A Novel Molecular Test to Diagnose Canine Visceral Leishmaniasis at the Point of Care

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Abstract. Dogs are the principal reservoir hosts of zoonotic visceral leishmaniasis (VL) but current serological methods are not sensitive enough to detect all subclinically infected animals, which is crucial to VL control programs. Polymerase chain reaction (PCR) methods have greater sensitivity but require expensive equipment and trained personnel, impairing its implementation in endemic areas. We developed a diagnostic test that uses isothermal recombinase polymerase amplification (RPA) to detect *Leishmania infantum*. This method was coupled with lateral flow (LF) reading with the naked eye to be adapted as a point-of-care test. The *L. infantum* RPA-LF had an analytical sensitivity similar to real-time-PCR, detecting DNA of 0.1 parasites spiked in dog blood, which was equivalent to 40 parasites/mL. There was no cross amplification with dog or human DNA or with *Leishmania braziliensis*, *Leishmania amazonensis*, or *Trypanosoma cruzi*. The test also amplified *Leishmania donovani* strains ($N = 7$). In a group of clinically normal dogs ($N = 30$), RPA-LF detected more subclinical infections than rK39 strip test, a standard serological method (50% versus 13.3% positivity, respectively; $P = 0.005$). Also, RPA-LF detected *L. infantum* in noninvasive mucosal samples of dogs with a sensitivity comparable to blood samples. This novel molecular test may have a positive impact in leishmaniasis control programs.

INTRODUCTION

Visceral leishmaniasis (VL), which is caused by *Leishmania infantum* (both in the New and Old World) or *Leishmania donovani* (only in the Old World) accounts for 200–400,000 new cases each year. An undetermined proportion of people are subclinically infected and millions of people are at risk.^{1,2} VL is one of the group of “Neglected Tropical Diseases” identified by the World Health Organization as requiring major international efforts to reduce its negative impact on humankind. In the Americas, VL is a zoonotic disease transmitted between dogs and humans by sand flies, principally *Lutzomyia longipalpis*. This sand fly species has efficiently adapted to urban environments by feeding on domestic animals. Therefore, urbanization of the transmission cycle has positioned dogs as the principal reservoir hosts.³ Infected dogs develop progressive disease that is associated with increased infectivity to sand fly vectors.⁴ However, asymptomatic infectious dogs are difficult to detect and thus represent a latent risk of VL infection to the humans in endemic areas. Consequently, the identification of infected dogs using sensitive diagnostic methods and subsequent removal from the endemic area could significantly contribute to VL control.⁵

Humans are considered to be both victims and reservoir hosts of *L. donovani* and, to a lesser extent *L. infantum*.^{6,7} Thus, early diagnosis and treatment of patients may constitute an efficacious strategy to decrease morbidity and interrupt transmission.^{2,8} A major constraint in diagnosing VL is that the disease occurs in remote or resource-poor areas where basic health infrastructure and/or access to care is limited. In addition, current parasitological diagnosis requires invasive,

potentially dangerous procedures (bone marrow or spleen aspirates), which must be performed in hospital settings not accessible to the majority of patients. A variety of serological methods (ELISA, DAT, IFAT, K39, K26) have been used to identify infected dogs, but a significant proportion of seronegative dogs could still be infected and represent a risk to humans.⁹ Standard polymerase chain reaction (PCR) and real-time PCR have demonstrated greater sensitivity to detect *Leishmania* infections compared with serology.^{10,11} However, the need for sophisticated, expensive equipment, infrastructure, and trained personnel makes this approach impossible in resource-limited endemic areas.¹² The availability of inexpensive, sensitive, and field-applicable diagnostic tests is essential to improve the effectiveness of current VL control programs.

