics of sand

Competing interests: The authors have declared that no competing interests exist.

flies in a military training camp located in a remnant of Atlantic rainforest in northeastern Brazil, where outbreaks of cutaneous leishmaniasis have sporadically been described. In total, 24,606 sand flies belonging to 25 species were identified. Sand flies were present during all months, being more numerous in March and April 2013. *Lutzomyia choti* was the most abundant species and three pools of females belonging to this species were found to be positive for *Leishmania braziliensis* DNA. Our results suggest that the risk of cutaneous leishmaniasis by *Leishmania braziliensis* in Atlantic rainforest remnants is permanent and thus not dictated by sand fly population peaks. People overnighting in Atlantic rainforest remnants should adopt preventative measures such as the use of repellents on bare skin or clothes and insecticide-treated tents.

Introduction

Cutaneous leishmaniasis is a neglected tropical disease highly prevalent in Central and South America. Indeed, Brazil, Costa Rica and Peru are among the 10 countries accounting for 70–75% of the global estimated cutaneous leishmaniasis incidence [1]. Only in Brazil, 554,475 cases of cutaneous leishmaniasis were notified to public health authorities between 1988 and 2007, with an average incidence of 17.3 cases per 100,000 inhabitants [1]. The disease is widespread in this country occurring mainly in rural areas and forest environments, affecting mainly individuals older than 10 years and males [1, 2].

While widespread in Brazil, cutaneous leishmaniasis is more prevalent in the Amazon forest and Atlantic forest regions [1]. The Amazon forest biome covers 48% of the Brazilian territory (4,245,024 km^2), but the Atlantic forest biome has been over explored since the arrival of the first Portuguese settlers and in the past centuries its original cover has been reduced to almost nothing. Today, only 2–5% of its original area is considered to be in its original state (http://www.fao.org/3/a-i3825e/i3825e6.pdf). Indeed, the Atlantic forest region presently encompasses an area of 1,129,760 km^2, extending along the Atlantic coast of Brazil, from Rio Grande do Norte (in the north) to Rio Grande do Sul (in the south). The largest cities (e.g., Rio de Janeiro and São Paulo) and industries in the country are located in the Atlantic forest region, which houses 70% of the Brazilians and accounts for about 80% of its gross domestic product. Nonetheless, the Atlantic forest biome is still home to about 2,000 species of animals and 20,000 species of plants; a biological diversity similar to that found in the Amazon region (http://www.nature.org/ourinitiatives/regions/southamerica/brazil/placesweprotect/atlantic-forest.xml).

Cutaneous leishmaniasis in the Atlantic forest region usually affects rural and forest workers that live near or literally within forest fragments, as well as people developing nocturnal activities in these environments. For instance, several cases of cutaneous leishmaniasis have been reported among soldiers after periods of nocturnal training activities in remnants of Atlantic forest in northeastern Brazil [3–6]. Indeed, the presence of proven and putative sand fly vectors of parasites such as *Leishmania braziliensis*, the most widespread causative agent of cutaneous leishmaniasis in the Americas, is acknowledged in these areas [3, 7–11].

Nonetheless, our knowledge on the ecology of sand flies (Phlebotomine) and its relationship with cutaneous leishmaniasis incidence in the Atlantic forest region is still fragmentary. In this study, we investigated the sand fly fauna in a military training camp located in a remnant of Atlantic rainforest in northeastern Brazil, where outbreaks of cutaneous leishmaniasis have been sporadically described. Our hypothesis was that the temporal dynamics of sand flies

was correlated with climate variables and the incidence of cutaneous leishmaniasis in this region.

Materials and methods

Ethics statement

This study used secondary data (i.e., anonymized data that has previously been collected in the course of normal care) on human cutaneous leishmaniasis obtained from Reference Service on Leishmaniasis of the Aggeu Magalhães Institute, Oswaldo Cruz Foundation (Fiocruz). No ethical approval was required.

Study sites

This study was carried out in the Campo de Instrução Militar Marechal Newton Cavalcanti (CIMNC), located in the Atlantic forest zone of Pernambuco State. This military training camp comprises an area of 7,324 hectares (Fig 1), distributed in five municipalities: Araçoiaba, Paulista, Igarassu, Paudalho and Tracunhaém. The camp possesses has a central pavilion of command, two villages with 16 adjoining houses, a school, a chapel, 14 houses portholes, eight sheds used for any troops under training and six areas for military training and exercises.

The climate of this region is rainy tropical type with dry summer. The vegetation is represented by the Atlantic rainforest distributed into two main types: open ombrophilous forest

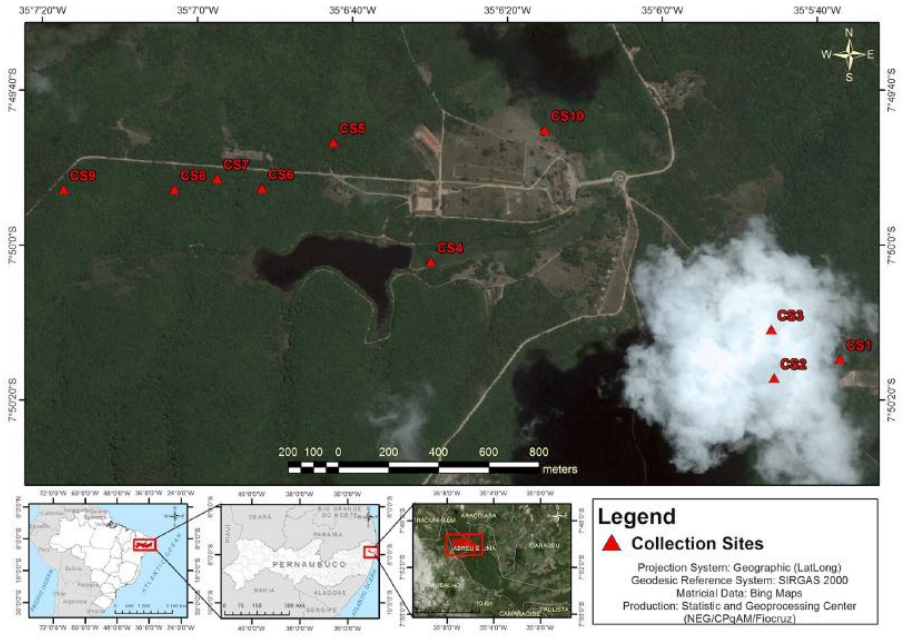

Fig 1. Location of the study area.

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Table 1. Brief description of collection sites selected in this study.

Collection site	Geographical coordinates	Altitude	Description
CS1	7˚ 50.25' S	133 m	Area near the forest edge, with predominant shrubby vegetation and fruit trees; thin litter layer
	35˚ 05.62' W		
CS2	7˚ 50.29' S	139 m	Interior of the forest, with dense shrubby-arboreal vegetation; thick litter layer
	35˚ 05.76' W		
CS3	7˚ 50.18' S	162 m	Interior of the forest, with some clearings; presence of a large tingling, animal burrows and decaying trees
	35˚ 05.77' W		
CS4	7˚ 50.04' S	116 m	Area near the forest edge, with a dam and water streams; thin litter layer
	35˚ 06.50' W		
CS5	7˚ 49.78' S	174 m	Forest interior, with shrubby-arboreal vegetation, mainly lianas; area used for military training activities
	35˚ 06.71' W		
CS6	7˚ 49.88' S	178 m	Forest interior, with shrubby-arboreal vegetation; thick litter layer
	35˚ 06.86' W		
CS7	7˚ 49.86' S	169 m	Forest interior, with shrubby-arboreal vegetation; thick litter layer
	35˚ 06.96' W		
CS8	7˚ 49.88' S	151 m	Forest interior, with shrubby-arboreal vegetation; thick litter layer; animal burrows and decaying trees
	35˚ 07.05' W		
CS9	7˚ 49.88' S	173 m	Forest interior, with shrubby-arboreal vegetation; thick litter layer
	35˚ 07.29' W		
CS10	7˚ 49.75' S	125 m	Sheep and goat stable, located in an open area near the military camp headquarter; grass fields and shrubby vegetation
	35˚ 06.25' W		

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and seasonal semidecidual. Outbreaks of cutaneous leishmaniasis are sporadically reported in this region [3–5].

Sand fly collection and identification

Sand fly collections were carried out monthly, for four consecutive nights, from July 2012 to July 2014 in 10 pre-selected collection sites (CS1-CS10), totaling 96 actual nights and 960 total trap-nights (Table 1). In each site, a CDC-type light trap was placed *ca.* 1.5 m above the ground, operating from 5:00 pm to 6:00 am. Collection sites were typically near animal holes, trunks and roots of large trees, usually in shady and humid environments. Collection sites were also near the places where the soldiers use to camp overnight during training periods. The only exception was CS10, which was a sheep and goat stable, located near the military camp headquarter.

Collected sand flies were separated from other insects and placed in labeled vials containing 70% ethanol until processing for morphological identification [12]. For females, the head (containing the cibarium) and the last three abdominal segments (containing the spermathecae) were used for morphological identification, whereas the thorax and the remaining part of the abdomen were used for DNA extraction and PCR testing.

DNA extraction and *Leishmania* spp. detection

DNA extraction was performed from 1,003 females (grouped in 108 pools) belonging to four species: *Lu. choti* (688 females grouped in 71 pools), *Lu. sordellii* (197 females grouped in 24 pools), *Lu. complexa/wellcomei* (113 females grouped in 12 pools) and *Lu. amazonensis* (one pool of five females). Each pool contained 5–10 specimens of the same species and separated according to date and place of collection. DNA extraction was performed using the Chelex 100

resin as described elsewhere [13]. Extracted DNA samples were kept frozen at –20˚C until testing.

Real-time PCR testing for detecting *Leishmania* kinetoplast DNA was performed using the primers LEISH-1 (5'-AACTTTTCTGGTCCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCC AGTTTCCCGCC-3') and the TaqMan probe FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB, as described elsewhere [14]. All real-time PCR assays were run on a QuantStudio 5 Real-Time PCR machine (Applied Biosystems) and contained a standard curve of 10-fold serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg per reaction mixture) from DNA of *L. infantum* and a no-template control (DNA-free water). The real-time PCR results were analyzed using QuantStudio Design and Analysis Software v1.4 (Applied Biosystems).

DNA was also extracted from human patients using QIAamp DNA mini kit (Qiagen), according to the manufacturer's recommendations. Conventional PCR for the detection of *Leishmania* (*Viannia*) DNA in human samples was performed as described elsewhere [15].

Leishmania characterization by restriction enzyme analysis

PCR products (10 μl) were digested with HaeIII (1 μl) (New England BioLabs) for 1 h, at 37˚C and then electrophoresed in a 3% high-fidelity agarose gel (Invitrogen). Bands were stained with ethidium bromide. Positive controls (amplified standard DNA from both *L. infantum* and *L. braziliensis*) were included in each analysis. The species of *Leishmania* detected in each sand fly pool was determined by comparing the banding profile with the ones obtained with positive controls. A 100 bp ladder (Invitrogen) was used as molecular weight.

Cutaneous leishmaniasis among soldiers

Secondary data on human cases of cutaneous leishmaniasis were obtained from the Reference Service on Leishmaniasis, Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Brazil. All cases whose patients were soldiers involved in training activities in the study area during 2012–2014 were included in this study. In brief, these patients presented skin lesions suggestive of cutaneous leishmaniasis and were referred to a local military hospital in Recife. At the hospital, the physician in charge determined the diagnosis, initially based on clinic-epidemiological data, and then by leishmanin skin test (Montenegro skin test) and skin-scraping cytology, as recommended by Ministry of Health of Brazil [16]. Skin samples from these patients were seeded in tubes containing biphasic Novy-MacNeal-Nicolle culture medium at 26˚C. Isolates obtained were identified using multilocus enzyme electrophoresis [17] at the Oswaldo Cruz Institute (Rio de Janeiro, Brazil) and using monoclonal antibodies [18] at the Evandro Chagas Institute (Belém do Pará, Brazil). Geographical coordinates and altitude of each patient's house were recorded using a Garmin eTrex Venture HC GPS (Garmin International Ltd, US).

Meteorological data

Meteorological data (i.e., relative humidity, monthly average temperature, and precipitation) for the whole period of study were obtained from Instituto de Tecnologia de Pernambuco (ITEP) (meteorological station: 82900). The saturation deficit (SD) was calculated as follows: $SD = (1 - RH/100) \times 4.9463 \times e^{0.0621 \times T}$, where RH is relative humidity and T is temperature.

Data analysis

The correlation between climatic variables and the number of sand flies collected monthly or daily was done using Spearman's (rs) or Pearson's (r) correlation coefficients, as appropriate.

Normality of data was assessed using Lilliefors. The number of sand flies of each species collected according to collection sites or months was compared using Kruskal-Wallis. The significance level was set at $P < 0.05$. Statistical analyses were performed using BioEstat, version 5.3 [19]. The diversity indices (Species richness, Shannon's diversity index and Pielou's equitability index) and abundance were calculated using PAST, version 2.16 [20]. The standardized index of species abundance (SISA) was as described elsewhere [21].

Results

Sand fly species diversity

A total of 24,606 sand flies (Table 2), being 12,683 males and 11,923 females (overall sex ratio close to unity), belonging to 25 species (i.e., *Lutzomyia choti, Lutzomyia longispina, Lutzomyia complexa, Lutzomyia sordellii, Lutzomyia amazonensis, Lutzomyia walkeri, Lutzomyia wellcomei, Lutzomyia quinquefer, Lutzomyia evandroi, Lutzomyia barrettoi barrettoi, Lutzomyia ayrozai, Lutzomyia capixaba, Lutzomyia naftalekatzi, Lutzomyia claustrei, Lutzomyia schreiberi, Lutzomyia umbratilis, Lutzomyia whitmani, Lutzomyia brasiliensis, Lutzomyia viannamartinsi,*

Table 2. Sand fly species collected according to sex and Standardized Index of Species Abundance (SISA), July 2012–July 2014.

Species	Males		Females		Total		SISA
	n	%	*n*	%	*n*	%	
Lu. amazonensis	102	0.80%	141	1.29%	243	1.03%	0.78
Lu. aragaoi	0	0.00%	1	0.01%	1	0.00%	0.03
Lu. ayrozai	2	0.02%	46	0.42%	48	0.20%	0.36
Lu. barrettoi barrettoi	3	0.02%	46	0.42%	49	0.21%	0.29
Lu. brasiliensis	1	0.01%	5	0.05%	6	0.03%	0.14
Lu. choti	9,990	78.77%	7,961	72.58%	17,951	75.90%	1.00
Lu. capixaba	4	0.03%	36	0.33%	40	0.17%	0.49
Lu. claustrei	14	0.11%	17	0.15%	31	0.13%	0.43
Lu. complexa	356	2.81%	0	0.00%	356	1.51%	0.73
Lu. evandroi	28	0.22%	68	0.62%	96	0.41%	0.53
Lu. furcata	1	0.01%	0	0.00%	1	0.00%	0.03
Lu. longispina	1,622	12.79%	1,794	16.36%	3,416	14.44%	0.93
Lu. migonei	1	0.01%	0	0.00%	1	0.00%	0.01
Lu. naftalekatzi	20	0.16%	14	0.13%	34	0.14%	0.42
Lu. sordellii	266	2.10%	613	5.59%	879	3.72%	0.89
Lu. oswaldoi	1	0.01%	0	0.00%	1	0.00%	0.04
Lu. quinquefer	12	0.09%	108	0.98%	120	0.51%	0.58
Lu. schreiberi	7	0.06%	12	0.11%	19	0.08%	0.25
Lu. shannoni complex	1	0.01%	3	0.03%	4	0.02%	0.13
Lu. walkeri	109	0.86%	77	0.70%	186	0.79%	0.73
Lu. wellcomei	130	1.02%	0	0.00%	130	0.55%	0.55
Lu. whitmani	11	0.09%	4	0.04%	15	0.06%	0.19
Lu. umbratilis	0	0.00%	16	0.15%	16	0.07%	0.10
Lu. viannamartinsi	2	0.02%	3	0.03%	5	0.02%	0.10
Lu. yuilli pajoti	0	0.00%	3	0.03%	3	0.01%	0.04
Total	12,683	53.63%	10,968	46.37%	23,651[a]	100.00%	NA

[a] Females identified as *Lutzomyia complexa/wellcomei* ($n = 955$) are not included in this table.

NA: not applicable; *Lu.*: *Lutzomyia*.

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Table 3. Species richness (S), Shannon's diversity index (H') and Pielou's equitability index (J) according to collection site in the Atlantic Forest fragment, northeastern Brazil.

Collection site	Richness (S)	Shannon's diversity index (H')	Pielou's equitability index (J)
CS1	17	0.8588	0.3031
CS2	20	0.6733	0.2248
CS3	17	0.8297	0.2928
CS4	12	0.9518	0.383
CS5	15	0.82	0.3028
CS6	13	1.079	0.4209
CS7	15	1.076	0.3974
CS8	16	1.083	0.3904
CS9	16	1.254	0.4524
CS10	11	0.6667	0.2781

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Lutzomyia shannoni complex, *Lutzomyia yuilli pajoti*, *Lutzomyia aragaoi*, *Lutzomyia furcata*, *Lutzomyia migonei* and *Lutzomyia oswaldoi*) were identified.

