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TITLE: **A Novel Field-Deployable Point-of-Care Diagnostic Test for Cutaneous Leishmaniasis**

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CONTRACTING ORGANIZATION: University of Texas Medical Branch, Galveston
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14. ABSTRACT **Leishmaniasis is caused by the protozoan Leishmania and is generally transmitted by the bite of sand flies of the genus Lutzomyia or Phlebotomus, The disease has significant global impact, producing 10-20 million cases of leishmaniasis worldwide. Cutaneous leishmaniasis (CL) is characterized by chronic skin ulcers that can impact the individual's functional status, lead to expensive and untimely treatment, and result in disfiguring scarring. Military training and combat operations resulted in cases of CL in soldiers (USA, UK) deployed to Central America. More recently (2003-2004), CL was reported in almost 1,200 members of the U.S. Armed Forces deployed to Iraq and Afghanistan, and the infection is an ongoing concern in the OEF/OIF veteran population. To date, there is no field-standardized molecular method based on sensitive DNA amplification coupled with Lateral Flow reading to detect leishmaniasis. Isothermal amplification by RPA (Recombinase Polymerase Amplification) is a novel strategy to diagnose infectious diseases that can be used at the POC because it is highly sensitive, fast, inexpensive and able to work at most ambient temperatures.**

15. SUBJECT TERMS

Nothing listed

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1. INTRODUCTION:

Leishmaniasis is caused by the protozoan *Leishmania* and is generally transmitted by the bite of sand flies of the genus *Lutzomyia* or *Phlebotomus,* The disease has significant global impact, producing 10-20 million cases of leishmaniasis worldwide. Cutaneous leishmaniasis (CL) is characterized by chronic skin ulcers that can impact the individual's functional status, lead to expensive and untimely treatment, and result in disfiguring scarring. Military training and combat operations resulted in cases of CL in soldiers (USA, UK) deployed to Central America. More recently (2003-2004), CL was reported in almost 1,200 members of the U.S. Armed Forces deployed to Iraq and Afghanistan, and the infection is an ongoing concern in the OEF/OIF veteran population. To date, there is no field-standardized molecular method based on sensitive DNA amplification coupled with Lateral Flow reading to detect leishmaniasis. Isothermal amplification by RPA (Recombinase Polymerase Amplification) is a novel strategy to diagnose infectious diseases that can be used at the POC because it is highly sensitive, fast, inexpensive and able to work at most ambient temperatures.

1. KEYWORDS:

Cutaneous leishmaniasis-diagnosis-point of care-DNA amplification-field applicable-isothermal amplification-protozoan parasite

2. ACCOMPLISHMENTS

Specific Aim	Month	% completion
Aim 1: To use simulated field conditions to optimize and produce the established RPA lateral flow diagnostic test for POC deployment.		
Sub-Aim 1.2: To determine if a simple DNA extraction method will provide adequate sensitivity for optimal test function under field conditions. Comparison of DNA yield, sufficient for RPA-LF test using a DNA mini-extractor vs. Whatman FTA filter paper utilizing dermal tissues spiked with *Leishmania* grown in the lab	1-3	80% Lab assays completed. Clinical samples from the field still require optimization of DNA purification
Sub-Aim 1.3: To determine if subgenus- and/or species-specific primer-probe sets can achieve the same analytical sensitivity and specificity as the genus specific primer-probe set using *Leishmania* isolates and clinical specimens from the field sites.	3-12	100% The analytical sensitivity of the RPA-LF was established for *Leishmania Viannia* spp., *L. major* and *L. enriettii*
Kickoff Coordination Meeting of participating institutions	3	100% A UTMB meeting was organized with participants of all three study sites
Protocol submission for local IRB approval and HRPO approval	3	N-6 100% N-3 90% Ghana IRBs completed; pending final N-6 approval
Implementation of molecular laboratory in Madre de Dios and technology transfer of kDNA PCR procedures from Lima to Madre de Dios for on-site Leishmaniasis diagnosis in the endemic area	6-12	80% Training completed and equipment purchased. Lab set up is awaiting final construction of dedicated facilities.
Milestone Achieved: Local IRB and HRPO approved protocols	6	UTMB 100% NAMRU-6 100% NAMRU-3 80%
Milestone(s) Achieved: □ Coordination meeting completed		

□ Approvals of IRBs in place to initiate field studies in human populations □ RPA-Lateral Flow test fully adapted for field application □ On-site molecular diagnosis of cutaneous leishmaniasis in Madre de Dios	12	**(see specific items described above within the table)**

- We standardized the amplification conditions of RPA-LF to detect the principal Leishmania species producing cutaneous and muco-cutaneous disease in Latin America.