Isothermal amplification of DNA by recombinase polymerase amplification (RPA) is a novel strategy to diagnose infectious diseases that can be adapted as a point-of-care (POC) diagnostic test.¹³ In RPA, a recombinase and its cofactor form a nucleoprotein complex with oligonucleotide primers and scan for homologous sequences in a DNA template. Recognition of a specific homologous sequence leads to the initiation of strand invasion, and the opposing oligonucleotides are then extended by isothermal strand displacement amplification via *Sau* polymerase (*Staphylococcus aureus*).¹⁴ This results in amplification of the double-stranded DNA without the need for thermal or chemical melting of the DNA. As opposed to loop-mediated amplification (LAMP) or other isothermal amplification methods, the RPA is less complex and can be adapted easily to lateral flow (LF) detection of the amplification product. Recent publications have confirmed the applicability of this technology to detect viral, bacterial, and parasitic infections.^{15–19} We developed the RPA as a potential point-of-care diagnostic test for *L. infantum* infection because of its simplicity and adaptability to LF detection (instrument-free, one tube reaction, dry reagents, and naked eye reading). In this study, we show for the first time an RPA-based assay to detect *L. infantum* under isothermal amplification conditions with potential application in VL control programs.

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MATERIALS AND METHODS

Collection of dog samples. Uninfected blood was obtained from dogs living in a non-endemic area of the United States. Samples from infected dogs were collected at the Municipal Institute of Animal Health (IMUSA) in the city of Posadas, where the first endemic focus of human VL in Argentina was described.²⁰ IMUSA periodically collect stray dogs as part of the ongoing VL control program of the municipality; this procedure follows the Argentine Animal Welfare guidelines and is consistent with the recommendations of the International Companion Animal Management (ICAM Coalition; <http://icam-coalition.org/>). Dogs are euthanized if tested positive for VL or given for adoption if shown to be uninfected. Blood samples were obtained from 30 clinically normal mongrel dogs (21 female, 9 male) and nine symptomatic dogs (five female, four male), with ages ranging from 0.5 to 10 years (average = 4 years). These samples were used principally to determine the capacity of RPA-LF to detect subclinically infected dogs as compared with rK39 serological test. In addition, blood was collected from *L. infantum*-infected dogs that were positive for *Leishmania* amastigotes by microscopic examination of Giemsa-stained smears of lymph node aspirate. A small number of mucosal samples were also collected from dogs ($N = 7$) with symptoms compatible with VL. This was done by gently scraping 2–3 cm of the gums with a disposable miniature plastic spoon and resuspending the scraping in 0.1 mL of sterile phosphate buffered saline (PBS). One hundred microliters of laboratory (uninfected dog blood, parasite-spiked dog blood) or clinical samples (infected dog blood or mucosal scrapings in PBS), was absorbed in Whatman FTA[®] filter paper (Sigma-Aldrich, St Louis, MO) and dried at room temperature. The filter paper was stored at room temperature until use.

Parasites and DNA extraction. *Leishmania infantum* (MCAN/COL/98/CATIRE), *Leishmania braziliensis* (MHOM/CO/85/1132), *Leishmania amazonensis* (MRAT/BA/74/LV78), *Leishmania major* (MHOM/IL/81/Friedlin), and *Trypanosoma cruzi* were grown using M199 culture medium supplemented with 20% fetal calf serum (FCS). *Leishmania donovani* reference strain (MHOM/IN/80/DD8) and six other strains of this species preserved in our cryobank were thawed for subsequent DNA purification. DNA from *Giardia intestinalis* was purchased from American Type Culture Collection (ATCC; 30888[™] Portland-1 Isolation), human DNA was extracted from HCT-8 cells (ATCC CCL-244), and dog DNA was obtained from blood of healthy dogs from a non-endemic area. To estimate the analytical sensitivity of quantitative real-time PCR (qPCR) and RPA-LF, DNA was extracted from 1×10^5 cultured promastigotes spiked in dog blood and then serially diluted to the equivalent of 0.01 parasites per reaction. DNA was isolated from blood or tissue samples using the QIAGEN DNeasy blood and tissue extraction kit (Qiagen, Valencia, CA) following the instructions of the vendor. In brief, DNA was isolated from Whatman FTA paper as follows: two 6-mm disposable skin biopsy punches were used to cut the absorbed/dried samples and the filter disc was resuspended in 200 μ L water and placed in a heat block at 96°C for 2 minutes. Then, 20 μ L proteinase K solution and 200 μ L lysis buffer were added to the samples and incubated at 56°C for 10 minutes. After removal of the filter papers 200 μ L of 100% ethanol was added to the lysis buffer. Samples were placed in columns, washed following manufacturer's recommendations and eluted in 25 (blood) or