Lutzomyia choti, *Lu. longispina*, *Lu. complexa* and *Lu. sordellii* were the most frequently collected species, representing together 95.8% of the total collections. Yet, *Lu. barrettoi barrettoi*, *Lu. ayrozai*, *Lu. capixaba*, *Lu. naftalekatzi*, *Lu. claustrei*, *Lu. schreiberi*, *Lu. umbratilis*, *Lu. whitmani*, *Lu. brasiliensis*, *Lu. viannamartinsi*, *Lu. shannoni* complex, *Lu. yuilli pajoti*, *Lu. aragaoi*, *Lu. furcata*, *Lu. migonei and Lu. oswaldoi* were rarely collected, representing together 1.1% of the total.

The species richness was higher in CS2 (20 spp.), followed by CS1 (17 spp.), CS3 (17 spp.), CS8 (16 spp.) and CS9 (16 spp.), respectively. The highest Shannon's diversity and Pielou's equitability indexes were found in CS9 and CS6 (Table 3). Overall, no significant difference was found in the number of specimens from each species in relation to the site of collection ($H = 11.874$; $df = 9$, $P = 0.221$).

Sand fly temporal dynamics and climate variables

Overall, the sand fly population studied herein presented a unimodal temporal distribution pattern, peaking in the first semester of each year. The number of sand flies collected monthly during the whole study period ranged from 271 to 3,324, peaking in March (1,691) and April (3,324) 2013 and April (1,512), May (1,217) and June (1,619) 2014 (Fig 2). No significant variation was found in the number of specimens from each species in relation to the month of collection ($H = 14.404$; $df = 24$, $P = 0.937$).

No correlation was found between the monthly number of sand flies collected and climate variables ($rs = 0.29$, $P = 0.167$, for temperature; $rs = 0.14$, $P = 0.519$, for precipitation; $rs = -0.05$, $P = 0.829$, for relative humidity; and $rs = 0.11$, $P = 0.608$, for saturation deficit). However, comparing daily data, the number of sand flies collected was significantly correlated with temperature ($rs = 0.29$, $P = 0.003$), but not with precipitation ($rs = -0.185$, $P = 0.066$), relative humidity ($rs = -0.153$, $P = 0.128$) and saturation deficit ($rs = 0.181$, $P = 0.072$). Considering only the most abundant species (i.e., *Lu. choti*), the daily number of specimens collected was significantly correlated with temperature ($rs = 0.44$, $P < 0.001$), precipitation ($rs = -0.242$, $P = 0.015$), relative humidity ($rs = -0.25$, $P = 0.012$) and saturation deficit ($rs = 0.293$, $P = 0.003$) (Fig 3).

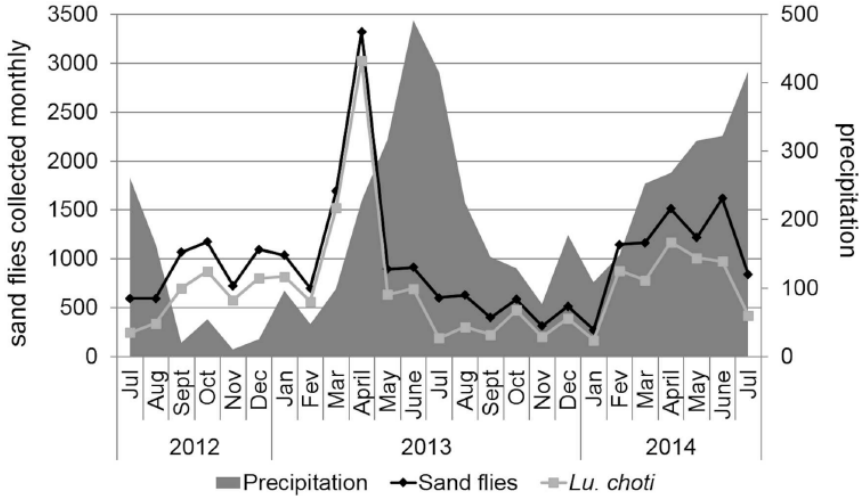

Fig 2. Monthly number of sand flies collected, July 2012–July 2014. Average monthly precipitation is also depicted.

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Cutaneous leishmaniasis among soldiers

Forty male soldiers (age range, 19–22 years) were diagnosed with cutaneous leishmaniasis in a regional military hospital from January 2012 to December 2014. Most of the soldiers were involved in nocturnal training activities in the study area in September 2013 ($n = 7$), October 2013 ($n = 16$), and September 2014 ($n = 11$). Among the remaining soldiers, two were training in June 2013, two in July 2013, one in September 2011 and one in October 2011. One of the soldiers that were training in September 2013 was also in the forest in June 2013.

Most of them were diagnosed with cutaneous leishmaniasis in November ($n = 22$), December ($n = 10$) or October ($n = 6$); the remaining two cases were diagnosed with cutaneous leishmaniasis in January 2012. All suspected cases but three were confirmed by one or more diagnostic test: 100% (30/30; eight refused to do the test) were positive at the leishmanin skin test, 31.6% (12/38) at cytology, 28.9% (11/38) at PCR, and 21.1% (8/38) at culture. All eight isolates obtained from those patients were all characterized as *L. braziliensis*.

Patients presented localized ulcers on legs, forearm, hands and neck. In most of the cases (84.2%), the diagnosis was made 1–2 months after the training period in the forest. All patients were successfully treated with n-methyl-glucamine antimoniate (Glucantime), except one whose lesion healed spontaneously without treatment.

Leishmania detection by real-time PCR and characterization by restriction enzyme analysis

Out of 1,003 females tested, six pools (10 females each) of *Lu. choti* were positive at real-time PCR, which gives an overall minimum infection rate of 0.6%. Considering only *Lu. choti*, the minimum infection rate was 0.87% (6/688). Three pools (i.e., F968, F651, and F1002) presented a HaeIII restriction profile identical to *L. braziliensis* (Fig 4). The three remaining

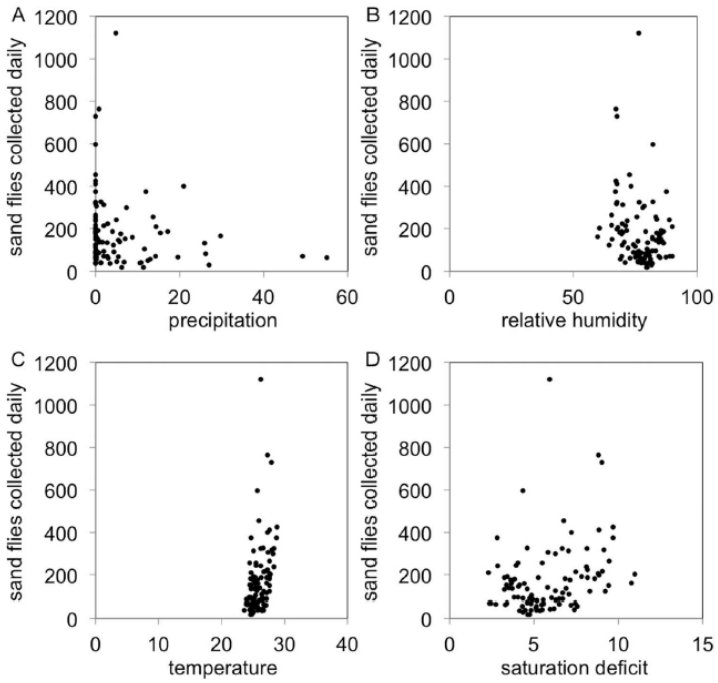

Fig 3. Scatter plot with the number of *Lutzomyia choti* captured versus climatic variables. (A) Precipitation. (B) Relative humidity. (C) Temperature. (D) Saturation deficit.

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positive pools did not show any pattern probably due to the very low amount of DNA detected by real-time PCR.

Discussion

A high diversity of sand flies was recorded in the studied Atlantic forest remnant studied herein. Our results, together with data available in the literature [11, 22, 23], suggest that most sand fly species found in Atlantic forest remnants are still more adapted to the forest than to human-modified environments; i.e., only 11 out 25 species identified in the study were collected in the animal stable, and typically in low numbers. Indeed, in some areas where cutaneous leishmaniasis by *L. braziliensis* is endemic, some vector species appear to be almost exclusively found in the forest interior or forest edge, rather than in the peridomicile [11, 22, 23]. Nonetheless, it is acknowledged that some vector species like *Lu. whitmani* are well adapted to human-dwellings [11]. This is in agreement with data obtained in the current study, where *Lu. whitmani* was found in very low numbers (15 specimens in the whole study period) and mostly in the horse stable (66.7% of the specimens collected).

The sand fly population studied herein presented a well-defined unimodal temporal distribution pattern, peaking in the first semester of each year. The highest peak was recorded in

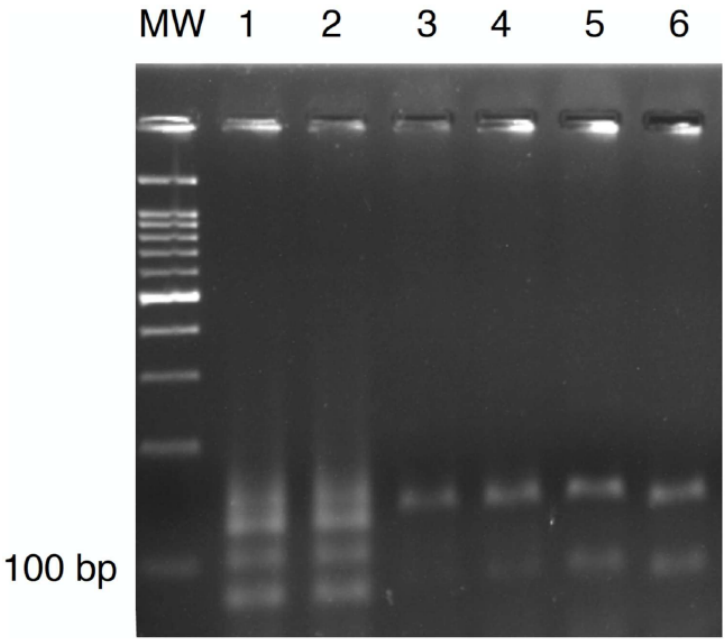

Fig 4. Restriction enzyme (HaeIII) analysis. MW, Molecular weight (100 bp); 1–2, *Leishmania infantum* (positive control); 3–4, *Leishmania braziliensis* (positive control); 5–6, F968.

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April 2013, with over 3,324 sand flies collected. This finding is congruent with a preliminary 1-year study conducted in 2003 in the same area [8]. These results clearly indicate that this sand fly population displays a relatively stable temporal distribution pattern, throughout the years. In turn, this suggests that the studied Atlantic forest remnant has not been much modified during the last 10 years or that possible modification that may have occurred during this period did not influence the sand fly population, neither negatively nor positively.

Still regarding the population dynamics, it is important to state that the heavy rains observed during some capture nights may have reduced the efficiency of our light traps during these nights. While obvious, this is not usually considered in the analysis of studies on sand fly population dynamics. It is crucial to consider this aspect because an apparent decline in the population may not be a real decline but merely an artifactual reduction of collection due to heavy rains or strong winds occurring during trapping nights. For instance, the reduced number of sand flies collected during some months (e.g., June and July 2013) may be a result of heavy rains recorded in these months. So, the common assertion that sand fly populations present a peak after rainy periods may be an artifact in some Brazilian regions. Indeed, it is expected that in drier areas sand flies (e.g., *Lu. longipalpis*) may be more active after rainy periods, when the relative humidity becomes higher [24]. However, in Atlantic forest remnants, where environmental conditions are optimal to sand flies throughout the year, population peaks after rainy periods may be actually due to adults that resume their activity after periods

of unfavorable weather conditions rather than due to the emergence of new adults. What do adult sand flies do when it rains is uncertain. Small fliers appear to be more robust than we think to in-flight perturbations [25]. Mosquitoes, which are larger than sand flies, have a strong exoskeleton and low mass that make them impervious to falling drops [25]. Water resistant hairs cover the wings and the whole body of sand flies and this may also protect them from falling raindrops.

Lutzomyia choti was the most abundant species and its daily number was positively correlated with temperature and saturation deficit, which is in line with a recent investigation conducted in low-density residential rural area, with mixed forest/agricultural exploitation [11]. This sand fly species was the only one found infected by *Leishmania* in the present study, with an estimated minimum infection rate of 0.87%. Real-time PCR-positive females were collected in January (one pool), February (two pools), April (one pool) and June (two pools). Whether by coincidence or not, all infected pools where collected in the first semester of the year, in parallel to the main sand fly population peak recorded in this study. *Lutzomyia choti* is one of the most common sand fly species in Atlantic forest remnants in Brazil [8, 10, 11]. It is also willing to feed on humans [9]. Altogether, these findings may indicate that *Lu. choti* may be an important vector of *L. braziliensis* in remnants of Atlantic forest in Brazil.

The period of the year when there is highest risk of *Leishmania* spp. transmission is always an issue of debate. In fact, one may say that the risk is higher when the sand fly population peaks up; higher biting rates. Other may argue that the highest risk would be later, when the vector population drops down and gets older; lower biting rates but higher infection rates in sand flies. Indeed, after emergence from the pupae, adult females need to take a meal to become infected, mature the infection in its gut to be able to transmit the parasites to a susceptible host. Taking into account our data on sand fly temporal distribution pattern and *Leishmania* infections in both sand flies and soldiers, we may speculate that the risk of cutaneous leishmaniasis may be permanent, but probably higher in the second semester. Nonetheless, this is a hypothesis that needs further investigations to be confirmed.

Cases of cutaneous leishmaniasis have been sporadically detected in the study area [3–5]. These cases are usually associated to soldiers or other military personnel that were involved in nocturnal activities in the forested environment. Indeed, the risk of infection by *L. braziliensis* in Atlantic forest remnants is eminent, as sand fly vectors [7–11] and reservoirs (e.g., small rodents) of this parasite are abundant in this biome [26–28]. It means that anyone overnighting in Atlantic forest remnants should seriously consider the adoption of protective measures, such as the use of repellents on bare skin or clothes and insecticide-treated tents. This should help reducing the risk of cutaneous leishmaniasis, especially among individuals like soldiers and forest workers that cannot avoid the contact with forested environments during the night.

The Atlantic forest biome has been reduced to almost nothing of its original land cover [29]. Nonetheless, this biome is still home to a great diversity of animals and plants. Among animals living in Atlantic forest remnants, there are arthropods that may act as vectors and small mammals that may act as reservoirs of disease agents (e.g., *Leishmania* spp. and *Rickettsia* spp.). It has been demonstrated that habitat fragmentation and biodiversity loss can increase the risk of pathogen transmission to humans through the so-called dilution effect [30–32]. It is yet to be investigated whether the almost complete destruction of the Atlantic forest biome has played a role on the epidemiology of cutaneous leishmaniasis in Brazil.

Concluding remarks

This study demonstrates that the temporal dynamics of sand flies is correlated to some extent to climate variables, with some species contrasts. The finding of *Lu. choti* females infected with

L. braziliensis, along with its known anthropophyly and high abundance in Atlantic forest fragments in Brazil, highlight the need for further studies to assess the vector competence of this sand fly for transmitting *L. braziliensis* under experimental conditions. Finally, people overnighting in Atlantic rainforest remnants should adopt preventative measures such as the use of repellents on bare skin or clothes and insecticide-treated tents to reduce their exposure to sand flies and other potential disease vectors.

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3.2 Artigo 2

O artigo intitulado "*Leishmania*-FAST15: A rapid, sensitive and low-cost real-time PCR assay for the detection of *Leishmania infantum* and *Leishmania braziliensis* kinetoplast DNA in canine blood samples" foi publicado em 2017 na revista Molecular and Cellular Probes (doi.org/10.1016/j.mcp.2016.08.006), o qual descrevemos um ensaio de PCR em tempo real para a detecção e quantificação de DNA de *L. infantum* e *L. braziliensis* em amostras de sangue de cão.

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Leishmania-FAST15: A rapid, sensitive and low-cost real-time PCR assay for the detection of *Leishmania infantum* and *Leishmania braziliensis* kinetoplast DNA in canine blood samples

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ABSTRACT

We describe an improved real-time PCR assay (designated as "*Leishmania*-FAST15") for the detection and quantification of *Leishmania infantum* and *Leishmania braziliensis* kinetoplast DNA minicircles in canine blood samples. The analytical sensitivity of this technique is 0.1 fg of DNA, which is equivalent to 0.002 parasite per reaction. This assay uses a small reaction volume (15 μl) and is rapid to perform, with the results being available in less than 34 min. This improved assay might also be suitable for detecting and quantifying *L. infantum* and *L. braziliensis* DNA in other tissues, such as bone marrow and lymph nodes.

1. Introduction

Leishmaniases are a group of neglected diseases of major public health significance in all continents, except Antarctica and Oceania. The burden of these diseases is disproportionally greater amongst the poorest of the poor people, with 0.9–1.6 million cases estimated to occur each year in 98 countries and three territories around the globe [1]. Visceral leishmaniasis is the most severe form of the disease and recent estimates suggest that 20,000 to 40,000 people die from leishmaniasis each year [1]. These figures indicate that new tools for diagnosing, treating and controlling leishmaniasis are needed.

Leishmania infantum is the causative agent of zoonotic visceral leishmaniasis [2]. Dogs are frequently infected by this parasite and are considered as the primary reservoirs in the peri-domestic transmission cycle [3]. Hence, early diagnosis and proper management of *L. infantum* infection in dogs is important, not only from a veterinary perspective, but also from a One Health viewpoint [3,4].