- The analytical sensitivity indicated that the RPA-LF test could amplify DNA equivalent to 0.1 parasites per microliter which in the laboratory is equivalent to qPCR used as gold standard.

- A major effort was carried out to include species and strains for Leishmania from different countries in South America. This was possible thanks to additional collaborations with Colombia (CIDEIM, Cali) and Brazil (FIOCRUZ, Rio de Janeiro), which allowed us to evaluate the test on strains covering a wide geographical range.

- The RPA-LF test could now be read using two different lateral flow strips. However, more consistent results were obtained with UStar strips than with Milenia strips. For this reason, future field evaluations will be done with the former strips.

- The RPA-LF test was also evaluated in its capacity to amplify L. major. Collaborations with Drs. McMahon-Pratt (Yale University) and Paul Bates (Lancaster University, UK) allowed us to validate the specific primers and probe for this species. Furthermore, previous work between NAMRU-3 and Dr. Bates allowed us to evaluate strains isolated from the study site in Ghana. This is a novel species (*L. enriettii*) not reported before infecting humans. Our RPA-LF test was capable of amplifying also this species. Consequently, we have an RPA-LF test that will detect both L. major and the newly identified *L. enriettii* that infect patients in the Jo district of Ghana.

Training Activities

Erika Costa, PhD student from FIOCRUZ, Brazil received training on isothermal amplification methods (RPA-LF) when one of UTMB's investigators (O. Saldarriaga) evaluated the diagnostic test in a large number of *Leishmania* species and strains. This training was carried out as part of newly established

collaborations with Dr. Renato Porrozzi at FIOCRUZ which expanded our capacity to evaluate our RPA-LF diagnostic tool.

Maxy de Los Santos, PhD from NAMRU-6 received training in RPA-LF diagnosis upon the technical visit of Alejandro Castellanos-Gonzalez, PhD from UTMB. During this training several species and strains and *Leishmania* were evaluated showing the capacity of RPA-LF to amplify the principal species circulating in Peru.

NAMRU-6 investigators trained Puerto Maldonado personnel in culture parasite isolation, DNA extraction, and kDNA-PCR to identify Leishmania-positive samples at the genus level using kinetoplast DNA (kDNA) from different sample types (by filter paper imprint and scraping lancet). Personnel traveled from Puerto Maldonado to Lima to be trained in these techniques. Prior to Departure from Lima (after training), personnel were confirmed proficient in the methodologies through the use of a series of positive and negative controls.

Results disseminated to communities

Nothing to report

Plans for the next reporting period

We plan to optimize the purification of DNA from patient samples with the goal of launching the field evaluations of the RPA-LF diagnostic test in Peru and Ghana. These evaluations are described in Aim 2 and will be developed during years 2 and 3 of the project.

4. IMPACT

Impact on the development of the principal discipline(s) of the project:

The diagnostic method that is being optimized (RPA-LF) is a novel approach to identify patients suffering cutaneous leishmaniasis, a parasitic disease of worldwide distribution. It demonstrates that a sensitive and specific molecular method could be utilized in the field without the need for expensive equipment and complex health infrastructures. It represents an innovative diagnostic tool that will be ready to use during military field deployment or in resource-limited endemic areas of cutaneous leishmaniasis.

Impact on other disciplines:

This diagnostic method, which is based on the isothermal amplification of DNA, is impacting the field of molecular biology. It is expanding the concept of instrument-free diagnosis of infectious disease and amplification of DNA for multiple purposes in biology and medicine.

Impact on technology transfer:

Once the RPA-LF has been validated in the field it will likely be transferred to a commercial company and subsequently make available to the public.