50 μ L (mucosa) water. DNA samples were stored at –20°C until used in either downstream applications, for example, SYBR green real-time PCR or RPA/RPA-LF. The DNA extraction of dogs from the endemic area was carried out by boiling 0.1 mL blood absorbed on FTA filter paper at 96°C for 2 minutes in 0.5 mL DNase-free water. Then, 2.5 μ L were taken for subsequent RPA amplification (see RPA amplification and LF detection below).

Primers and probe design. We selected the kinetoplast DNA (kDNA) minicircles as targets for designing RPA primers because of the high copy number of this extrachromosomal DNA.²¹ To design specific primers, we used the ClustalW program (GNU Lesser General Public License, www.clustal.org) to align different *Leishmania* sequences reported in GenBank for minicircle kDNA. The set of primers (each 30 nt [nucleotides]) were designed from a target region with moderate variability, obtaining an RPA amplicon of 182 bp in agarose gels. The forward (FW) and reverse (RV) primers (5'-3') were FW-CCATAGCGCTTTAGAATAGTTCTGACTCCGA; RV-biotin-ATCGGTATAGATATTACTACTACACACAGC.

RPA amplification and LF detection. For all RPA reactions we used the commercial TwistAmp[®] RPA kit (TwistDx Ltd., Cambridge, United Kingdom). The RPA was run as recommended by the vendor with the exception that we determined the optimal conditions for the specific *L. infantum* test using temperatures between 37°C and 42°C at variable reaction times ranging between 15 and 70 minutes. We designed the specific 45-nt labeled probe to target a region inside the forward and reverse primer sequences (5'-FAM-ATAACTGACATTACTCGTACACTATAA-THF-TATTATGTTTAATATAT-3'). The 30-nt reverse primer was biotinylated at the 5' end. The RPA amplicon (*L. infantum* DNA) was detected using LF oligochromatographic dipstick paper as recommended by the vendor (Milenia hybridtec, Gießen, Germany). In brief, the dipstick was immersed in a 1:20 dilution of RPA reaction and dipstick assay buffer, and the result read at room temperature in 2–5 minutes. The amplicon containing dual-labeled ends (6-carboxyfluorescein [FAM] and biotin) moves through the strip by capillarity and is recognized by an anti-FAM gold-labeled specific antibody. Then, as the amplicon-gold complex migrates it is immobilized by an anti-biotin-specific antibody generating a positive band at the center of the strip; the excess of unbound gold flow over the test band and is trapped by a species-specific antibody at the end of the strip (control band).

Quantitative PCR. The RPA-LF sensitivity was compared with SYBRgreen[®] real-time PCR (Qiagen) using the primers described by Pita-Pereira and others.²²

Serological testing. The rK39 serological test (Kalazar Detect[™]; InBios Seattle, WA), which is an antibody-mediated immunochromatographic test used in several endemic countries to diagnose VL in humans and dogs,^{23,24} was performed according to the manufacturer's instructions using sera from dogs inhabiting the endemic area in Argentina.

Statistical analysis. The sensitivity of RPA-LF versus qPCR was evaluated using kappa statistic (2015; GraphPad Inc., La Jolla, CA), which is the choice for estimating the agreement between tests with a dichotomous scale of measurement (positive or negative). Kappa statistic corrects for the agreement expected by chance and ranges from 0 to 1 ($\kappa = 0$ represents no agreement whereas $\kappa = 0.8$ –1.0 represents excellent agreement). The correlation of qPCR results between blood and

mucosal tissues was evaluated using Pearson's test, and the sensitivity of RPA-LF versus rK39 to identify subclinically infected dogs was determined using Fisher's exact test (GraphPad InStat 3.0).