In their daily routine in endemic areas, veterinary practitioners use ordinary parasitological, serological and, to a lesser extent, molecular methods for the diagnosis of *L. infantum* infection in dogs. On the other hand, public health officials usually use serological tests (e.g., indirect fluorescence antibody assays, enzyme-linked immunosorbent assays and/or rapid immunochromatographic tests). The reasons for the unavailability of molecular tools in official leishmaniasis control programs in many endemic countries include a high cost of laboratory infrastructure, equipment and reagents, and a lack of personnel skilled in the application of molecular techniques.

Numerous polymerase chain reaction (PCR) assays have been published in the literature in the past decade (e.g., [5–15]). These assays have used different genetic markers (e.g., internal transcribed spacers, *β-tubulin*, locus *gp63*, *hsp70* and *cysteine proteinase* genes, and kinetoplast DNA minicircles), for application to different types of samples (e.g., bone marrow, lymph nodes, skin, blood, hair and nasal, oral, ear, and ocular swabs). For instance, Francino et al. [5] developed a highly sensitive real-time PCR assay to detect and quantify *L. infantum* DNA in canine blood samples. This assay has been used for different purposes [16–21], including the detection

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of *L. infantum* DNA in HIV-infected patients [22]. In this study, we report an improved real-time PCR assay (designated as "*Leishmania*-FAST15") for the detection and quantification of *L. infantum* and *Leishmania braziliensis* kinetoplast DNA, using the same primers and probe developed by Francino et al. [5]. The main advantages of the new assay are its lower reaction volume and shorter running time, thus minimizing costs and time necessary to obtain the results.

2. Material and methods

2.1. Strains and clinical samples

Three reference strains were used in this study: *L. infantum* (MHOM/BR/76/M4192), *L. braziliensis* (MHOM/BR/1975/M2903), and *Trypanosoma cruzi* (Y strain). Additionally, total of 40 EDTA-treated blood samples from dogs living in the city of Goiana, Pernambuco state, north-eastern Brazil, were included in the study. All procedures were conducted in accordance with international ethical principles of animal use previously approved by the Animal Ethics Committee (CEUA; project no. 66/2014) of the Centro de Pesquisas Aggeu Magalhães, Oswaldo Cruz Foundation, Recife, Pernambuco, Brazil.

2.2. DNA extraction from reference strains and clinical samples

Genomic DNA extraction from reference strains and blood samples was performed using the PureLink® Genomic DNA Mini Kit (Invitrogen), following the manufacturer's instructions, using 200 µl of blood, treating with RNase and eluting in 100 µl of buffer. The extracted genomic DNA was frozen at −20 °C. DNA concentration and purity (absorbance ratio at 260/280 nm and at 260/230 nm) was assessed using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

2.3. Primers, probe and assay conditions

The primers LEISH-1 (5′-AACTTTTCTGGTCCTCCGGGTAG-3′) and LEISH-2 (5′-ACCCCCAGTTTCCCGCC-3′) and the TaqMan® probe FAM-5′-AAAAATGGGTGCAGAAAT-3′-non-fluorescent quencher-MGB were used. According to Francino et al. [5], these primers and probe were designed to target conserved regions of the kinetoplast DNA minicircle of *L. infantum* and amplify a fragment of 120 base pairs.

The original real-time PCR assay was performed strictly according to the conditions described previously [5]. The primers and the TaqMan® probe were added at 900 nM and 200 nM, respectively, to a 25 µl final volume reaction mixture, containing 12.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems). The thermocycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min (estimated running time = 90 min and 41 s).

Our improved real-time PCR assay was performed using the abovementioned primers and probe at the same concentrations mentioned above. A total of 7.5 µl of TaqMan® Fast Advanced Master Mix (Applied Biosystems) was used with the reaction mixture being adjusted to a final volume of 15 µl. The thermocycling profile was 95 °C for 20 s, 40 cycles at 95 °C for 1 s and 60 °C for 20 s (estimated running time: 33.5 min).

The features of each assay are given in Table 1. Each amplification run contained a reference standard and a no-template control (NTC). All real-time PCR reactions were performed in triplicate, and the experiments were repeated three times. All real-time PCR were run on a QuantStudio® 5 Real-Time PCR system (Applied Biosystems).

2.4. Amplification efficiency, detection limit and specificity

Standard curves were prepared using nine 10-fold serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg per reaction) of genomic DNA of *L. infantum* (MHOM/BR/76/M4192) to assess the analytical sensitivity (detection limit), amplification efficiency (ε), correlation coefficient (R^2) and slope of the assays. The detection limit was defined as the lowest amount of DNA consistently detected in a given assay. DNA-free water was used as NTC. The specificity of the primers was initially checked *in silico* using Primer-BLAST [23] and MEGA 7.0.18 software. Then, the specificity of the assays was empirically tested using genomic DNA from *L. braziliensis* (MHOM/BR/1975/M2903) and *T. cruzi* (Y strain). The cut-off point of our improved assay was defined as the Ct (threshold cycle) value that corresponds to its lower detection limit [24].

2.5. Quantification of "parasite load"

Parasite load was estimated through the absolute quantification method, using a standard curve of nine 10-fold serial dilutions of genomic DNA of *L. infantum* as calibrators. Based on these

Table 2
Parasite load categories.

Category	Parasites per ml of blood
Negative	0
Low positive	>0–10
Medium positive	>10–100
High positive	>100–1000
Very high positive	>1000

Table 1
Real-time PCR assays.

Variables	Original assay[a]	*Leishmania*-FAST15
Forward primer (LEISH-1)	2.25 µl	1.35 µl
Reverse primer (LEISH-2)	2.25 µl	1.35 µl
TaqMan® probe (NFQ-MGB)	0.5 µl	0.3 µl
TaqMan® Master mix	12.5 µl	7.5 µl
DNA-free water	5.5 µl	2.5 µl
DNA template	2 µl	2 µl
Final volume	25 µl	15 µl
Thermocycling conditions	50 °C/2 min, 95°C/10 min, 40 cycles at 95 °C/15 s and 60°C/1 min	95 °C/20 s, 40 cycles at 95 °C/1 s and 60 °C/20 s
Total running time	90 min and 41 s	33 min and 30 s
Cost per sample (US Dollar)	4.00	3.50

NFQ, nonfluorescent quencher; MGB, minor groove binder.
[a] Francino et al. [5].

estimates, parasite loads in blood samples were classified into five categories (Table 2), adapted from Martínez et al. [18] The conversion of DNA amount to parasite number was done considering that a haploid genome size of *L. infantum* (*i.e.*, 32,134,935 bp) [25], which corresponds to ~ 65 fg for its diploid genome.

2.6. Data analysis

Real-time PCR results were analysed using QuantStudio® Design and Analysis Software v1.3.1 (Applied Biosystems). The amplification efficiency of each assay was calculated using the equation: $E = 10^{(-1/slope)} - 1$. The agreement of results between the two assays was assessed using Kappa statistics, with a significance level set at $P < 0.05$. The strength of agreement was interpreted as no better than chance (Kappa value = 0), slight (Kappa value = 0.01–0.20), fair (Kappa value = 0.21–0.40), moderate (Kappa value = 0.41–0.60), substantial (Kappa value = 0.61–0.80), almost perfect (Kappa value = 0.81–0.99) or perfect (Kappa value = 1.00) [26].

3. Results

The results showed that both the original and the improved assays achieved high correlation coefficients, high amplification efficiency (Fig. 1) and consistency across replicate reactions (Table 3). In particular, both assays were able to consistently detect 0.1 fg (mean Ct value, 35.5) of *L. infantum* DNA per reaction, which is equivalent to 0.002 parasite. The improved assay detected even less genomic DNA (*i.e.*, 0.01 fg per reaction), but not as consistently as 0.1 fg per reaction. Typically, very low amounts (<0.1 fg) of *L. infantum* genomic DNA presented high Ct values (*i.e.*, 37–39) (see Fig. 2).

According to our *in silico* analysis, the primers and probe used herein are complementary to sequences of the kinetoplast DNA minicircle of species the *Leishmania donovani* complex (*i.e.*, *L. donovani* and *L. infantum*). They are also partially complementary to sequences of other species (e.g., *L. braziliensis*). However, they are not complementary to the kinetoplast DNA minicircle of *T. cruzi*. Indeed, our improved assay was also able to detect *L. braziliensis* DNA (from 1 ng down to 0.1 fg per reaction), but not *T. cruzi* DNA.

By testing 40 blood samples from dogs from a visceral leishmaniasis-endemic area, 18 were positive to one or both assays, of which 16 were simultaneously detected by both assays (Table 4). Among positive dogs, three dogs were classified as medium positive and the remaining as low positive. The strength of agreement between the results obtained with both assays was almost perfect (Kappa value = 0.90, $P < 0.0001$).

4. Discussion

We describe a rapid, high-sensitive, low cost real-time PCR assay to detect and quantify *L. infantum* and *L. braziliensis* DNA in blood samples from dogs. This assay may also be useful for detecting *L. infantum* and *L. braziliensis* DNA in other types of samples (*e.g.*, bone marrow and lymph nodes) and from different hosts. Blood is not considered the best choice in terms of sensitivity, due to the relatively limited amount of parasite DNA present in this sample type as compared with, for example, bone marrow and lymph nodes [27]. Nonetheless, blood samples are easy to obtain and thus may be an option for large-scale studies [27]. Moreover, the real-

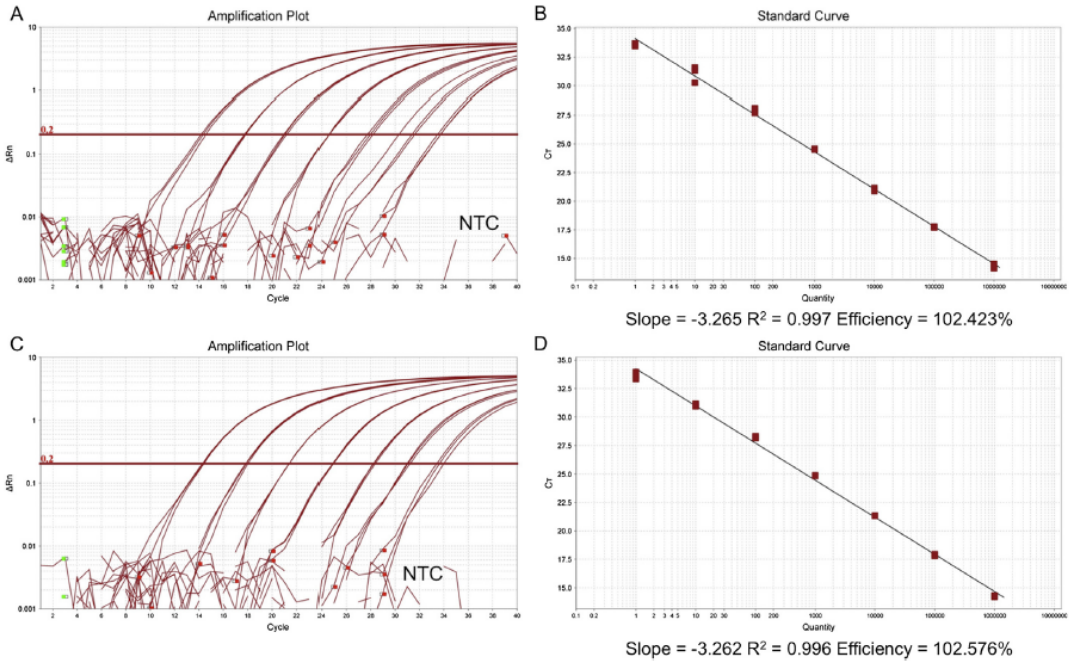

Fig. 1. Representative amplification plots and standard curves (from 1 ng down to 1 fg) for *Leishmania infantum* DNA obtained using the improved (A and B) and original (C and D) real-time PCR assays. NTC, no-template control.

Table 3
Amplification efficiency (ε), correlation coefficient (R^2), slope and detection limit of the real-time PCR assays[a].

Assay	ε (%)	R^2	Slope	Detection limit
Original assay	104.45 ± 1.60	0.995 ± 0.002	−3.21 ± 0.03	0.1 fg (0.002 parasite per reaction)
Leishmania-FAST15	105.49 ± 3.12	0.994 ± 0.002	−3.19 ± 0.06	0.1 fg (0.002 parasite per reaction)

[a] Results are presented as mean ± standard deviation calculated based on three experiments, with each point of the standard curve being tested in triplicate.

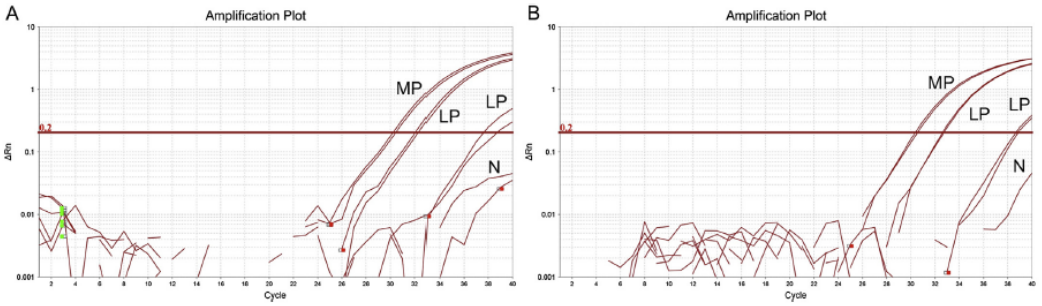

Fig. 2. Detection and quantification of *Leishmania infantum* in blood samples from dogs with the improved (A) and original (B) real-time PCR assays. LP, low positive. MP, medium positive. N, negative.

Table 4
Quantification of parasite loads in 18 dogs that were positive at each real-time PCR assay.

Sample ID	*Leishmania*-FAST15		Original assay	
	Ct value (mean)	Parasites per ml of blood	Ct value (mean)	Parasites per ml of blood
RR05	37.68	0.05[a]	37.98	0.04[a]
RR07	30.28	34.26[b]	29.74	44.48[b]
RR12	30.31	33.31[b]	29.73	43.69[b]
RR17	32.11	9.03[a]	31.97	8.90[a]
RR24	37.24	0.07[a]	0	0
RR30	30.45	31.01[b]	29.90	38.80[b]
RR31	36.58	0.11[a]	37.92	0.04[a]
RR34	35.59	0.22[a]	36.53	0.11[a]
RR35	37.33	0.04[a]	38.64	0.02[a]
RR36	37.11	0.09[a]	37.68	0.05[a]
RR41	0	0	37.07	0.08[a]
RR43	32.72	5.88[a]	32.23	8.18[a]
RR47	32.95	4.89[a]	33.12	3.94[a]
RR50	34.33	3.00[a]	34.59	1.45[a]
TA17	37.48	0.06[a]	37.28	0.06[a]
TA18	34.61	1.64[a]	34.99	1.15[a]
TA21	34.11	2.10[a]	33.20	3.84[a]
TA27	38.10	0.04[a]	38.18	0.07[a]

[a] Low positive.
[b] Medium positive.

time PCR assay described herein has a very high sensitivity, allowing the detection of minute amounts of *Leishmania* DNA, equivalent to less than a single parasite per test sample. In the case of the assays used herein, one of the reasons for their high sensitivity is the genetic target: the kinetoplast DNA minicircles. Due to their very high copy number (*i.e.*, 10,000 copies per parasite cell) [28], kinetoplast DNA minicircles are often the first choice for leishmaniasis diagnosis. In addition to variable regions, kinetoplast DNA minicircles contain three highly conserved sequence blocks (CSB-1, CBS-2 and CSB-3) [29], which certainly represent an obstacle to be overcome when trying to design primers that are species-specific.

Both *in silico* and empirical analyses confirmed that the primers and probe designed by Francino et al. [5] might also detect DNA from other species of *Leishmania* (e.g., *L. braziliensis*), but not from *T. cruzi*. This broadens the scope of this assay, which might therefore be used to screen dogs in areas where *L. braziliensis* is endemic, such as in several rural areas in South America [30]. On the other hand, this means that this assay may not confirm the identity of the DNA found as pertaining to *L. infantum*. Indeed, in areas where both *L. infantum* and *L. braziliensis* occur [31], this assay may still be useful for screening dogs, provided that positive samples can be properly identified through, for example, DNA sequencing. It remains to be tested whether our improved assay can also detect DNA from other *Leishmania* spp. (e.g., *L. amazonensis* and *L. panamensis*) found in dogs in South America.

In addition to a cost reduction, a 63% decrease in the overall cycling time was achieved with the improved assay. In practice, it means that the results are obtained in ~34 min, that is, almost a third of the time required for the original assay. This may also help

speed up treatment decisions for both canine and human patients.

It is always challenging to establish the cut-off point of a real-time PCR assay. The cut-off point might be defined as the Ct value that corresponds to the lower detection limit of the assay [24]. Our improved real-time PCR assay consistently detected 0.1 fg of DNA with Ct values ranging from 34 to 36 (average, 35.5). However, later amplifications (*i.e.*, Ct $\geq$ 37) were obtained with lower amounts of parasite DNA (*e.g.*, 0.01 fg per reaction) and with blood samples from newly infected dogs living in a visceral leishmaniasis-endemic area (data not shown). Therefore, while the detection limit of the *Leishmania*-FAST15 assay was 0.1 fg of DNA per reaction, specific amplifications of lower template concentrations may eventually be achieved at later cycles (*i.e.*, up to 39) and these results should be regarded as true-positives.