Impact on society:

The development of this diagnostic method, which is sensitive and requires minimal training, will improve the quality of life of populations living in endemic areas. The availability of RPA-LF in economically depressed regions will improve the diagnostic capacity. This will lead to early treatment which will significantly decrease the negative impact of disease.

5. CHANGES/PROBLEMS

The IRB approvals for NAMRU-3 (Ghana Detachment) were delayed due to a series of reviews requested by the corresponding boards. This prevented the scientists of NAMRU-3 to obtain patient samples for the initial lab phase of the RPA-LF optimization. Nevertheless, the availability of *Leishmania major* from other sources and access to three strains originally isolated from Ghana (*L. enriettii*) allowed us to confirm the capacity of the test to detect both species of *Leishmania* that circulate in the endemic area.

We have delayed the transfer of RPA-LF protocols necessary for the second phase of the project which involves the field evaluation. We wanted to provide our co-investigators with the best sample collection method that will yield high DNA concentrations for its easy extraction and amplification by RPA-LF. Different samples collection methods are currently being tested between NAMRU-6 and UTMB and expect to have this issue resolved in the next several weeks.

NAMRU-6 has purchased all the equipment required to perform culture parasite isolation, microscopy, DNA extraction and identification of Leishmania parasite by kDNA-PCR in Puerto Maldonado, and we are currently storing the equipment in our main laboratory in Lima, Peru. Implementation of molecular biology methodologies in the NAMRU-6 remote Puerto Maldonado laboratory has been delayed due to setbacks in construction upgrades (at our Puerto Maldonado facility). Completion of these facility upgrades is necessary to perform the molecular assays. Nevertheless, NAMRU-6 has initiated these construction upgrades (which include electrical and plumbing upgrades) in Puerto Maldonado and should be finished during FY2016. Following completion of these minor facilities upgrades we will send the required equipment for these techniques including microscopy for cultures, DNA extraction systems, thermocycler and electrophoresis equipment to our trained Puerto Maldonado personnel to achieve on-site Leishmania diagnostic capacity for this project.

6. PRODUCTS

Presentation at Military meeting:

Military Health System Research Symposium (MHSRS).Fort Lauderdale, August 17-20, 2015
Development of a Point-Of-Care Molecular Diagnostic Test for Cutaneous Leishmaniasis.
Bruno L. Travi, DVM, PhD1*, Omar A. Saldarriaga DVM, PhD1, Alejandro Castellanos, PhD1, Gerald C. Baldeviano, PhD2, Maxy B. De los Santos, PhD2, Peter C. Melby, MD1, Andrés G. Lescano, PhD2
1University of Texas Medical Branch, Galveston, TX; 2US Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Peru.

Presentation at international meeting:

ASTMH Meeting, Philadelphia, October 25-29, 2015
A field-applicable molecular tool to diagnose American cutaneous leishmaniasis.
Travi BL1, Saldarriaga OA1, Castellanos A1, Baldeviano GC2, De los Santos MB2, Melby PC1, Lescano AG2.
1University of Texas Medical Branch, Galveston, Texas; 2US Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Peru.

The PI (B. Travi) was selected to give an oral presentation of the work supported by this award (see appendices)

The basis of this research project is the development and validation of a novel method to diagnose cutaneous leishmaniasis. Therefore, we designed specific primers and probes to identify species of *Leishmania* of the subgenus *Viannia*, *Leishmania major* and *Leishmania enriettii*. These products are available, initially for the development of the ongoing research, but should become broadly available through future commercialization strategies.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Bruno Travi*
Project Role:	*PI*
Researcher Identifier	eRA Commons (NIH) BrunoTravi
Nearest person month worked:	*4*
Contribution to Project:	*Overall scientific supervision and administration of project*
Funding Support:	

Name:	*Alejandro Castellanos-Gonzalez*

Project Role:	*Co-I*
Researcher Identifier	eRA Commons (NIH) ALCASTEL
Nearest person month worked:	*3*
Contribution to Project:	*Participated in lab evaluations of RAP-LF and collaborated in the evaluation of strains from Peru together with NAMRU-6 investigators*
Funding Support:	