RESULTS

Development of the *L. infantum* RPA-LF test. We targeted the high copy number kDNA by designing multiple primer sets, typically 30–35 nt long, specific for *L. infantum* (but not other *Leishmania*) sequences found in GenBank. From these we identified one primer set that provided high analytical sensitivity and specificity. After RPA amplification for 40 minutes at 42°C we obtained the expected 182-bp amplicon from *L. infantum* strains, but no amplicon from *L. braziliensis*, *L. amazonensis*, or *T. cruzi*, which are other kinetoplastid pathogens known to circulate in VL-endemic areas in the Americas (Figure 1). Also, we found no cross reaction with *L. major*, human or dog DNA. The *L. infantum* RPA-LF, which included a species-specific probe, had an analytical sensitivity similar to qPCR. Both detected DNA from 0.1 parasites spiked in dog blood, which was equivalent to 40 parasites/mL (Figure 2).

Seven different strains of *L. donovani* were also amplified when the RPA-LF test was carried out under the same time and temperature conditions established for *L. infantum*, dem-

onstrating the potential utility of this test for diagnosing VL in the Old World (data not shown).

Sensitivity and specificity of the *L. infantum* RPA-LF test for detection of infected dogs. RPA-LF and qPCR were run in parallel to detect *L. infantum* in dried blood spots from a small group of dogs ($N = 6$) with confirmed parasite-positive lymph node aspirates and negative controls from a non-endemic region ($N = 6$). We found 100% agreement between qPCR and RPA-LF results. All known negative dogs had no DNA detectable by qPCR (Ct value of 37.6 ± 2.5 ; no template control C_t value of 38) or RPA-LF (no visible band), whereas all the known positive dogs had DNA detectable in the blood by qPCR (mean Ct value 29.1 ± 4.5) and RPA-LF (clearly visible band; Figure 3).

Detection of *L. infantum* by RPA-LF in noninvasive mucosal samples. In another small group of symptomatic dogs (suspected of suffering VL) from the same endemic area, we evaluated the potential of RPA-LF to identify *L. infantum* infections in mucosal samples. Mucosal scrapings were dried on filter paper, stored at room temperature, and DNA was extracted and qPCR and RPA-LF were run in parallel. We found a 100% agreement ($\kappa = 1.0$) between positive and negative samples (Figure 4). No additional blood samples were available to run RPA-LF after qPCR was carried out in this group of dogs. However, the correlation between qPCR Ct values of blood and mucosa suggested that mucosal samples were as good as blood samples (from the same dogs) for detection of *L. infantum* DNA (Pearson $r = 0.773$, $P = 0.0406$). One of the symptomatic dogs (no. 5), which tested negative by RPA-LF in mucosal sample, also had high Ct value by qPCR suggesting that it was not infected with *L. infantum* (Figure 4).

Identification of subclinically infected dogs by RPA-LF and rK39. In collaboration with the health authorities of Posadas, the city with the highest recorded prevalence of canine and human VL in Argentina, we compared the sensitivity of RPA-LF and rK39 serological test in 30 clinically normal dogs and nine symptomatic dogs. Blood samples were absorbed in Whatman FTA paper to preserve DNA, and plasma was separated for anti-rK39 antibodies. We found that RPA-LF detected significantly more subclinically infected dogs (no signs or symptoms of VL) than rK39, the standard diagnostic method currently used in the field (50% versus 13.3% positivity, respectively; Fisher's exact test, $P = 0.005$). The strength of agreement between the tests was "poor" for asymptomatic dogs ($\kappa = -0.218$) and "fair" for symptomatic dogs ($\kappa = 0.250$) as determined by kappa analysis (Supplemental Table 1).