5. Conclusion

In conclusion, the real-time PCR assay described herein represents a rapid, low-cost molecular tool for the detection and quantification of *L. infantum* and *L. braziliensis* DNA in blood samples from dogs. It could be useful not only by veterinary practitioners, but also by public health authorities, as its low cost allows for its use in leishmaniasis control programs. Finally, it should also be useful for clinical trials, permitting the use of blood rather than samples (e.g., bone marrow) obtained via invasive methods, which is desirable, particularly for large-scale epidemiological studies.

Author contributions

FDT designed the study and performed the *in silico* analyses. KGSS and LGS performed the experiments. FDT, KGSS, LGS, DO and LAF discussed and interpreted the results. FDT wrote the manuscript. DO and LAF reviewed the manuscript. All authors read and approved the final manuscript.

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3.3 Artigo 3

O artigo intitulado “Home sweet home: sand flies find a refuge in remote indigenous villages in north-eastern Brazil, where leishmaniasis is endemic” foi publicado em 2019 na revista Parasites and Vectors (doi: 10.1186/s13071-019-3383-1), o qual investigamos a presença de flebotomíneos dentro de habitações humanas e também a exposição de cães a *Leishmania* spp. em aldeias indígenas no nordeste do Brasil.

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Parasites & Vectors

RESEARCH Open Access

Home sweet home: sand flies find a refuge in remote indigenous villages in north-eastern Brazil, where leishmaniasis is endemic

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Abstract

Background: From 2012 to 2013, an outbreak of cutaneous leishmaniasis by *Leishmania braziliensis* was detected in indigenous villages located in a remote rural area of Pernambuco state, north-eastern Brazil. Considering that the principal activities of this indigenous community are farming and crop plantation, and also that the outbreak involved many children, we investigated the presence of sand fly vectors inside human houses and also the exposure of dogs to leishmanial parasites. Our general objective was to gather epidemiological data that could indicate the occurrence of a peri-domestic/domestic transmission cycle of *L. braziliensis* in these indigenous villages.

Methods: From March 2015 to March 2016, sand flies were collected using light traps in the indoor and immediate outdoor environments in the three indigenous villages that reported the most cutaneous leishmaniasis cases during the 2012–2013 outbreak. Moreover, samples obtained from 300 dogs living in the outbreak villages and two nearby villages were tested by a rapid immunochromatographic test and by a real-time PCR for detecting anti-*Leishmania* antibodies and *Leishmania* DNA, respectively.

Results: In total, 5640 sand flies belonging to 11 species were identified. Males ($n = 3540$) predominated over females ($n = 2100$). *Migonemyia migonei* (84.3%) was the most abundant species, followed by *Evandromyia lenti* (5.5%), *Lutzomyia longipalpis* (4.1%), *Nyssomyia intermedia* (1.6%) and *Micropygomyia capixaba* (1.4%), representing together ~97% of the sand flies collected. Nine out of the 11 species identified in this study were found indoors, including *M. migonei*, *L. longipalpis* and *N. intermedia*, which are proven vectors of *Leishmania* spp. Out of 300 dogs tested, 26 (8.7%) presented anti-*Leishmania* antibodies and six (2%) were *Leishmania* DNA-positive. The level of exposure in dogs living in the indigenous villages where the 2012–2013 outbreak of human CL was detected was almost 2-fold higher than in the two nearby villages (11.0 *vs* 6.2% for serology and 2.6 *vs* 1.4% for real-time PCR).

Conclusions: The results suggest that different sand fly vectors may be adapted to human dwellings, thus increasing the risk of transmission in the indoor and immediate outdoor environments. The adaptation of sand flies to the indoor environment in the studied indigenous villages may be partly explained by the poor housing conditions and the proximity of the houses to crop plantations and forest fragments.

Keywords: Cutaneous leishmaniasis, Dogs, Phlebotomine sand fly, Rural area

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Background

Leishmaniases are neglected tropical diseases, which cause significant morbidity and mortality in endemic areas, particularly in tropical and subtropical regions of the world [1]. Brazil, India, Bangladesh, Sudan, South Sudan and Ethiopia account for more than 90% of the global cases of visceral leishmaniasis (VL), with an estimated 200,000 to 400,000 new cases per year [2]. About 75% of the global incidence of cutaneous leishmaniasis (CL) occurs in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica and Peru, with an estimated 0.7 to 1.2 million new cases per year [2]. From a global perspective, Brazil is one of the main foci of leishmaniases, with an annual average incidence of 1.7 and 8.0 new VL and CL cases per 100,000 population, respectively, during the period 2013–2017 [3].

CL and VL are primarily zoonoses, with wild animals (e.g. forest rodents) and domestic dogs, respectively, being involved as reservoirs in their zoonotic transmission cycles [4, 5]. Outbreaks of zoonotic CL in Brazil are commonly detected among males at working age who enter the forest for various reasons, such as for military training [6]. Deforestation (e.g. for road construction and crop plantation) and population movements from non-endemic to endemic areas (and *vice versa*) are also risk factors for both CL and VL [7]. In recent years, CL and VL have spread across different Brazilian regions, indicating the ineffectiveness of control measures to reduce the burden of the disease in both rural and urban areas [8, 9].

Degradation of natural habitats may force sand fly vectors to adapt to the modified environment [10]. Indeed, blood-feeding insects such as sand flies are commonly attracted to human dwellings, where they may find food sources (e.g. domestic animals and humans) [11], resting places and breeding sites [12]. Certainly, the adaptation of sand fly vectors to human dwellings may increase the risk of *Leishmania* spp. transmission in the peri-domestic and domestic environments.

The increasing interaction between humans, domestic and wild animals, in wild, peri-domestic and domestic environments has caused profound changes in the epidemiology of leishmaniases in the past decades [7]. For instance, CL caused by *Leishmania braziliensis* is a zoonosis maintained by multitude of small mammals (e.g. forest rodents and marsupials) and sand fly vectors, which have adapted to the peri-domestic and domestic environments. In endemic areas, dogs are frequently exposed to sand fly vectors and are often infected by *L. braziliensis* [13]. While dogs play no role as reservoirs of this parasite [14], they can play a useful role as a sentinel host [15].

From 2012 to 2013, an outbreak of CL was detected in indigenous villages located in a remote rural area of Pernambuco state, north-eastern Brazil. These villages are settled in legally-expropriated lands and are presently home to the tribe Xukuru de Ororubá. While many ancient indigenous traditions are maintained by the Xukuru people, their principal working activities are farming and crop plantation for their own subsistence. Bearing this in mind and also considering that the outbreak detected in 2012–2013 involved many children, we investigated the presence of sand fly vectors inside human houses and also the exposure of dogs to leishmanial parasites. Our general objective was to gather epidemiological data that could indicate the occurrence of a peri-domestic/domestic transmission cycle of *L. braziliensis* in these indigenous villages.

Methods

Study area

This study was carried out in the municipality of Pesqueira, which is located in the agreste region of Pernambuco, a narrow zone between the Atlantic forest zone (zona da mata) and the semiarid region (sertão). In particular, three indigenous villages were surveyed in this study: Guarda (V1; 8°21′49.6″S, 36°48′47.1″W, altitude: 844 m above sea level), Santana (V2; 8°20′12.3″S, 36°43′38.1″W, altitude: 850 m) and Afetos (V3; 8°19′06.1″S, 36°42′37.3″W, altitude: 965 m) (Fig. 1). Geographical coordinates and altitude of each village were recorded using a Garmin eTrex Venture HC GPS (Garmin International, Olathe, USA).

These villages are part of the 24 indigenous villages of the tribe Xukuru de Ororubá, which occupy approximately 27,000 hectares of a chain of mountains named Serra do Ororubá. The villages are located in a rural area and the native vegetation is represented by semi-deciduous and deciduous forests, although most of the original forest coverage has been substituted by crop plantations [16]. The landscape is represented by a xeric shrubland and thorn forest, which consists primarily of small, thorny trees that shed their leaves seasonally. The ground layer is made up of cacti, thick-stemmed plants, thorny brush and arid-adapted grasses. The climate of this area is semiarid, characterized by low humidity and little rainfall. The raining period ranges from February to July, with an annual average temperature of 26 °C (range, 24–27 °C), average relative humidity of 76% (range, 69–86%) and average precipitation of 700 mm^3.

The population of the villages is currently around 2720 [17]. The local economy is mostly based on agriculture, with plantations of bananas, beans, cassava, corn and vegetables, as well as dairy cattle and goat farming [18]. Many of the houses are precarious and lack basic

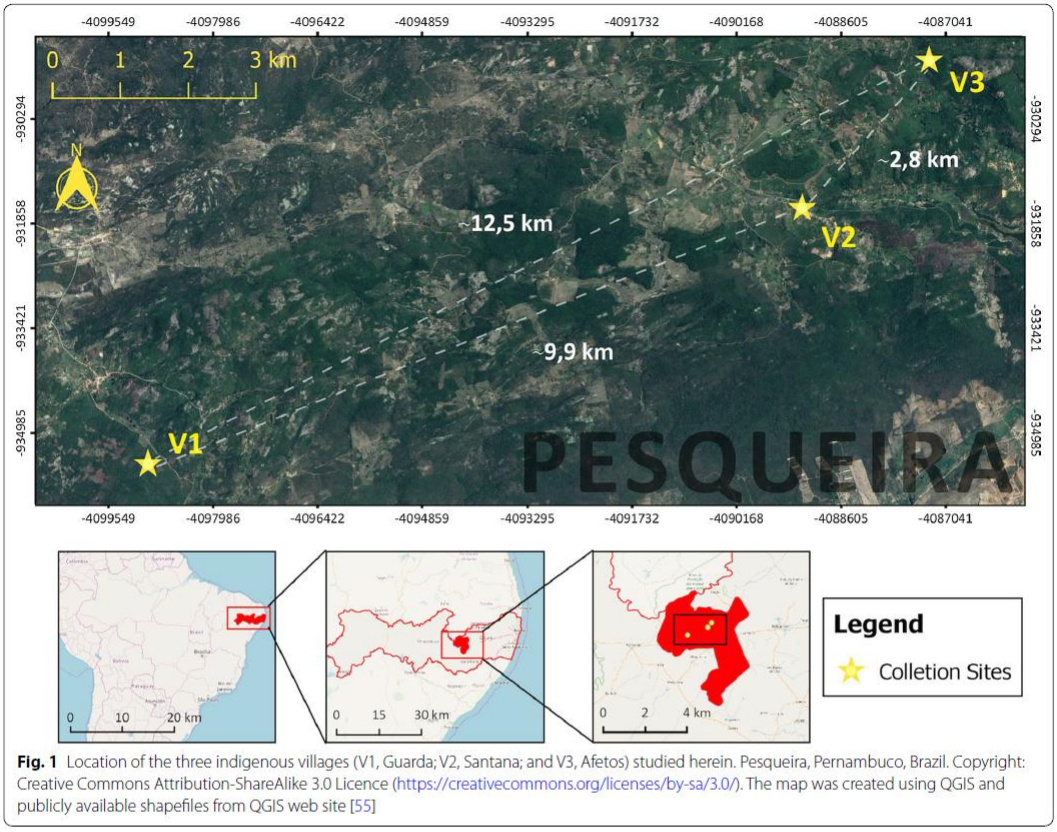

Fig. 1 Location of the three indigenous villages (V1, Guarda; V2, Santana; and V3, Afetos) studied herein. Pesqueira, Pernambuco, Brazil. Copyright: Creative Commons Attribution-ShareAlike 3.0 Licence (https://creativecommons.org/licenses/by-sa/3.0/). The map was created using QGIS and publicly available shapefiles from QGIS web site [55]

sanitation. Children and teenagers typically go to school in the morning and afternoon, respectively. In their spare time they play and are at home early in the evening, particularly young children. Domestic animals (e.g. dogs, cats and chickens) are common both indoors and outdoors of human houses.

Collection and morphological identification of sand flies

Sand fly collections were carried out monthly, from March 2015 to March 2016 (except in October 2015, for logistic reasons) for two to three consecutive nights. Collection sites (houses) were chosen based on the occurrence of human cases of CL. Some of the houses were made of mud walls and thatched roofs, with obvious openings that may facilitate the entrance of insects during the night. Moreover, some of the houses were surrounded by native vegetation.

Each night, one to four CDC light traps were installed in each village, operating from 18:00 h to 6:00 h, for a total of 253 traps installed and 3036 cumulative hours of trapping. Each trap was positioned 1.5 m above the ground in two types of environments: indoor (living rooms and bedrooms) and outdoor (backyards with chicken coop, goats and/or dogs). All specimens collected were transferred to labelled vials containing 70% ethanol and subsequently identified using morphological keys for American sand flies based on characters of male genitalia, female spermathecae and pharyngeal armature [19]. The nomenclature of sand fly species followed Galati's proposal [20]. Females collected indoors and outdoors during the last seven months of collection (from August 2015 to March 2016) were classified as engorged (blood in the abdomen, total or partial) or unfed (no visible blood in the abdomen).

Meteorological data

Monthly average temperature (°C), relative humidity (%) and rainfall (mm) data were obtained from station 82900 of the Technology Institute of Pernambuco (ITEP). The saturation deficit (SD) was calculated

as follows: $SD = (1 - RH/100) \times 4.9463 \times e^{0.0621 \times T}$, where RH is relative humidity and T is temperature [6].

Canine blood collection and diagnostic procedures

Dogs living in the investigated indigenous villages (Guarda, Santana and Afetos) and two nearby villages (Cimbres and São José) were chosen as sentinel hosts. From March to June 2015, blood samples were collected (~5 ml) from 300 privately-owned dogs. Aliquots of ~2 ml and ~3 ml were added to EDTA tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) and gel serum separator tubes (Greiner Bio-One GmbH), respectively. Gel serum separator tubes were centrifuged at 2000×*g* for 10 min for serum separation. The obtained sera and blood samples were stored at -20 °C.

Genomic DNA extraction from EDTA-blood samples was performed using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quantity and purity of the extracted DNA were assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted DNA samples were stored at -20 °C until testing.

Real-time PCR reactions were performed using the primers LEISH-1 (5′-AAC TTT TCT GGT CCT CCG GGT AG-3′) and LEISH-2 (5′-ACC CCC AGT TTC CCG CC-3′) and the TaqMan® probe FAM-5′-AAA AAT GGG TGC AGA AAT-3′-non-fluorescent quencher-MGB, as described elsewhere [21]. The final reaction volume was adjusted to 25 µl containing 5.5 µl of type 1 (ultrapure) water, 2.25 µl of each primer (900 nM), 0.5 µl of probe (200 nM), 12.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and 2 µl of DNA template. PCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. All samples were tested in duplicate and no template control (NTC) and positive controls (DNA extracted from cultured *L. infantum* promastigotes) were included in each PCR run. Reactions were run on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and the results analysed using the 7500 software v.2.0.5.

Dog sera were tested using DPP LVC (Bio-Manguinhos, Rio de Janeiro, RJ, Brazil), according to the manufacturer's instructions. This test uses recombinant antigens and it is the official screening test used by public health authorities in Brazil [22]. Results were read after 10 min and interpreted as follows: negative (only control red line present), positive (control and sample red lines present) and invalid (control red line absent).

Human cases

Secondary data regarding human cases of CL detected during the 2007–2017 in the indigenous villages were obtained from the Brazilian Information System on Diseases of Compulsory Declaration [3]. In particular, we were interested in all CL cases detected in the outbreak of 2012–2013. Variables of interest were year and month of notification, gender, place of residence, and age. Data were obtained and processed anonymously.

Diversity indices and statistical analyses

Sand fly species richness and diversity were assessed using the following parameters: species richness (*S*), number of individuals (*n*), Shannon's diversity index (*H*′) and Pielou's equitability index (*J*′). We also computed the species accumulation curve (sample-based rarefaction) as a function of number of samples using Mao's tau, with standard deviation; in the graphical plot, the standard errors were converted to 95% confidence intervals. Diversity indices and species accumulation curve were calculated using PAST, v.3.23 for Mac OS [23].

Before statistical analysis, normality of data was assessed using Lilliefors. Pearson's (*r*) or Spearman's (r_s) correlation coefficients were used to determine the correlation between the meteorological variables and the relative frequencies of sand flies (i.e. number of individuals per hour of trapping). Student's t-test was used for comparing the relative frequencies of sand flies collected monthly indoors *vs* outdoors. To compare the abundance of each species indoors *vs* outdoors, we calculated the index of species abundance (ISA), which was then converted to a scale of zero to one, through the standardized index of species abundance (SISA), where the value 1.00 represents the most abundant species [24]. The Kruskal-Wallis H-test (with Dunn's *post-hoc* test) was used to compare the relative frequencies of sand flies collected monthly in the three surveyed indigenous villages. Chi-square test was used to assess whether positivity to *Leishmania* spp. varied according to dog data including sex (male, female), age ($\leq$ 1 year, > 1 year), clinical status (healthy, sick) and housing condition (domiciled, semi-domiciled). We also used the Chi-square test to determine whether there was a significant difference between the frequencies of engorged and unfed females collected indoors *vs* outdoors. Statistical analyses were performed using BioEstat v.5.3 (Mamirauá Institute of Sustainable Development, Tefé, AM, Brazil) and $P \leq 0.05$ was considered statistically significant.