Name:	*Omar Saldarriaga*
Project Role:	*Post-doc*
Researcher Identifier	eRA Commons (NIH) OMSALDAR
Nearest person month worked:	*6*
Contribution to Project:	*Participated in lab evaluations of RAP-LF and evaluated strains from Brazil together with collaborators in FIOCRUZ*
Funding Support:	

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

Nothing to Report

(**note**: the Partnering PI at NAMRU-6, Andres Lescano PhD was replaced by Robert V. Gerbasi LT USN; this change will be reflected in NAMRU-6 annual report)

Other organizations involved as partners

Organization Name: Fundacion Oswaldo Cruz-FIOCRUZ
Location of Organization: Brazil
Partner's contribution to the project
In-kind support: Species and strains of *Leishmania* isolated from patients in endemic areas of cutaneous leishmaniasis
Facilities: Laboratory facilities of Dr. Renato Porrozzi at FIOCRUZ to carry out *Leishmania* identification using the RPA-LF test.
Collaboration: FIOCRUZ staff (PhD student) collaborated in the evaluation of *Leishmania* strains

Organization Name: Centro Internacional de Entrenamiento e Investigaciones Médicas-CIDEIM
Location of Organization: Colombia
Partner's contribution to the project
In-kind support: Delivery from the lab of Dr. Nancy Gore Saravia of *Leishmania* strains isolated from patients in endemic areas of cutaneous leishmaniasis

Organization Name: Yale School of Public health
Partner's contribution to the project
In-kind support: Delivery of *Leishmania major* strains from the lab of Dr. Diane McMahon-Pratt

Organization Name: Lancaster University
Location of Organization: UK
Partner's contribution to the project
In-kind support: Delivery of *Leishmania major* and *Leishmania enriettii* strains from the lab of Professor Paul Bates

A NOVEL FIELD-DEPLOYABLE POINT OF CARE DIAGNOSTIC TEST FOR CUTANEOUS LEISHMANIASIS.

Log # PR130282

PI: Bruno L. TRAVI **Org: University of Texas Medical Branch - Award Amount: 397,101**

Study Aim(s)

Aim 1: To use simulated field conditions to optimize and produce the established RPA lateral flow diagnostic test for POC deployment.

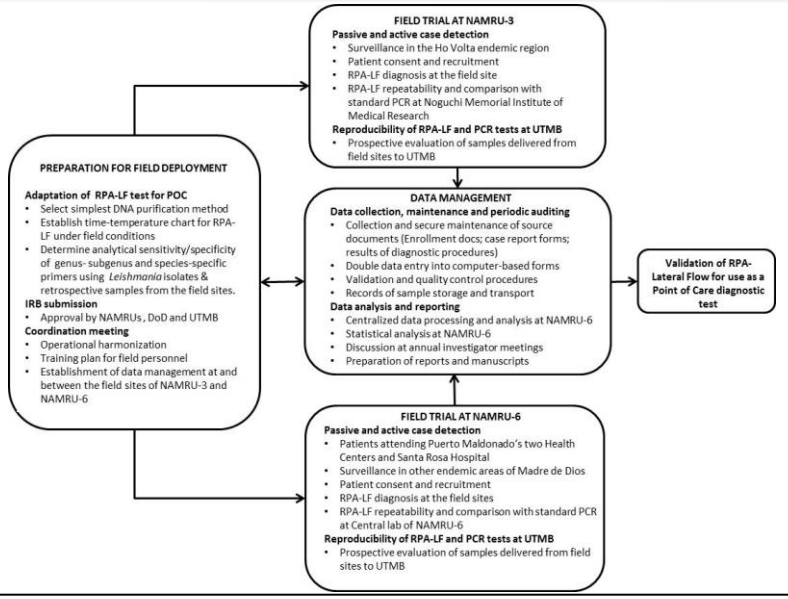
Aim 2: To prospectively determine the diagnostic sensitivity and specificity of the RPA-lateral flow test for diagnosis of cutaneous leishmaniasis.

Approach

Sub-Aim 1.1: To identify time-temperature constraints for optimal test function under field conditions. **Sub-Aim 1.2:** To determine if a simple DNA extraction method will provide adequate sensitivity for optimal test function under field conditions.