DISCUSSION

We developed a novel, field-applicable test to detect *L. infantum* infections in dogs by isothermal amplification of kDNA coupled with LF detection. This test also detected *L. donovani* so may be useful to diagnose VL patients or subclinically infected individuals at risk of developing overt disease in either the Old or New World. The RPA-LF test had an analytical sensitivity similar to that of our qPCR. It was able to detect as few as 0.1 parasite in the reaction (40 parasites/mL blood), which is in the range of what is considered a low level of parasitemia for patients with active VL.²⁵ Similar to qPCR, the RPA-LF test detected parasite

FIGURE 1. Specificity of RPA and RPA-LF (recombinase polymerase amplification–lateral flow) for detecting *Leishmania infantum*. DNA amplification was carried out at 42°C during 40 minutes and the products were run in a 2% agarose gel. A 182 pb band corresponding to *L. infantum* can be clearly observed (upper panel). The amplification products were then applied to LF strips and read at 5 minutes obtaining similar specificity results (lower panel). 1) *Leishmania braziliensis*, 2) *Leishmania amazonensis*, 3) *Leishmania major*, 4) *L. infantum*, 5) *Trypanosoma cruzi*, 6) human DNA, 7) dog DNA, 8) *Giardia lamblia*, and 9) *Cryptosporidium parvum*. This is a representative figure of four similar assays.

FIGURE 2. Sensitivity of recombinase polymerase amplification–lateral flow (RPA-LF) to detect *Leishmania infantum* compared with real-time polymerase chain reaction (PCR) used as gold standard. Tenfold serial dilutions of *L. infantum* promastigotes in dog blood were extracted using Qiagen® DNeasy blood and tissue kit and detected by real-time quantitative PCR (SYBRgreen) or RPA-LF. Parasite dilutions: 1 = 10^5 , 2 = 10^4 , 3 = 10^3 , 4 = 10^2 , 5 = 10, 6 = 1, and 7 = 0.1 parasites and BI = uninfected dog blood. The top band is the control band; the lower band is the test band. This is a representative figure of two similar assays.

DNA in blood of all dogs found to be infected by lymph node aspirate, and in none of the dogs from a non-endemic area. We also found that RPA-LF detected *L. infantum* DNA from noninvasive mucosal samples of infected dogs matching the qPCR results. The RPA-LF and qPCR data suggested that mucosal samples were similar to blood samples (from the same dogs) for detection of *L. infantum* DNA, in agreement with other molecular studies.^{26,27} The RPA-LF did not detect DNA from other *Leishmania* spp. suggesting that it could distinguish between infection caused by *L. (Leishmania) infantum* and *Leishmania (Viannia)* spp. since in some regions of South and Central America these species can produce cutaneous lesions.²⁸ A future larger study of dogs with different clinical presentations and parasite burdens should confirm the usefulness of this approach. Preliminary work with blood samples spiked with serial parasite dilutions absorbed in filter paper showed that DNA extraction using the boiling method or portable DNA extractor (QuickGene Mini80, Autogen, Holliston, MA) yielded comparable results, indicating that this

method should be incorporated to the field evaluation of RPA-LF (Supplemental Figure 1).

The evaluation of dogs from a VL-endemic area in Argentina, although limited in sample size, confirmed the low sensitivity of the rK39 serological method^{4,29} and higher sensitivity of RPA-LF to identify dogs during the asymptomatic phase of infection. The poor strength of agreement between the tests indicated differences in sensitivity probably due to infections with humoral immune responses below the threshold of the serological test.

The similar sensitivity of RPA-LF and the reference test (qPCR) is consistent with that reported for PCR-based detection in dogs naturally infected with *L. infantum*.³⁰ Also, we found that the collection and subsequent processing of biological samples preserved in Whatman FTA paper is an excellent method for DNA isolation under field conditions. The good

FIGURE 3. Agreement between recombinase polymerase amplification–lateral flow (RPA-LF) test and parasitologically positive and negative dogs. Negative blood samples (*) were obtained from dogs of a non-endemic area. Positive *Leishmania infantum* blood samples (1–6) were collected from dogs parasitologically positive by lymph node aspirate. This is a representative figure of two similar assays.