Table 1 Number (*n*) and percentage (%) of sand flies collected indoors and outdoors in the surveyed indigenous villages (V1–V3) in Pesqueira, Pernambuco, Brazil, from March 2015 to March 2016, according to species and sex. Sex ratio (female:male) is also provided

Species	Indoor							Outdoor						
	Female		Male		Total		Sex ratio	Female		Male		Total		Sex ratio
	n	%	*n*	%	*n*	%		*n*	%	*n*	%	*n*	%	
Micropygomyia capixaba	46	59.0	3	3.8	49	62.8	15.3	24	30.8	5	6.4	29	37.2	4.8
Evandromyia evandroi	5	7.8	1	1.6	6	9.4	5.0	53	82.8	5	7.8	58	90.6	10.6
Micropygomyia villelai	4	11.1	10	27.8	14	38.9	0.4	16	44.4	6	16.7	22	61.1	2.7
Nyssomyia intermedia[a]	3	3.4	12	13.5	15	16.9	0.3	34	38.2	40	44.9	74	83.1	0.9
Evandromyia lenti	18	5.8	30	9.7	48	15.6	0.6	72	23.4	188	61.0	260	84.4	0.4
Lutzomyia longipalpis[a]	25	10.9	46	20.0	71	30.9	0.5	50	21.7	109	47.4	159	69.1	0.5
Migonemyia migonei[a]	438	9.2	620	13.0	1058	22.3	0.7	1272	26.8	2423	51.0	3695	77.7	0.5
Evandromyia sallesi	0	0.0	0	0.0	0	0.0	nc	2	66.7	1	33.3	3	100.0	2.0
Micropygomyia schreiberi	0	0.0	0	0.0	0	0.0	nc	3	100.0	0	0.0	3	100.0	nc
Sciopemyia sordellii	0	0.0	7	24.1	7	24.1	0.0	9	31.0	13	44.8	22	75.9	0.7
Micropygomyia trinidadensis	10	21.3	6	12.8	16	34.0	1.7	16	34.0	15	31.9	31	66.0	1.1
Total	549	42.8	735	57.2	1284	22.8	0.7	1551	35.6	2805	64.4	4356	77.2	0.6

[a] Proven vector species [24]

Abbreviation: nc, not calculated

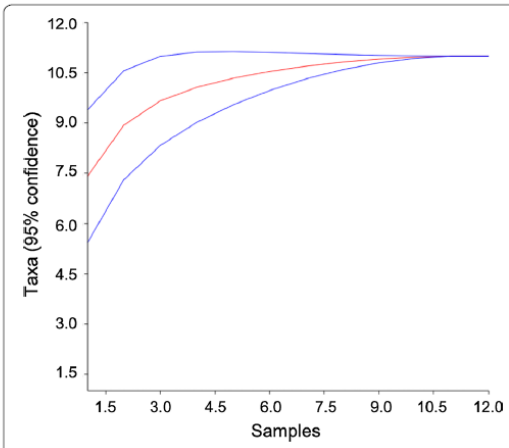

Fig. 2 Species accumulation curve (Mao's tau function) (red line) and 95% confidence interval (blue lines) for sand flies in Pesqueira, Pernambuco, Brazil

Results

Sand fly species and numbers

A total of 5640 sand flies were collected and morphologically identified (Table 1). The species accumulation curve reached saturation at the 10th sampling event (Fig. 2), with 11 species identified.

The percentage of females (37.2%, $n = 2100$) was lower than that of males (62.8%, $n = 3540$), with an overall female:male ratio of 0.6:1. A positive correlation between the monthly number of males and females collected during the study ($r_{(10)} = 0.86$, $P < 0.001$) was found. However, the sex ratio varied according to sand fly species, type of environment (indoors *vs* outdoors) (Table 1) and village (Table 2).

Most sand flies were collected outdoors (77.2%; $n = 4356$; mean = 2.36 sand flies per hour of trapping) as compared to indoors (22.8%; $n = 1284$; mean = 1.08 sand flies per hour of trapping) (Student's t-test, $t_{(22)} = 3.14$, $P = 0.005$). However, 9 out of 11 species found in this study were collected inside the houses. Some sand fly species (i.e. *Evandromyia lenti*, *Migonemyia migonei* and *Lutzomyia longipalpis*) were consistently collected indoors throughout the entire study period (Fig. 3, Additional file 1: Table S1). According to data recorded from August 2015 to March 2016, 28 out of 232 (12.1%) *M. migonei* females collected indoors and 98 out of 890 (11.0%) those collected outdoors were engorged ($\chi^2 = 0.21$, $df = 1$, $P = 0.650$). Additionally, 2 out of 32 (6.3%) *Nyssomyia intermedia* females collected outdoors were also engorged. All other females belonging to other species, which were collected in the aforementioned period, were unfed.

Migonemyia migonei was the most abundant species both indoors (SISA = 1.00) and outdoors (SISA = 1.00). In the outdoor environment, *L. longipalpis* (SISA = 0.90) was the second most abundant species, followed by *E. lenti* (SISA = 0.67), *Evandromyia evandroi* (SISA = 0.67), *N. intermedia* (SISA = 0.58), *Sciopemyia sordellii* (SISA = 0.43), and other less abundant species (Additional file 2: Figure S1). In the indoor environment,

Table 2 Number (*n*) and percentage (%) of sand flies collected in three indigenous villages (V1–V3) in Pesqueira, Pernambuco, Brazil, from March 2015 to March 2016, according to species and sex. Sex ratio (female:male) is also provided

Species	Guarda (V1)							Santana (V2)							Afetos (V3)						
	Female		Male		Total		Sex ratio	Female		Male		Total		Sex ratio	Female		Male		Total		Sex ratio
	n	%	*n*	%	*n*	%		*n*	%	*n*	%	*n*	%		*n*	%	*n*	%	*n*	%	
Micropygomyia capixaba	31	4.5	7	0.7	38	2.2	4.4	3	1.6	0	0.0	3	0.8	nc	36	3.0	1	0.0	37	1.0	36.0
Evandromyia evandroi	32	4.6	2	0.2	34	2.0	16.0	9	4.8	1	0.5	10	2.5	9.0	17	1.4	3	0.1	20	0.6	5.7
Micropygomyia villelai	4	0.6	11	1.1	15	0.9	0.4	3	1.6	0	0.0	3	0.8	nc	13	1.1	5	0.2	18	0.5	2.6
Nyssomyia intermedia[a]	10	1.4	14	1.4	24	1.4	0.7	1	0.5	2	1.0	3	0.8	0.5	26	2.1	36	1.5	62	1.7	0.7
Evandromyia lenti	50	7.2	125	12.5	175	10.3	0.4	12	6.4	28	13.6	40	10.2	0.4	28	2.3	65	2.8	93	2.6	0.4
Lutzomyia longipalpis[a]	70	10.1	126	12.6	196	11.6	0.6	0	0.0	5	2.4	5	1.3	0.0	5	0.4	24	1.0	29	0.8	0.2
Migonemyia migonei[a]	483	69.8	704	70.5	1187	70.2	0.7	154	82.4	163	79.1	317	80.7	0.9	1073	88.0	2176	93.1	3249	91.4	0.5
Evandromyia sallesi	0	0.0	0	0.0	0	0.0	nc	2	1.1	1	0.5	3	0.8	2.0	0	0.0	0	0.0	0	0.0	nc
Micropygomyia schreiberi	0	0.0	0	0.0	0	0.0	nc	1	0.5	0	0.0	1	0.3	nc	2	0.2	0	0.0	2	0.1	nc
Sciopemyia sordellii	7	1.0	6	0.6	13	0.8	1.2	2	1.1	4	1.9	6	1.5	0.5	0	0.0	10	0.4	10	0.3	0.0
Micropygomyia trinidadensis	5	0.7	4	0.4	9	0.5	1.3	0	0.0	2	1.0	2	0.5	0.0	19	1.6	17	0.7	36	1.0	1.1
Total	692	40.9	999	59.1	1691	30.0	0.7	187	47.6	206	52.4	393	7.0	0.9	1219	34.3	2337	65.72	3556	63.0	0.5

[a] Proven vector species [24]

Abbreviation: nc, not calculated

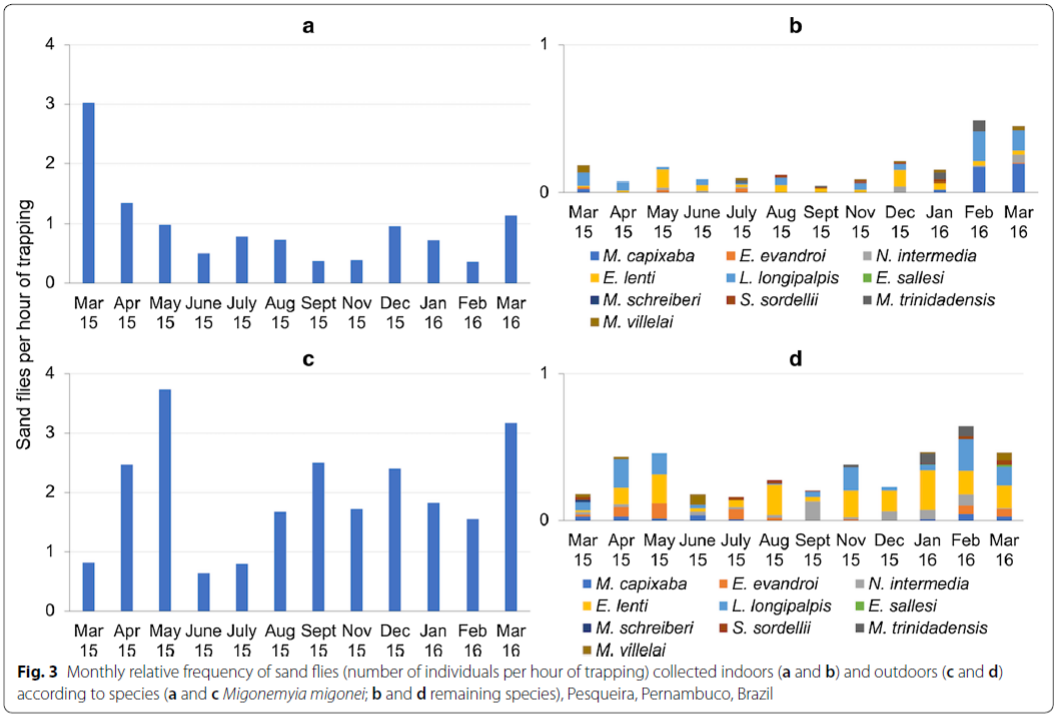

Fig. 3 Monthly relative frequency of sand flies (number of individuals per hour of trapping) collected indoors (**a** and **b**) and outdoors (**c** and **d**) according to species (**a** and **c** *Migonemyia migonei*; **b** and **d** remaining species), Pesqueira, Pernambuco, Brazil

Micropygomyia capixaba (SISA = 0.81) was the second most abundant species, followed by *E. lenti* (SISA = 0.78), *Micropygomyia trinidadensis* (SISA = 0.54), *L. longipalpis* (SISA = 0.48), *Micropygomyia villelai* (SISA = 0.46), and other less abundant species (Additional file 2: Figure S1).

Among the species collected, *M. migonei* (84.3%), *E. lenti* (5.5%), *L. longipalpis* (4.1%), *N. intermedia* (1.6%) and *M. capixaba* (1.4%) were the most frequent, representing together ~97% of the total sand flies captured. These three species were consistently trapped during the whole study period and in the three studied villages. However, *M. migonei* specimens were more frequently collected in V3, whereas most *E. lenti* and *L. longipalpis* in V1. *Evandromyia sallesi* ($n = 3$) and *Micropygomyia schreiberi* ($n = 3$) were the least representative species, being found only in V2 and V3 (Table 2).

The relative frequencies of sand flies collected monthly varied according to village (Kruskal-Wallis H-test, $H = 18.24$, $df = 2$, $P < 0.001$; Dunn's *post-hoc* test, $P < 0.05$ for V1 *vs* V2 and V2 *vs* V3, and $P > 0.05$ for V1 *vs* V3). The highest number of sand flies was recorded in village V3 (63.0%; $n = 3556$; mean = 2.77 sand flies per hour of trapping), followed by V1 (30.0%; $n = 1691$; mean = 1.68 sand flies per hour of trapping) and V2 (7.0%; $n = 393$; mean = 0.53 sand flies per hour of trapping). The highest species richness was found in V2, where all 11 species found in this study were present (Table 3). The species diversity and equitability were higher in V1 ($H' = 1.06$, $J' = 0.48$) and V2 ($H' = 0.81$, $J' = 0.34$), corresponding to the villages with lower altitude (i.e. 844 and 850 m, respectively).

Table 3 Diversity indices in three indigenous villages in Pesqueira, Pernambuco, Brazil, from March 2015 to March 2016

Index	Guarda (V1)	Santana (V2)	Afetos (V3)
Species richness	9	11	10
Individuals (*n*)	1691	393	3556
Shannon (*H'*)	1.06	0.81	0.46
Equitability (*J'*)	0.48	0.34	0.20

The months with the greatest number of sand flies collected were May 2015 and March 2016 (Fig. 4), when the monthly average precipitations were 179 and 69.2 mm, monthly average temperatures 26.4 and 27.4 °C and monthly average relative humidity 77.3 and 74%, respectively (Fig. 5).

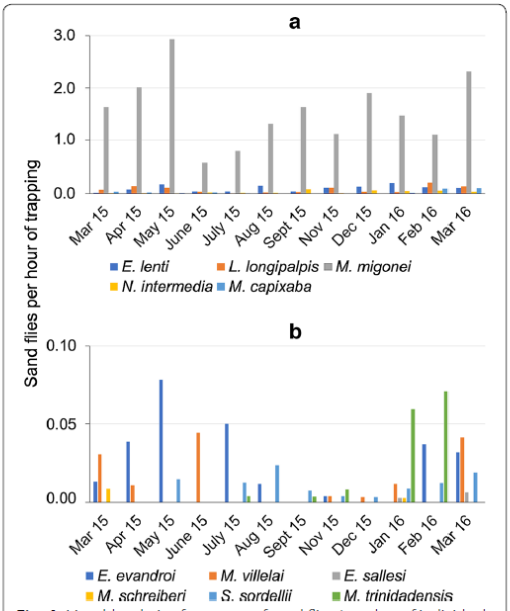

Fig. 4 Monthly relative frequency of sand flies (number of individuals per hour of trapping) collected in Pesqueira, Pernambuco, Brazil (**a** the five most common species; **b** the remaining species)

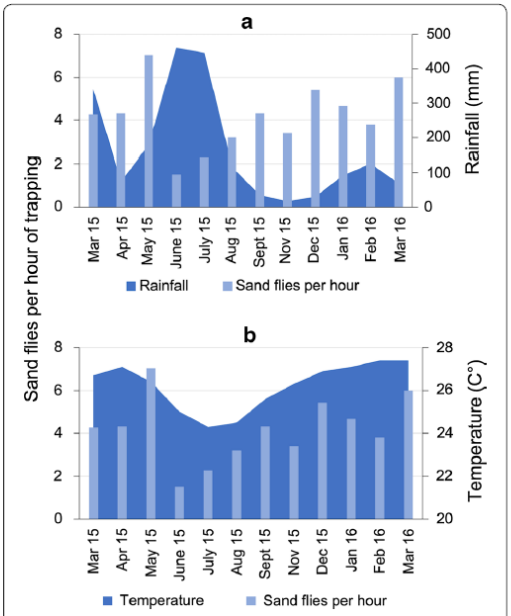

Fig. 5 Monthly relative frequency of sand flies (number of individuals per hour of trapping) in relation to rainfall (**a**) and temperature (**b**) in Pesqueira, Pernambuco, Brazil

The months with higher temperatures and lower relative humidity coincided with peaks in sand fly population. Indeed, the relative frequency of sand flies (i.e. sand flies per hour of trapping) collected monthly was positively correlated with temperature ($r_{(10)} = 0.62$, $P = 0.033$). On the other hand, no significant correlation was found between the relative frequency of sand flies with relative humidity ($r_{(10)} = -0.44$, $P = 0.152$), precipitation ($r_s = -0.32$, $P = 0.308$) or saturation deficit ($r_{(10)} = 0.49$, $P = 0.107$). The most abundant species collected (i.e. *M. migonei*, *E. lenti* and *L. longipalpis*) were collected mainly in months with an average relative humidity < 75%.

Canine exposure to *Leishmania* spp

Out of 300 dogs tested, 26 (8.7%) were positive by serology and six (2%) by real-time PCR. Six dogs were simultaneously positive for both the tests. The highest positivity rates for both serology and real-time PCR were recorded in the outbreak villages (serology = 11.0%, 17/155; real-time PCR = 2.6%, 4/155), as compared with nearby villages (serology = 6.2%, 9/145; real-time PCR = 1.4%, 2/145). Most of the dogs presented apparent clinical signs ($n = 179$) and were semi-domiciled ($n = 265$). No significant association was found between positivity (serology and/or real-time PCR) and variables such as the sex ($\chi^2 = 1.11$, $df = 1$, $P = 0.293$), age ($\chi^2 = 2.78$, $df = 1$, $P = 0.096$), clinical status ($\chi^2 = 3.52$, $df = 1$; $P = 0.061$) and housing condition ($\chi^2 = 1.69$, $df = 1$, $P = 0.194$).