Sub-Aim 1.3: To determine if subgenus- and/or species-specific primer-probe sets can achieve the same analytical sensitivity and specificity as the genus-specific primer-probe set using *Leishmania* isolates and clinical specimens from the field. **Sub-aim 2.1.** NAMRU-6; Lima, Puerto Maldonado in Madre de Dios and Iquitos in Loreto, Peru

Sub-aim 2.2. NAMRU-3, Ghana detachment, Noguchi Memorial Institute for Medical Research, Ho Volta region.



Timeline and Cost

Activities years	14	15	16	17
Aim 1: To use simulated field conditions to optimize and produce the established RPA lateral flow diagnostic test for POC deployment				
Aim 2: To prospectively determine the diagnostic sensitivity and specificity of the RPA-lateral flow test for diagnosis of cutaneous leishmaniasis (NAMRU-3, NAMRU-6, UTMB).				
Estimated Budget	66,359	132,367	132,367	66,358

Updated: Oct. 30, 2015

Goals/Milestones

Month 6: Local IRB and HRPO approved protocols (month 6) Coordination meeting completed- **On Track**

Month 12: Approvals of IRBs in place to initiate field studies in human populations in place. **NAMRU-6 on Track; NAMRU-3 in progress** RPA-Lateral Flow test fully adapted for field application On-site molecular diagnosis of cutaneous leishmaniasis in Madre de Dios, Peru set up. **Training completed. Awaiting lab setup**

YEARS 2-3: Month 14: Technical meeting at NAMRU-3, Ghana

Month 36: Updated epidemiological assessment of cutaneous leishmaniasis in the endemic areas of Peru and Ghana available. New point-of-care diagnostic test for cutaneous leishmaniasis ready for submission to obtain FDA clearance.Final Report to DoD and scientific publications of results. (**upon completion of study**).

Comment: Lab evaluations of test completed. DNA extraction for point of care still under evaluation. Field sites awaiting final POC protocol. Once standardized, technical visit to N-3 Ghana will be scheduled

MHSRS meeting August 17-20, 2015
Marriott Harbor Beach Resort, Ft. Lauderdale, FL

Development of a point-of-care molecular diagnostic test for cutaneous leishmaniasis.

Bruno L. Travi, DVM PhD[1], Omar A. Saldarriaga DVM PhD[1], Alejandro Castellanos, PhD[1], Gerald C. Baldeviano, PhD[2], Maxy B. De los Santos, PhD[2], Peter C. Melby, MD[1], Andrés G. Lescano, PhD[2].

[1]University of Texas Medical Branch, Galveston, Texas; [2]US Naval Medical Research Unit No. 6 (NAMRU-6), Lima,Peru.

Cutaneous leishmaniasis (CL) is widely distributed in the Old and New World accounting for >1.2 million annual cases. Military training and combat operations resulted in cases of CL in soldiers deployed to Central America and more recently (2003-2004) it was reported in almost 1,200 members of the U.S. Armed Forces deployed to Iraq and Afghanistan. Microscopy is the most common diagnostic method used in endemic regions but its sensitivity is low (≤70%) and tends to decrease further with disease chronicity. Serology is variable and does not distinguish between current and past infections. Conventional or quantitative PCR from dermal or mucosal samples have good sensitivity (≈87-98%) and specificity (≥87%). However, it requires expensive equipment, trained personnel and lab facilities beyond the possibilities of military field operations or health infrastructure of endemic areas. We developed an innovative point of care molecular test to diagnose dermal leishmaniasis produced by Leishmania Viannia spp., which are responsible for the majority of cases in the Americas. We designed primers and probes that targeted the kinetoplast DNA minicircles. Leishmania DNA was extracted using the Qiagen® kit and detected by isothermal Recombinase Polymerase Amplification (42 ºC, 30 min) coupled with a lateral flow immunochromatographic strip (RPA-LF). The test has sensitivity similar to real time PCR (gold standard) detecting as few as 0.1 parasites per reaction. It does not require expensive equipment and the results are read with the naked eye in < 1 hour. The RPA-LF specificity was confirmed by the amplification of L. braziliensis, L. panamensis, L. guyanensis, L. peruviana and L. lainsoni. There was no cross amplification with L. chagasi, L. major, L. mexicana, L. amazonensis or T. cruzi. Preliminary data indicated that RPA-LF has excellent agreement with PCR as determined in parasite isolates from endemic areas of Peru. Also, for diagnosis of Old World CL, we designed RPA primer sets targeting kinetoplast DNA of L. major which produced 111 bp or 121 bp bands in 1% agarose gel. Different specific probes have been designed that will allow us detecting this parasite species also by lateral flow reading. This novel method fills the need for a field applicable diagnostic tool critical to cutaneous and mucosal leishmaniasis management in civilians and military personnel.