Mucosa qPCR C_t values vs. RPA-LF									
C_t	28	22	26	30	35	30	21	20	32
Nº	1	2	3	4	5	6	7	8	9

FIGURE 4. Agreement between recombinase polymerase amplification–lateral flow (RPA-LF) and real-time quantitative polymerase chain reaction (qPCR) to detect *Leishmania infantum* in mucosal samples. Lanes 1–7 correspond to different dogs suspected of suffering visceral leishmaniasis (VL) inhabiting an endemic area in Argentina; 8 and 9 are positive and negative controls (*L. infantum* DNA and no template, respectively). Dogs with higher parasite burdens (low qPCR C_t values) showed stronger bands in the RPA-LF test. This is a representative figure of two similar assays.

sensitivity of RPA-LF in noninvasive mucosal samples indicates that this sampling approach is well suited for field application and should be further evaluated in larger field studies. Collectively, the study showed that this novel molecular test offers several advantages over invasive sampling methods and sophisticated molecular tests and may have a positive impact in leishmaniasis control programs. The sensitivity of RPA-LF compared with serological methods should allow identifying a larger pool of infected dogs, some of them at early stages of infection. With this information in hand, health authorities could recommend removing dogs from the endemic area or subject them to euthanasia, which although not mandatory, is a standard practice in Latin America. Alternatively, dog owners could choose to use insecticide-impregnated collars and maintain their pets inside the household at night to protect them from sand fly bites thereby impairing *L. infantum* transmission.³¹ This approach has to be accompanied by community education to ensure compliance.

There are several reasons why a field-adapted molecular diagnostic test such as RPA-LF could positively impact leishmaniasis control compared with serological diagnosis. First, antibody-mediated tests such as rK39 to identify VL cases will delay the diagnosis and limit early interventions related to reservoir control and reduction of zoonotic or anthroponotic transmission.^{32,33} In humans, serological tests remain positive for prolonged periods after successful therapy, but RPA-LF is likely to provide a more definitive parasitological status of treated patients.³⁴ This has implications for both clinical and disease elimination efforts. Second, the regions where VL is endemic are characterized by extreme poverty, where rudimentary housing open to vector activity, undernutrition, and difficult access to primary health care are commonplace. Therefore, the field applicable RPA-LF test, which does not require expensive equipment and could be used by minimally trained personnel, is the ideal tool for individual diagnosis or epidemiological studies of human or canine VL.

For control programs, focusing only on the diagnosis of clinically suspicious cases is epidemiologically inadequate because studies in leishmaniasis have indicated that subclinical infections represent the largest proportion of individuals in endemic areas and an unknown number of them may also act as reservoir hosts.^{33,35,36} Spatiotemporal and genetic analyses showed clustering of cases in "hot spots" and increased risk of infection and disease in household members of VL cases, underscoring the need for active search of subclinical infections in areas highly endemic for VL.^{37–39} In a VL endemic focus of Brazil, > 70% of persons were considered to have asymptomatic infections, and around 12% either showed some symptoms of VL or progressed toward overt disease.³⁵ Asymptomatic infections also could be an important risk factor for recipients of blood and organs from donors inhabiting endemic areas.⁴⁰ On the other hand, in an endemic area of India, 69% of individuals subclinically infected with *L. donovani* developed overt disease within a 1-year period, stressing the importance of early diagnosis.⁴¹ Therefore, a strategy aimed at identifying subclinical (parasite positive) infections using a sensitive, field-applicable diagnostic test such as RPA-LF is essential for the control of VL. These punctual, preliminary results suggest that further laboratory and field evaluations of RPA-LF for *L. infantum* and *L. donovani* diagnosis are warranted.

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