Human cases

From 2007 to 2017, 49 human cases of CL were notified in 12 indigenous villages of Pesqueira. Of these, 40 cases were diagnosed from February 2012 to December 2013, with 77.5% ($n = 31$) of the cases being diagnosed in 2012. During this outbreak, 67.5% of the cases were detected in V1 (50%, $n = 20$), V2 (12.5%, $n = 5$) and V3 (5%, $n = 2$). Cases were notified during almost all months of the year, except in May and June. The months with more cases recorded during outbreak were: March 2012 ($n = 7$) and October 2012 ($n = 14$). Men ($n = 29$) were more frequently affected than women ($n = 11$), and most cases (62.5%) were from 3 to 10 years-old (25%) and 11 to 17 years-old (37.5%), with age ranging from 3 to 66 years.

Discussion

Throughout the study period, we identified 11 sand fly species in the investigated indigenous villages. *Migonemyia migonei*, *E. lenti*, *L. longipalpis*, *N. intermedia* and *M. capixaba* were the most abundant species, present in

all villages, both indoors and outdoors. From an epidemiological point of view, this finding is very important because it may suggest a year-long risk for the transmission of *Leishmania infantum* and *L. braziliensis* in this region. *Lutzomyia longipalpis* and *M. migonei* are incriminated as vectors of *L. infantum* and *L. braziliensis*, respectively, in Brazil and other Latin American countries [25]. In addition, *M. migonei* has been strongly suggested as a vector of *L. infantum* in some foci [26, 27] and a recent laboratory study reinforced this hypothesis [28]. Furthermore, *N. intermedia* is also a proven vector of *L. braziliensis* in Brazil [25]. Considering the presence of potential vectors during the whole year, further research focused on detecting DNA (by PCR) or promastigotes (by dissection and microscopical examination) of *Leishmania* spp. in sand flies could provide valuable data on the transmission pattern in this area, which may include the participation of multiple vectors.

Our results support our initial hypothesis that sand flies may be adapted to human dwellings in the studied indigenous villages. For instance, *M. capixaba* is a sylvatic species, generally found in forests and marginal areas [29]. However, in the present study, this species was the second most abundant species indoors (SISA = 0.81), after *M. migonei* (SISA = 1.00). Notably, 46 out of 49 *M. capixaba* specimens caught indoors were female, which could suggest an endophilic behaviour, although none of them were engorged. Future studies, with a larger number of specimens, are needed to assess the blood meals of *M. capixaba* females collected indoors and outdoors in these villages. The houses where *M. capixaba* were found indoors have openings, as did most of the houses in the studied indigenous villages. Moreover, some of the houses were surrounded by native vegetation, which may have favoured the encounter of *M. capixaba* in their interior. A study carried out in São Vicente Férrer (agreste region of Pernambuco) [30] reported a single female in the peridomicile and 24 males and 31 females in the forest environment. In Caruaru (agreste region of Pernambuco) [31] reported only three females of *M. capixaba* in the intradomicile. These findings suggest that sylvatic sand fly species (e.g. *M. capixaba*) may find a home inside human houses in the studied indigenous villages. Indeed, also sand flies incriminated as vectors of *Leishmania* spp. (i.e. *M. migonei*, *N. intermedia* and *L. longipalpis*) were consistently collected inside the investigated houses during this study. Moreover, 12.1% of *M. migonei* females caught indoors contained fresh blood in their abdomen, suggesting an endophilic behaviour. Overall, these findings may indicate a constant, close contact between sand fly vectors, domestic animals and humans, potentially increasing the risk of *Leishmania* spp. transmission. Several factors may drive the adaptation of sand flies to human dwellings, including deforestation, construction of houses close to forest fragments, poor housing conditions and presence of animal sheds in the backyards. All these factors were observed in the indigenous villages surveyed in this study.

Similar studies conducted in Pernambuco reported a species richness ranging between 4–25 species [6, 27, 30–32]. Until this study, 41 sand fly species were considered to be present in Pernambuco [29]. With the record of *M. trinidadensis*, this study increases the number of sand fly species of Pernambuco to 42, corresponding to approximately 4.3 species per 10,000 km^2. Pernambuco has a rich sand fly fauna as compared with other Brazilian states [29], such as Alagoas (3.2 per 10,000 km^2) and São Paulo (3.1 per 10,000 km^2). Incidentally, some authors have mentioned the presence of *M. trinidadensis* in Pernambuco [33], but provided no evidence or reference supporting this statement. Indeed, this species was not considered in subsequent sand fly species checklists of this state [13, 29].

The distribution of CL appears to be influenced by altitude. We observed a high species diversity and lower species dominance in the villages with altitude ~850 m (V1 and V2), where 62.5% of the CL cases reported from 2007 to 2017 were concentrated. A study conducted in south-eastern Brazil showed that the number of CL cases decreased progressively with altitude [34]; most cases occurred at 650–750 m and no case occurred at 850–950 m. This is in partial agreement with our results, since CL cases were detected in V3, which is located at an altitude of 965 m. It is worth noting that the highest number of the potential vectors *M. migonei* and *N. intermedia* were found exactly in V3, where only two CL cases were reported in ten years. This suggests that the risk of CL in this area may not be directly correlated with sand fly abundance.

The overall number of males was higher than females, as reported in different studies conducted in other regions of Brazil [35–38]. It is acknowledged that the sex ratio may be influenced by trapping methods, with light traps usually attracting more males than females [39]. However, the sex ratio varied widely according to species and environment (indoors *vs* outdoors, e.g. *M. villelai*), being close to unity in some (e.g. *M. trinidadensis*) and female-biased in others (e.g. *M. capixaba* and *E. evandroi*). This indicates that females of some species may be more phototropic than others, as emphasized elsewhere [39].

The highest sand fly population peaks occurred in May 2015 and March 2016, corresponding to the pre-rainy season in 2015 and following the first rains in February 2016, respectively. The number of sand flies collected was positively correlated with temperature. It means

that sand flies were more frequently trapped during hot months. It is worth noting that in the agreste region of Pernambuco the rains are unevenly distributed throughout the year, occurring mainly from February to July. This factor may be an important driver of the seasonality of sand flies in this region, similarly to what occurs in the semiarid region of Ceará state [40]. The decline in the sand fly collections during torrential rains (June and July) may also be attributed to inherent difficulties in collecting sand flies using light traps under the rain [6]. In other words, the lower trapping success during this period probably related to a reduced flying activity of sand flies during the raining nights rather than due to their absence. In a study conducted in Passira, another municipality located in the agreste region of Pernambuco, the authors reported that 82.4% of the *L. longipalpis* specimens were collected in months with relative humidity surpassing 75% [31]. In the present study, most *L. longipalpis* specimens (79.1%) were collected in months with a relative humidity less than 75%. These divergent results reinforce the hypothesis that *L. longipalpis* in north-eastern Brazil is less dependent on climate [31] or that the relationship between climate and *L. longipalpis* population may vary locally.

It is worth noting that 50% of the CL cases reported during the outbreak from 2012 to 2013 in Pesqueira were concentrated at V1. Interestingly, most *M. migonei* females (50.2%) collected indoors in this study were collected in V1. While this species displays a sylvatic behaviour in some Brazilian regions, it is recognized that in north-eastern Brazil this species is adapted to different environments, including forest fragments, animal sheds and human houses [41, 42].

In general, most human cases of CL diagnosed in rural and/or forested areas in north-eastern Brazil are males involved in occupational activities that increase their exposure to sand flies [6, 43–45]. In rural settings where children are frequently affected by CL, the transmission cycle of *L. braziliensis* is probably taking place in the indoor or immediate outdoor environments [46], where domestic dogs are also frequently exposed. In the present study, a relatively low (8.7%) overall level of exposure to *Leishmania* spp. infection was detected among dogs living in the studied indigenous villages and two nearby villages, as compared to other studies conducted in Pernambuco [47–50] or elsewhere in Brazil [51, 52]. While the overall level of exposure to *Leishmania* spp. infection in dogs was relatively low, data from notified CL cases and from our serological survey suggest that transmission is taking place in the peri-domestic and/or domestic environments. Indeed, these dogs are typically free roaming during the day, but stay around or inside the houses during the night. Furthermore, considering that remarkably anthropophilic vector species (e.g. *N. intermedia*) [10] were found indoors, the risk of exposure to *L. braziliensis* may be ever higher in humans as compared to dogs. This could partially explain the 2012–2013 outbreak in humans and the relatively low exposure to leishmanial parasites in dogs. Nevertheless, the level of exposure in dogs living in the indigenous villages where the 2012–2013 outbreak of human CL was detected was almost 2-fold higher than in the two nearby villages (11.0 *vs* 6.2% for serology and 2.6 *vs* 1.4% for real-time PCR).

While this was not our primary objective for screening dogs, the low exposure to leishmanial parasites in dogs suggests that these animals are not playing any role as reservoirs of *L. braziliensis* in the study area, which is in line with the current notion that dogs are mere accidental hosts of this parasite [14]. Indeed, previous studies conducted in other endemic foci in Pernambuco have indicated sylvatic and synanthropic rodents as the reservoirs of *L. braziliensis* [4, 53], a hypothesis also supported by an experimental study [54].

Conclusions

In conclusion, we confirm that proven sand fly vectors are present in the indoor and immediate outdoor environments in indigenous villages where CL is endemic. The adaptation of sand flies to the indoor environment may be related to the poor housing conditions observed in these villages and the proximity of houses to green areas (e.g. crop plantations and forest fragments).

Additional files

Additional file 1: Table S1. Number of sand flies collected indoors and outdoors in each village (V1–V3) in Pesqueira, Pernambuco, Brazil, from March 2015 to March 2016.

Additional file 2: Figure S1. Standardized index of species abundance (SISA) of sand fly species collected indoors and outdoors in Pesqueira, Pernambuco, Brazil, from March 2015 to March 2016.

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Availability of data and materials
The data supporting the conclusions of this article are included within the article and its additional files. Raw data can be shared with other researchers upon specific request.

Authors' contributions
FDT conceived the study. KGSS, LAF and PLC conducted the fieldwork. KGSS and LAF conducted the laboratory testing. KGSS, DEOM and FJS processed and identified the sand flies. FDT and KGSS conducted the literature review and wrote the manuscript. SPBF participated in data interpretation and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The Committee on Ethics in the Use of Animals CEUA (number: 56/2013) of the Aggeu Magalhães Institute (Fiocruz) approved the procedures used in this study. Dog owners signed a consent form before the inclusion of their animals in this study. Residents of the houses where sand fly collections were conducted authorized the installation of the traps in their houses.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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3.4 Artigo 4

O artigo intitulado “Fast multiplex real-time PCR assay for simultaneous detection of dog and human blood and *Leishmania* parasites in sand flies” foi aceito para publicação na revista Parasites and Vectors, o qual descreve o um ensaio de PCR multiplex em tempo real baseado em TaqMan, capaz de detectar simultaneamente quantidades mínimas de DNA *Leishmania* spp., de cão e humano, em flebotomíneos. O artigo atualmente se encontra em revisão.

Fast multiplex real-time PCR assay for simultaneous detection of dog and human blood and *Leishmania* parasites in sand flies

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Abstract

Background: The blood feeding behaviour of female sand flies may increase their likelihood of acquiring and transmitting *Leishmania* parasites. Studies on the host usage by these insects may thus improve our understanding of the *Leishmania* transmission risk in leishmaniases-endemic areas. Here, we developed a fast multiplex real-time PCR assay for simultaneous detection of dog and human blood and *Leishmania* parasites in sand flies.

Methods: Primers and TaqMan probes targeting the mitochondrially encoded cytochrome *c* oxidase I and cytochrome b genes of dog and human, respectively, were combined in a multiplex assay, which also includes primers and a TaqMan probe targeting the *Leishmania* minicircle kinetoplast DNA.

Results: The multiplex assay was 100% specific, with analytical sensitivities of 10^3 fg/reaction for dog and human and 1 fg for *Leishmania*. By testing field-collected engorged female sand flies (95 *Migonemyia migonei* and two *Nyssomyia intermedia*), 50 *M. migonei* were positive for one or two targets (positivity rates: 45.4% for human, 4.1% for dog and 12.4% for *Leishmania* parasites).

Conclusions: This multiplex real-time PCR assay represents a novel fast assay for detecting dog and human blood and *Leishmania* parasites in female sand flies and therefore a tool for assessing the risk of *Leishmania* transmission to these hosts in areas of active transmission.

Keywords: Phlebotomine sand flies, Blood meal, Brazil, Real time PCR.

Background

Sand flies (Psychodidae, Phlebotominae) are insects of paramount medical and veterinary significance, mainly due to their competence in transmitting pathogens to many animal species, including humans [1]. Plant-derived carbohydrates (e.g., nectar, honeydew and phloem sap) are part of the diet of both male and female sand flies, being an essential source of energy for various activities, including flight and reproduction [2]. However, adult females are also blood feeders and require vertebrate blood as a source of protein for oogenesis [3]. Thus, the feeding behaviour of female sand flies may influence their likelihood of acquiring and transmitting pathogens, including *Leishmania* parasites (Kinetoplastida: Trypanosomatidae), the causative agents of leishmaniases. To date, over 1,000 sand fly species have been identified globally, of which more than 50% are exclusively found in the Neotropics [4]. About 98 species of sand flies have been listed as proven or suspected vectors of *Leishmania* spp. [1].

Leishmaniases are among the top ten neglected tropical diseases causing high levels of morbidity and mortality in endemic areas, mainly in tropical and subtropical regions of the world [5]. Brazil, India, Bangladesh, Sudan, South Sudan and Ethiopia concentrate 90% of the global incidence of visceral leishmaniasis (VL), whereas

Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica and Peru concentrate ~75% of the global incidence of cutaneous leishmaniasis (CL) [5].

An important factor influencing the dynamics of *Leishmania* parasite transmission in endemic areas is the feeding behaviour of female sand flies. For example, to be considered a 'good vector' for zoonotic *Leishmania* parasites, females from a given sand fly species should feed frequently on the reservoir host(s) and on humans as well. Therefore, investigations about the blood meals of various species of sand flies are crucial towards a better assessment of the risk of *Leishmania* transmission in areas where leishmaniasis are endemic. Several methodologies to identify blood meal in sand flies have been used, including enzyme-linked immunosorbent assay (ELISA), mass spectrometry, precipitin test and polymerase chain reaction (PCR) [6-11]. More recently, quantitative real-time PCR [12,13] and PCR followed by amplicon sequencing [14] demonstrated promising results, with high-level sensitivity. Although all these methods generated important information about feeding behaviour of sand flies, they may present several drawbacks, such as low sensitivity and specificity (e.g., ELISA and precipitin test) and high cost (e.g., mass spectrometry and PCR followed by amplicon sequencing) [12,15,16].

In this context, we developed a fast multiplex real-time PCR assay for simultaneous detection of dog and human blood meals and *Leishmania* parasites in sand flies, with high analytical sensitivity and specificity, as well as relatively low cost.

Methods

Blood samples and *Leishmania* parasites

Venous blood samples (2 ml) were withdrawn from a dog and a human in EDTA tubes (Vacuette K3EDTA tube, Greiner Bio-One, Kremsmünster, Austria) and frozen at –20 °C until DNA extraction. Reference strains of *Leishmania infantum* (MHOM/BR/76/M4192) and *Leishmania braziliensis* (MHOM/BR/1975/M2903) were obtained from the Leishmaniasis Reference Service of the Aggeu Magalhães Institute (Fiocruz-PE). Both blood samples and *Leishmania* parasites were used for the preparation of standard curves (see below). Additionally, two non-engorged females of *Migonemyia migonei* obtained from a laboratory colony established in the Aggeu Magalhães Institute (Fiocruz-PE) were used as negative controls.

99 **Nucleic acid extraction**

100 Genomic DNA extraction from sand flies, blood samples and *Leishmania* parasites was
101 performed using DNeasy Blood & Tissue kit (Qiagen, GmbH, Germany), according to
102 the manufacturer's instructions. All samples were eluted in 100 µl of Buffer AE (10mM
103 Tris Cl, 0.5 mM EDTA, pH 9.0), properly labelled and frozen at –20 °C. The quantity
104 and purity (absorbance ratio at 260/280 nm and at 260/230 nm) of the extracted DNA
105 were accessed using a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham,
106 USA).
107
108 **Primer and probe design**

109 Primers and TaqMan hydrolysis probes (Table 1) targeting dog and human DNA were
110 developed based on the sequences of the mitochondrially encoded cytochrome *c* oxidase
111 I (*MT-CO1*) and cytochrome b (*CYTB*) genes, respectively, available from GenBank
112 (accession numbers: NC_002008.4 and NC_012920.1), using the Primer3 v.0.4.0
113 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Primers and probes were designed
114 following the instructions of the *TaqMan Multiplex Optimization User Guide* [17] for
115 optimum assay efficiency. In particular, primers should have a GC content of 40–60%
116 and generate amplicons of 50–150 base pairs. The melting temperature (T_m) should be
117 similar for all primers; the T_m of the probes be ~10°C higher than the T_m of the primers.
118 With that in mind, primers (18–20 bp) and probes (13–25 bp) were designed to have T_m
119 of 58–60°C and 68–70°C, respectively (Table 1).