64th Annual Meeting of the American Society of Tropical Medicine and Hygiene, October 25-29, 2015. Philadelphia

A field-applicable molecular tool to diagnose American cutaneous leishmaniasis.

Travi BL[1], Saldarriaga OA[1], Castellanos A[1], Baldeviano GC[2], De los Santos MB[2], Melby PC[1], Lescano AG[2].

[1]University of Texas Medical Branch, Galveston, Texas; [2]US Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Peru.

Dermal and mucosal leishmaniasis is widely distributed in Central and South America affecting an estimated 190,000- 300,000 people annually. Microscopy is the most common diagnostic method used in endemic regions but its sensitivity is low (≤70%) and tends to decrease further with disease chronicity. Serology is variable and does not distinguish between current and past infections. Conventional or quantitative PCR from dermal or mucosal samples have high sensitivity (≈87-98%) and specificity (≥87%) but require expensive equipment, trained personnel and lab facilities beyond the possibilities of resource-limited health infrastructure of endemic areas. We developed a novel point of care molecular test to diagnose dermal and mucosal leishmaniasis produced by Leishmania Viannia spp., which are responsible for the majority of cases. We designed primers and probes that targeted the kinetoplast DNA minicircles. Leishmania DNA was extracted using the Qiagen® kit and detected by isothermal Recombinase Polymerase Amplification coupled with a lateral flow immunochromatographic strip (RPA-LF). The test has sensitivity similar to real time PCR (gold standard) detecting as few as 0.1 parasites per reaction. It does not require expensive equipment and the results are read with the naked eye in < 1 hour. The RPA-LF specificity for the L. Viannia subgenus was confirmed by the amplification of L. braziliensis, L. panamensis, L. guyanensis, L. peruviana and L. lainsoni. There was no cross amplification with L. chagasi, L. major, L. mexicana, L. amazonensis or T. cruzi. Preliminary data indicated that RPA-LF has an excellent agreement with PCR as determined in patient samples from endemic areas of Peru. We are evaluating additional primer sets capable of amplifying the Leishmania subgenus with the goal of developing an RPA- multiplex lateral flow test that encompasses all species that produce cutaneous leishmaniasis. This novel method could fill the need for a field applicable diagnostic tool critical to cutaneous and mucosal leishmaniasis management and control.

An innovative field-applicable molecular test to diagnose cutaneous *Leishmania Viannia spp.* infections

Short title: **molecular diagnostic test for cutaneous leishmaniasis**

Omar A. Saldarriaga[1], Alejandro Castellanos-Gonzalez[1,3,], Renato Porrozzi[4], Gerald C. Baldeviano[5], Andrés G. Lescano[5,7], Maxy B. de Los Santos[5], Olga L. Fernandez[6], Nancy G. Saravia[6], Erika Costa[4], Peter C. Melby[1,2,3], Bruno L. Travi[1,2,3*].

[1]Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch (UTMB), Galveston, Texas; [2]Department of Microbiology and Immunology, [3]Center for Tropical Diseases (CTD), University of Texas Medical Branch (UTMB), Galveston, Texas. [4]Laboratório de Pesquisa em Leishmaniose, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.[5]U.S. Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Perú. [6]Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM), Cali, Colombia. [7]Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru.

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INTRODUCTION

Dermal and mucosal leishmaniasis are widely distributed in Central and South America, affecting an estimated 190,000-300,000 people annually (1). Many different *Leishmania* species grouped under the subgenera *Leishmania* or *Viannia* can produce dermal leishmaniasis. Epidemiologically, *Viannia* is the most relevant subgenus in this region since it is highly

prevalent and also responsible for metastatic mucosal leishmaniasis (*L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis*), the severe form of tegumentary disease (2, 3).