120 To avoid nonspecific amplification, primers were submitted to BLAST/n of the
121 National Centre for Biotechnology Information (NCBI) to verify its specificity.
122 Furthermore, the formation of dimmers, hairpins, and T_m were assessed with the
123 OligoAnalyzer 3.1 software (https://eu.idtdna.com/calc/analyzer). The primers LEISH-1
124 and LEISH-2 and a TaqMan probe (Table 1) were used to detect a 120 bp fragment of
125 the *Leishmania* minicircle kinetoplast DNA (kDNA) [18].
126
127 **Optimization of singleplex real-time PCR assays**

128 Before optimizing the multiplex real-time PCR assay, singleplex real-time PCR assays
129 were optimized to specifically detect dog and human DNA. Additionally, a singleplex
130 real-time PCR assay targeting kDNA was performed as described elsewhere [19]. A
131 dilution matrix was made to determine optimal concentration of primers and probes
132 [17]. The reaction mixture contained 1.35 µl of each primer (final concentration of

900nM each), 0.3 µl of the probe (200 nM), 2.5 µl of water (DNAse and RNAse free), 7.5 µl of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, USA) and 2 µl of genomic DNA, in a final volume of 15 µl. Positive controls consisted DNA extracted from dog and human blood and from cultured promastigotes of *L. infantum*, whereas a master mix without DNA (no template control - NTC) and DNA from unfed female sand flies were used as negative controls. The real-time PCR thermal conditions were as follow: 20 s at 95°C and 35 cycles of 1 s at 95°C and 20 s at 60°C (estimated running time: 33.5 min). All singleplex real-time PCR were run on a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific, USA), with automatic baseline setting and automatic threshold. The reactions were performed in triplicate, with inconsistent or undetermined results between the replicates being regarded as negative.

Optimization of the multiplex real-time PCR assay

Sets of primers and probes targeting dog, human and kDNA were multiplexed by labelling each probe with a different dye (Table 1). The reaction mixture consisted of 0.675 µl of each primer (900nM), 0.15 µl of each probe (200 nM), 7.5 µl of TaqMan® Fast Advanced Master Mix (Applied Biosystems, Woodward, USA) and 3 µl of genomic DNA, in a final volume of 15 µl. Positive controls consisted of mixed DNA extracted from dog and human blood and cultured promastigotes of *L. infantum*. Negative controls and thermal conditions employed in the multiplex assay were the same used in the singleplex assays. All the three channels (reporter and quencher) for VIC-QSY, NED-NFQ/MGB and FAM-NFQ were selected. All multiplex real-time PCR assays were run on a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific, USA), with automatic baseline setting and automatic threshold. The reactions were performed in triplicate and repeated three times, with inconsistent or undetermined results between the replicates being regarded as negative.

Specificity, sensitivity, linearity and reproducibility

An *in silico* analysis of the specificity of the primers and probes was checked using program BLAST/n. The analytical specificity was assessed through cross-tests between the targets (dog, human, *L. braziliensis* and *L. infantum*) and unfed female sand flies. Standard curves were prepared using nine serial dilutions (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 fg per reaction) of genomic DNA from dog, human and *L. infantum* to

assess the analytical sensitivity (detection limit) of the assays. The analytical sensitivity was defined as the lowest amount of DNA detectable in a given assay. The cut-off point of an assay was defined as the threshold cycle (C_t) value corresponding to the detection limit [20].

The amplification efficiency (E) was calculated using the slope of the regression line in the standard curve through the equation: $E = 10^{(-1/\text{slope})} - 1$. The slope close to −3.33 was considered optimal. The correlation coefficient (R^2) value was automatically calculated using measure of the closeness of fit between the regression line and the individual C_t data points of the standard reactions [21]. The y-intercept value also automatically calculated and corresponds to the theoretical C_t value for a single copy of the target molecule. In intra-assays, triplicates were made in the same plate, whereas in inter-assay, triplicates were repeated in three independent assays performed in three different days within a week.

Assay of field-collected sand flies

A total of 97 engorged female sand flies collected in the context of a previous study [22] were tested individually by the newly developed multiplex real-time PCR. These females belonged to two species: *Migonemyia migonei* (n = 95) and *Nyssomyia intermedia* (n = 2). Details regarding sand fly collection, identification and processing are described elsewhere [22]. Part of these females were collected indoors (n = 23) and part outdoors (n = 74).

Data analysis

Real-time PCR results were analysed using QuantStudio Design & Analysis Software 1.3.1 (Thermo Fisher Scientific, USA). To assess intra- and inter-assay reproducibility, the percent coefficient of variation (%CV) was calculated for each set of triplicate reactions. The positivity rates for *Leishmania* parasites in female sand flies collected indoors and outdoors was compared using Fisher's exact test, considering P <0.05 as statistically significant. Statistical analysis and calculations were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA).

Results

Specificity, sensitivity and linearity of the singleplex and multiplex assays

The sets of primers and probes specific for dog *COX1*, human *MT-CYTB* and *Leishmania* kDNA detected only the expected target. Moreover, they did not produce any non-specific amplification in the cross-testing with non-target DNA, or with no template and negative controls. Likewise, there were no false positives due to cross-talk between dye signals from each assay. Thus, analytical specificity of the assays was considered to be 100%.

The analytical sensitivity of the singleplex real-time PCR assays for dog *COX1* and human *MT-CYTB* was 1000 fg, with C_t values of 34.2 ± 0.4 and 33.2 ± 0.1, respectively. Conversely, the analytical sensitivity for *Leishmania* kDNA was 1 fg (C_t 33.5 ± 0.1) (Table 2).

The linear regression analysis of standard curves confirmed linearity of the singleplex real-time PCR assays for dog *COX1* (R^2 = 0.999, *E* = 101.9, slope = −3.28, y-intercept = 44.1), human *MT-CYTB* (R^2 = 0.999, *E* = 108.9, slope = −3.12, y-intercept = 42.8) and *Leishmania* kDNA (R^2 = 0.991, *E* = 103.5, slope = −3.24, y-intercept = 35.2) (Fig. 1).

Similar results were found with the multiplex real-time PCR assay, being the detection limits for dog *COX1* and human *MT-CYTB* 1000 fg, with C_t values of 33.1 ± 1.0 and 30.6 ± 0.3, respectively. The detection limit for *Leishmania* kDNA was 1 fg (C_t value = 33.1 ± 0.3) (Table 2). Similarly, the linear regression analysis of standard curves confirmed linearity of the multiplex real-time PCR assay for dog *COX1* (R^2 = 0.996, *E* = 109.8, slope = −3.11, y-intercept = 42.5), human *MT-CYTB* (R^2 = 0.993, *E* = 116.0, slope = −2.99, y-intercept =39.8) and *Leishmania* kDNA (R^2 = 0.998, *E* = 109.9, slope = −3.10, y-intercept = 33.6) (Fig. 2).

Reproducibility of multiplex real-time PCR assay

The variability of the multiplex real-time PCR assay was assessed between and within runs based on standard curves. The coefficients of variation values of intra- and inter-assays were in the range of 0.16–3.34% (Table 3) and 0.92–7.44% (Table 4), respectively. Overall, the difference between C_t values intra- and inter-assay was ≤2, suggesting that the multiplex real-time PCR assay is reliable.

Evaluation of multiplex real-time PCR assay with field-collected sand flies

Fifty (51.6%) out of 97 engorged female sand flies tested by the multiplex real-time PCR assay were positive. All positive females belonged to the species *M. migonei*.

234 Forty (80.0%) females were positive for one target (i.e., human *MT-CYTB*, dog
235 *COX1* or *Leishmania* kDNA) and 10 (20.0%) for two targets. Among females that were
236 positive for one target (n = 40), 85.0% were positive for human *MT-CYTB*, 10.0% for
237 *Leishmania* kDNA and 5.0% for dog *COX1*. Among those positive for two targets (n =
238 10), 80.0% were simultaneously positive for human *MT-CYTB* and *Leishmania* kDNA
239 and 20.0% were simultaneously positive for human *MT-CYTB* and dog *COX1*.
240 Out of 12 *Leishmania*-positive females, eight were fed on humans and none
241 were fed on dogs. Four positive females were collected indoors and eight outdoors
242 (Fisher's exact test, P = 0.4704).
243

244 **Discussion**

245 In this study, we were interested in developing a tool that could generate information
246 about role of sand flies collected inside human houses and surrounding areas in the
247 transmission of *Leishmania* parasites to dogs and humans. Here, we developed a
248 TaqMan-based fast multiplex quantitative real-time PCR assay for the simultaneous
249 detection of dog and human blood meals and *Leishmania* parasites in female sand flies.
250 The addition of multiple primers and probes in a single reaction as well as changes in
251 the number of cycles and annealing temperature can affect the specificity, sensitivity
252 and efficiency of real-time PCR assays [23,24]. This is in fact one of the main obstacles
253 to overcome while developing a multiplex real-time PCR assay [17]. Although, many
254 singleplex assays have been successful in identifying blood meal and *Leishmania*
255 parasites in sand flies [12-15,25], none of them combined the detection of different host
256 and the parasite DNA in a one-step assay. The development of the multiplex real-time
257 PCR assay proposed by the present study resulted in a series of advantages compared to
258 other assays, such as the reduction in reagent consumption, labour time and the ability
259 to provide faster results (considering that regular real-time PCR assays take over 1 hour
260 to be done, e.g., ~90 min [12, 18], ~78 min [13]). In practice, this assay allows testing a
261 great number of sand flies for both dog and human blood meals and presence of
262 *Leishmania* spp. in a shorter period of time (~34 min), reducing overall costs.
263 Considering that female sand flies consume a small amount of blood (≤1 µl)
264 during blood feeding [26], one of the main technical challenges while developing a
265 molecular tool for detecting host blood and *Leishmania* parasites is the necessity to
266 detect and quantify a very limited amount of DNA [16]. In fact, the sensitivity of such

an assay depends directly on both the initial quantity of the target DNA in the sample and on the time span from blood ingestion, as there is a progressive degradation of the host DNA during blood digestion [12,16,27]. The multiplex real-time PCR assay developed in this study was shown to be specific and highly sensitive, without interference and competition between targets and dyes. Particularly, identical analytical sensitivities were obtained with singleplex and multiplex assays (i.e., 10^3 fg/reaction for dog *COX1* and human *MT-CYTB*, and 1 fg for *Leishmania* kDNA). These results are similar to those obtained with other assays for detecting blood meals of female sand flies, which reported a detection limit from 10^2 fg to 10^4 fg of host genomic DNA [6,12,27]. More recently, two SYBR Green-based real-time PCR assays were reported to have a detection limit of 26 fg for dog and 84 fg for human [13]. Despite the good analytical sensitivity of these assays, when non-target DNA samples were used some slight noise were reported in the melting curve analysis, though with a C_t value always higher than 30 [13]. In fact, non-specific signals are a known limiting factor of some SYBR Green-based real-time PCR assays [12,28], which may eventually obscure the interpretation of the results. The use of hydrolysis probes (e.g., TaqMan probes) may increase the specificity of real-time PCR assays as demonstrated elsewhere [29,30].

For *Leishmania* kDNA detection, the analytical sensitivity of our multiplex real-time PCR was similar to a singleplex assay using the same primers and probe [19], allowing the detection of less than a single parasite per sample. This high sensitivity may be partly attributed to the target used (i.e., kDNA), which is present in high number of copies (~10,000 copies of minicircle molecules), per parasite [31]. In fact, other real-time PCR assays using the same target gene reported very good analytical sensitivity as well [32,33].

Our multiplex real-time PCR assay was also successfully applied in field-collected samples. A total of 44 females of *M. migonei* (stored at −20°C for ~2 years) were positive for human blood. This sand fly species displays a remarkable degree of anthropophily, and it is a proven vector of *L. braziliensis* and a putative vector of *L. infantum* in Latin America [34,35]. Interestingly, eight out of 44 females fed on humans were also positive for *Leishmania* spp. *Leishmania*-positive sand flies were collected in human dwellings (four indoors and eight outdoors), where human cases of CL by *L. braziliensis* were previously recorded [22]. Altogether, these findings strongly suggest that *M. migonei* is a vector of *L. braziliensis* for humans in the indigenous villages, where sand flies were collected. While our multiplex real-time PCR assay was tested

with sand flies collected from a CL focus, it is also suitable for other epidemiological settings, namely VL endemic regions, considering that the primers and probe used also detected efficiently *L. infantum* kDNA [18,19].

The identification of humans as the most frequent host of *M. migonei* females in this study, also reinforces their high attractiveness for humans [34] as well as the hypothesis that this species may be adapted to feed indoors [22]. It has been shown that human CL patients that have been treated and clinically cured may harbour viable parasites in their scars [36] and it has been suggested that they could eventually act as a source of infection to sand flies [37]. Our multiplex real-time PCR assay may be a useful tool to assess the presence of *Leishmania* parasites and human blood in sand flies from other CL-endemic areas, ultimately to investigate their possible role in the transmission cycle of *L. braziliensis*.

The finding of eight *Leishmania*-positive females which previously fed on humans raises interesting questions regarding whether these females acquired the infection from a previous unknown host (e.g., small rodents) [38] or from humans themselves. Interestingly, four *Leishmania*-positive engorged female sand flies did not apparently feed on humans or dogs, further suggesting that they probably acquired the parasites from another host.

Conclusion

In conclusion, a novel TaqMan-based fast multiplex real-time PCR assay was developed, optimized and validated herein for simultaneous detection of dog and human blood meals and *Leishmania* parasites in female sand flies. This assay may represent a tool for assessing *Leishmania* parasite infection in female sand flies and for investigating whether and how often these females feed on dogs and humans, thereby allowing estimating the risk of infection in these hosts.

Abbreviations

VL: visceral leishmaniasis; CL: cutaneous leishmaniasis; PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; EDTA: ethylene diamine tetra acetic acid; DNA: deoxyribonucleic acid; kDNA: kinetoplast minicircle DNA; MT-CO1: mitochondrially encoded cytochrome c oxidase I; CYTB: cytochrome b; GC: Guanine/Cytosine; BLAST: Basic Local Alignment Search Tool; NCBI: National

Center for Biotechnology Information; NTC: no template control; Tm: melting temperature; Ct: threshold cycle; E: efficiency; %CV: percent coefficient of variation; SD: standard deviation.

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Declarations

Ethics approval and consent to participate

All methods were carried out in accordance with the recommendations of the Brazilian National Council of Animal experimentation (CONCEA) and Brazilian ethical guidelines for research involving humans (resolution: 466/2012). All experimental protocols were reviewed and approved by the Animal Ethics Committee (CEUA: 100/2016) and Research Ethics Committee (CEP: 56276916.0.0000.5190) of the Aggeu Magalhães Institute (Fiocruz-PE). Before blood collection, written informed consents were obtained from the human subject and from the dog owner, both of whom were adults.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the conclusions of this article are included within the article. Raw data can be shared with other researchers upon a specific request.

Competing interests

The authors declare that they have no competing interests.

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370
371 **Authors' contributions**
372 KGSS and FDT conceived and designed the experiments. KGSS conducted all
373 laboratory work. DEOM and LAF contributed to sample collection. MHSP, DO, KGSS
374 and FDT performed all data analysis. KGSS and FDT wrote the paper. All authors read
375 and approved the final manuscript.
376
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Legends to figures

Fig. 1 Amplification plots (**left**) and standard curves (**right**) of the singleplex real-time PCR assays, showing values of slope, correlation coefficient (R^2), efficiency (ε) and y-intercept (y) for each target (dog, human and *Leishmania* parasites). All DNA samples were tested in triplicate and curves below the threshold line are negative.

Fig. 2 Standard curve (**top**) and amplification plot (**bottom**) of the multiplex real-time PCR assay, showing values of slope, correlation coefficient (R^2), efficiency (ε) and y-intercept (y) for each target (dog, human and *Leishmania* DNA). All DNA samples were tested in triplicate.

533 **Tables**

534 **Table 1** Primers and TaqMan probes used in the singleplex and multiplex real-time PCR

535 assays.

Species	Target	Primers and probes	Sequence (5' – 3')	Product size (base pairs)	Reference
Canis familiaris	*MT-CO1* gene	KF/CF-F (forward)	GGGGCTTTGGAAACTGACTA	95	This study
		KF/CF-R (reverse)	TGGAGGAAGGAGTCAGAAGC		
		KF/CF-P (probe)	VIC-ATTGGTGCTCCGGACATGGCAT-QSY		
Homo sapiens	*CYTB* gene	KF/HS-F (forward)	CCACCCTCACACGATTCTTT	104	This study
		KF/HS-R (reverse)	GTTGTTTGATCCCGTTTCGT		
		KF/HS-P (probe)	NED-TGCAGCCCTAGCAACACTCCACC-NFQ-MGB		
Leishmania spp.	kDNA	LEISH-1 (forward)	AACTTTTCTGGTCCTCCGGGTAG	120	[18]
		LEISH-2 (reverse)	ACCCCCAGTTTCCCGCC		
		TaqMan-MGB (probe)	FAM-AAAAATGGGTGCAGAAAT-NFQ-MGB		

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548 **Table 2** Analytical sensitivity and corresponding threshold cycle (C_t) values from
549 singleplex and multiplex real-time PCR assays for each target.

DNA sample	Quantity (fg/reaction)	C_t value (mean ± SD)	
		Singleplex	Multiplex
Canis familiaris	10^8	17.92 ± 0.17	17.91 ± 0.15
	10^7	21.14 ± 0.06	20.38 ± 0.99
	10^6	24.43 ± 0.04	23.80 ± 0.04
	10^5	27.79 ± 0.10	27.09 ± 0.20
	10^4	31.09 ± 0.11	30.14 ± 0.19
	10^3	34.21 ± 0.40	33.14 ± 1.03
Homo sapiens	10^8	17.81 ± 0.05	16.11 ± 0.31
	10^7	20.84 ± 0.12	18.34 ± 0.14
	10^6	24.12 ± 0.04	21.73 ± 0.58
	10^5	27.43 ± 0.08	25.31 ± 0.23
	10^4	30.55 ± 0.24	27.90 ± 0.37
	10^3	33.20 ± 0.07	30.59 ± 0.28
Leishmania spp.	10^8	8.64 ± 0.05	9.18 ± 0.31
	10^7	12.38 ± 0.10	11.43 ± 0.14
	10^6	15.51 ± 0.12	14.61 ± 0.06
	10^5	19.11 ± 0.04	17.88 ± 0.16
	10^4	22.63 ± 0.07	21.45 ± 0.17
	10^3	26.20 ± 0.02	24.44 ± 0.23
	10^2	29.77 ± 0.13	27.46 ± 0.05
	10^1	32.33 ± 0.23	30.88 ± 0.25
	10^0	33.49 ± 0.09	33.11 ± 0.25

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562 **Table 3** Intra-assay reproducibility of the multiplex real-time PCR assay.

DNA sample	Quantity (fg/reaction)	C_t value			Mean ± SD	%CV
		R1	R2	R3		
Canis familiaris	10^8	17.27	17.27	17.01	17.18 ± 0.15	0.87
	10^7	19.23	19.17	19.30	19.24 ± 0.06	0.33
	10^6	22.98	22.22	21.73	22.31 ± 0.63	2.82
	10^5	25.06	25.44	26.14	25.55 ± 0.55	2.15
	10^4	28.94	29.02	28.74	28.90 ± 0.15	0.51
	10^3	33.61	32.99	34.48	33.69 ± 0.75	2.22
Homo sapiens	10^8	16.67	16.99	16.87	16.84 ± 0.16	0.96
	10^7	18.76	18.74	18.87	18.79 ± 0.07	0.39
	10^6	22.50	22.35	22.74	22.53 ± 0.20	0.88
	10^5	25.87	25.57	25.72	25.72 ± 0.15	0.58
	10^4	28.48	28.52	27.83	28.27 ± 0.38	1.36
	10^3	30.94	33.24	33.13	32.44 ± 1.30	4.01
Leishmania spp.	10^8	5.96	5.92	6.06	5.98 ± 0.07	1.23
	10^7	8.58	8.87	9.16	8.87 ± 0.29	3.28
	10^6	12.54	12.22	12.56	12.44 ± 0.19	1.53
	10^5	15.81	15.58	16.32	15.91 ± 0.38	2.39
	10^4	19.31	19.43	19.78	19.51 ± 0.25	1.26
	10^3	23.16	23.06	22.72	22.98 ± 0.23	1.02
	10^2	26.66	26.21	26.78	26.55 ± 0.30	1.13
	10^1	29.82	29.91	29.83	29.85 ± 0.05	0.16
	10^0	32.03	32.10	32.52	32.22 ± 0.26	0.81

563 Ct: cycle threshold; R: replicate; SD: standard deviation; %CV: percent coefficient of
564 variation.
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575 **Table 4** Inter-assay reproducibility of the multiplex real-time PCR assay. In each day,
576 DNA samples were tested in triplicate (mean values reported).

DNA sample	Quantity (fg/reaction)	C_t value (mean)			Mean ± SD	%CV
		D1	D2	D3		
Canis familiaris	10^8	17.18	17.61	17.29	17.36 ± 0.23	1.30
	10^7	19.24	19.80	19.38	19.47 ± 0.29	1.51
	10^6	22.31	23.30	22.80	22.8 ± 0.50	2.17
	10^5	25.55	26.62	26.18	26.12 ± 0.54	2.07
	10^4	28.90	29.95	29.57	29.47 ± 0.53	1.80
	10^3	33.69	34.42	33.54	33.88 ± 0.47	1.39
Homo sapiens	10^8	16.84	15.51	16.43	16.26 ± 0.68	4.19
	10^7	18.79	17.57	18.18	18.18 ± 0.61	3.35
	10^6	22.53	21.30	21.86	21.90 ± 0.62	2.82
	10^5	25.72	24.95	25.33	25.33 ± 0.39	1.53
	10^4	28.27	27.65	27.78	27.90 ± 0.33	1.18
	10^3	32.44	32.21	31.78	32.14 ± 0.34	1.04
Leishmania spp.	10^8	5.98	6.76	6.86	6.53 ± 0.48	7.37
	10^7	8.87	9.95	10.24	9.68 ± 0.72	7.44
	10^6	12.44	13.48	13.77	13.23 ± 0.70	5.28
	10^5	15.91	16.91	17.47	16.76 ± 0.79	4.73
	10^4	19.51	20.50	20.87	20.29 ± 0.70	3.47
	10^3	22.98	24.02	24.84	23.95 ± 0.93	3.89
	10^2	26.55	27.43	25.47	26.48 ± 0.98	3.71
	10^1	29.85	30.40	30.33	30.20 ± 0.30	0.99
	10^0	32.22	32.37	32.79	32.46 ± 0.30	0.92

577 C_t: cycle threshold; D: day; SD: standard deviation; %CV: percent coefficient of
578 variation.
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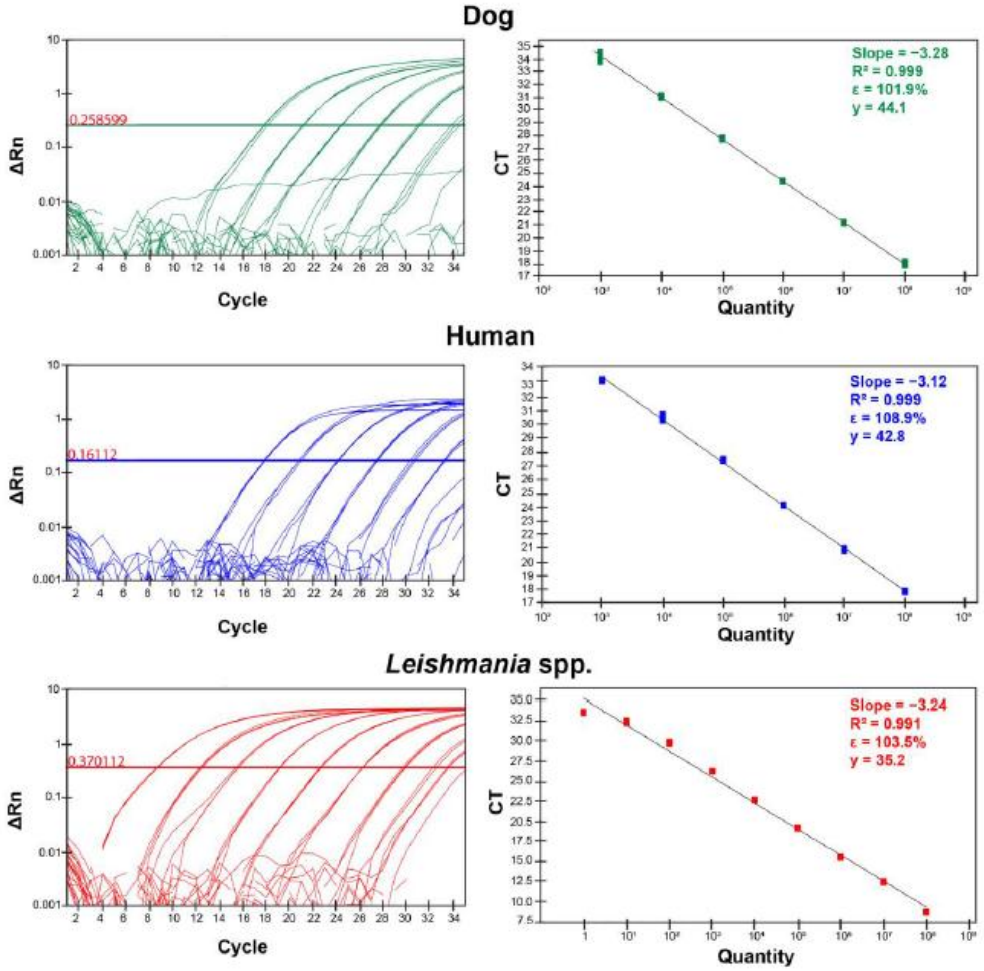

Fig 1. Amplification plots (**left**) and standard curves (**right**) of the singleplex real-time PCR assays, showing values of slope, correlation coefficient (R^2), efficiency (ε) and y-intercept (y) for each target (dog, human and *Leishmania* parasites). All DNA samples were tested in triplicate and curves below the threshold line are negative.

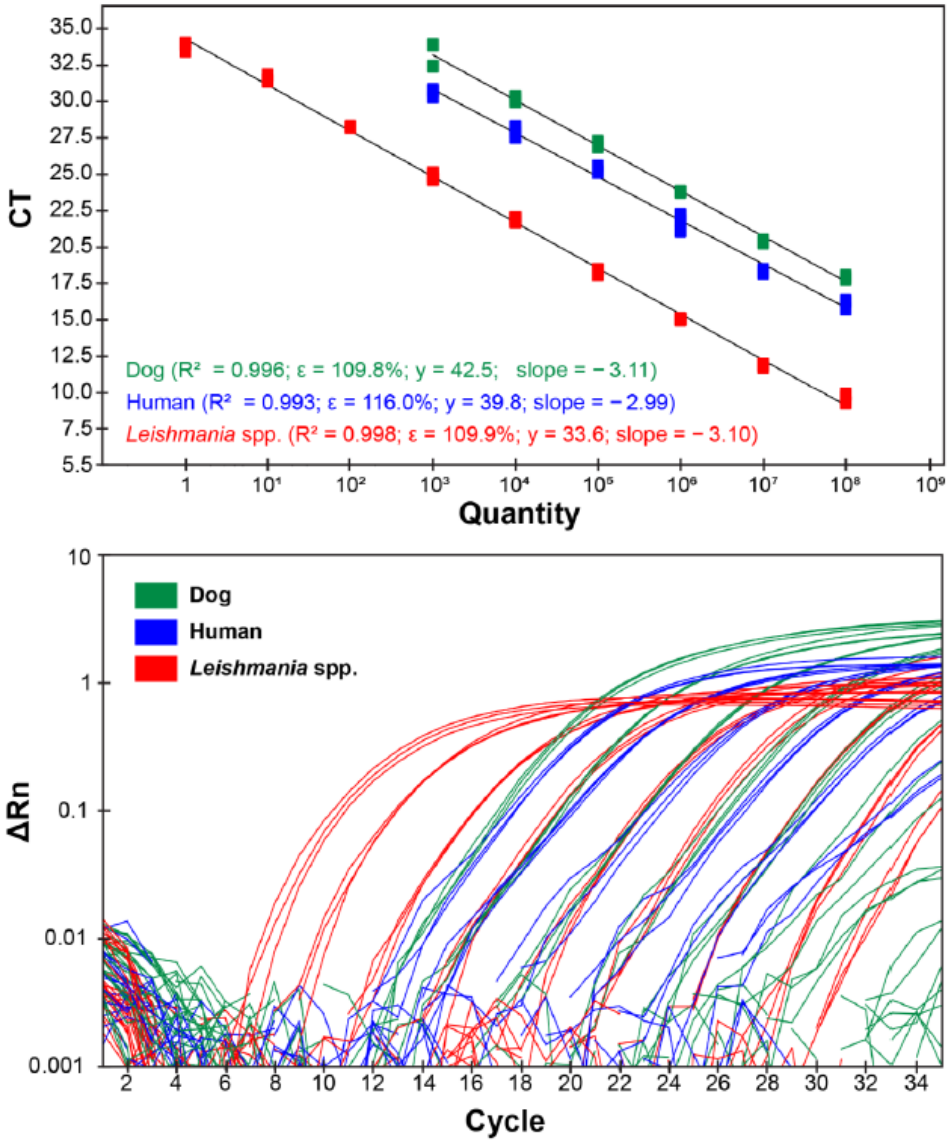

Fig 2. Standard curve (**top**) and amplification plot (**bottom**) of the multiplex real-time PCR assay, showing values of slope, correlation coefficient (R^2), efficiency (ε) and y-intercept (y) for each target (dog, human and *Leishmania* DNA). All DNA samples were tested in triplicate.

4 CONCLUSÕES

O novo ensaio de PCR multiplex em tempo real baseado na tecnologia TaqMan permitiu a detecção simultânea de pequenas quantidades de DNA de cão, humano e *Leishmania* spp. em fêmeas de flebotomíneos de campo. A PCR multiplex em tempo real foi capaz de detectar e quantificar o DNA dos hospedeiros em fêmeas de flebotomíneos com alta sensibilidade analítica e 100% especificidade. Esse ensaio constitue uma ferramenta para rastreios em grande escala para avaliar a infecção por *Leishmania* spp. em fêmeas de flebotomíneos e também para investigar se e com que frequência essas fêmeas se alimentam de cães e humanos. Como perspectiva, será de grande valia a realização de novos estudos que visem a aplicação desta ferramenta para avaliar o risco de transmissão de *Leishmania* spp. para cães e humanos em áreas de transmissão ativa.

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APÊNDICE A - TCLE

TERMO DE CONSENTIMENTO LIVRE E ESCLARESCIDO

Projeto: PCR multiplex em tempo real para detecção de fontes alimentares e de leishmania spp. em flebotomíneos.

Eu, _____, RG _____, proprietário do animal _____, espécie _____, residente à rua/Av. _____, bairro_____, cidade/estado _____, CEP:_____ aceito participar desse estudo, cujo objetivo é desenvolver um método de identificação de fonte alimentar de flebotomíneo ("mosquito palha"). Fui informado que meu animal será submetido à coleta de sangue para os testes em laboratório, sem que haja nenhum dano à saúde do mesmo. A coleta de sangue será realizada com uso de agulha e seringa e, o animal poderá sentir um desconforto no momento em que a agulha for introduzida na pele, sendo retirado de 3 mL de sangue, o equivalente a 1 colher de chá; este processo também poderá causar uma mancha roxa no local que, desaparecerá com o tempo de, aproximadamente, sete dias. Durante a assinatura deste termo fui informado que as amostras coletadas serão incorporadas ao Laboratório de Imunoparasitologia do Departamento de Imunologia do Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, podendo ser utilizados em pesquisas posteriores; e que tenho plena liberdade de recusar ou retirar o consentimento sem sofrer nenhum tipo de penalização ou pressão por tal. Estou ciente que não haverá incentivo financeiro para participação nesta pesquisa, que este documento é emitido em duas vias, uma ficará comigo e outra com a equipe de pesquisa, tenho garantida de que não haverá divulgação de minha identidade ou de meu animal nos produtos gerados por este projeto.

Contatos: **Dr. Filipe Dantas Torres, CPqAM/FIOCRUZ – Tel. (81) 2101-2413**

_____ _____

Sujeito da pesquisa Coordenador do projeto

Recife, ____/____/____

ANEXO A – CEUA

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **"PCR MULTIPLEX EM TEMPO REAL PARA A IDENTIFICAÇÃO DO REPASTO SANGUÍNEO E DETECÇÃO DE LEISHMANIA SPP. EM FLEBOTOMÍNEOS"** protocolado sob nº **100/2016** pelo (a) pesquisador (a) Dr Filipe Dantas Torres está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz (CEUA/CPqAM) em 07/10/2016. Na presente versão, este projeto está licenciado e tem validade até 07 de outubro de 2019.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Cão (*Canis familiaris*)	01 (macho ou fêmea)
TOTAL	**01**

We certify that project **"PCR MULTIPLEX EM TEMPO REAL PARA A IDENTIFICAÇÃO DO REPASTO SANGUÍNEO E DETECÇÃO DE LEISHMANIA SPP. EM FLEBOTOMÍNEOS"** (CEUA Protocol nº 100/2016) coordinated by **FILIPE DANTAS TORRES** is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz on october, 07, 2016. In present verson this project is licensed and valid until october 2019.

Recife (PE, BRAZIL) October, 07, 2016

Drª Sheilla Andrade de Oliveira
Coordenadora CEUA/CPqAM

Drª Sheilla Andrade de Oliveira
Coordenadora da Comissão de Ética no Uso de Animais – CEUA
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CPqAM/Fiocruz

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Recife - PE - CEP: 50.670-420
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www.cpqam.fiocruz.br

ANEXO B – CEP

CEP
Comitê de Ética em Pesquisa

Título do Projeto: "PCR multiplex em tempo real para a identificação do repasto sanguíneo e detecção de Leishmania spp. em flebotomíneos".

Pesquisador responsável: Filipe Dantas Torres

Instituição onde será realizado o projeto: CPqAM/Fiocruz

Data de apresentação ao CEP: 20/05/2016

Registro no CAAE: 56276916.0.0000.5190

Número do Parecer PlatBr: 1.951.868

PARECER

O Comitê avaliou e considera que os procedimentos metodológicos do Projeto em questão estão condizentes com a conduta ética que deve nortear pesquisas envolvendo seres humanos, de acordo com o Código de Ética, Resolução CNS 466/12, e complementares.

O projeto está aprovado para ser realizado em sua última formatação apresentada ao CEP.

O CEP/CPqAM reforça a necessidade de entrega de relatórios parcial e final, em comprimento a resolução 466/12, capítulo XI, artigo 2d.

Recife, 13 de março de 2017.

Janaina Campos de Miranda
Coordenadora do CEP/CPqAM/FIOCRUZ-PE

Janaina Campos de Miranda
Pesquisadora em Saúde Pública
Coordenadora
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