Microscopy is still the most common diagnostic method used in endemic regions but its sensitivity is not ideal and markedly affected by the experience of the microscopist (4). Furthermore, the sensitivity of this method tends to decrease with disease chronicity, which is characterized by a low number of amastigotes in the lesions (4). Serological tests were used in the past and the identification of new antigens and formats for serodiagnosis of American cutaneous leishmaniasis is still considered (5, 6). However, in general, they have proven to be of limited value due to the variable immune responses of patients and no clear distinction between current disease and past infections or exposure.

Conventional or quantitative PCR from dermal or mucosal samples have high diagnostic sensitivity (≈87-98%) and specificity (≥84%) (7). This molecular method is currently the gold standard in leishmaniasis reference centers or tertiary care facilities. However, the need for expensive equipment, trained personnel, and relatively complex laboratory facilities are beyond the capability of the typical health infrastructure in endemic areas.

Therefore, there is a clear need to provide primary health systems with diagnostic tools that are simple, easy to use and have good sensitivity and specificity. To address this critical gap, we developed a novel-point-of-care molecular test to diagnose dermal and mucosal leishmaniasis produced by *Leishmania Viannia* spp. We designed primers and probes that targeted the kinetoplast DNA minicircles, similar to the strategy we used previously to detect *L. infantum chagasi* (8). Leishmania DNA was amplified using isothermal Recombinase Polymerase Amplification (RPA) and detected in a lateral flow immunochromatographic strip (LF) which is read with the naked eye. Its analytical sensitivity and specificity indicated that it could be used

as a point-of-care diagnostic test for dermal and mucosal leishmaniasis in endemic areas of Latin America.

MATERIALS AND METHODS

Design of primers and probe. The primer sets for *Leishmania Viannia* are 30-35 nucleotides long and target conserved sequences identified by computational alignment of *L. Viannia* kDNA minicircle sequences reported in GenBank. Primers were designed with 40-60% GC content, few direct/inverted repeats, and absence of long homopolymer tracts. We focused principally on conserved regions and to a lesser extent on regions with moderate variability, obtaining a 120 bp RPA amplicon in agarose gels (not shown)**.** To enable detection by lateral flow, the reverse primer was biotinylated at the 5' end. We designed a 45bp conserved internal probe (Biosearch technologies -Petaluma, CA) that included FAM (5'-carboxy fluorescein amidite) at the 5' end, an internal dSpacer and a SpacerC3 in the 3' end, as suggested by the manufacturer (TwistDx). Forward Primer: *Fw- GATGAAAATGTACTCCCCGACATGCCTCTG*. Reverse Primer: *Rev-bio-CTAATTGTGCACGGGGAGGCCAAAAATAGCGA*. Internal Probe: The probe contains a 5'-fluorescein group (FAM), an internal (THF)-tetrahydrofuran residue, and a C3 spacer block at the 3' end. *Probe-**FAM**-GTAGGGGNGTTCTGCGAAAACCGAAAAATG[THF]CATACAGAAACCCCG[C3-spacer]*.

Parasite DNA isolation. Promastigote suspensions of reference strains or clinical strains thawed from cryopreserved stocks or absorbed in Whatman FTA® filter paper (Sigma-Aldrich) were subjected to 95ºC for 2 minutes in a dry bath to lyse the parasites. DNA purification was carried out using the DNeasy Blood & Tissue Kit (Qiagen®) following the recommendations of the vendor and adjusted to 10 ng/µL.

RPA reaction and lateral flow reading. The amplification mixture was comprised of: 1) forward primer, 2) biotinylated reverse primer, 3) FAM-labeled probe (stocks-5µM), 4) magnesium

acetate, and 5) the rehydrated cocktail (Twist amp nfo RPA kit -TwistDx, UK). Parasite DNA (5 - 25ng/µL) was immediately added to the mixture and subjected to amplification at 45ºC for 30 minutes using a dry bath. The RPA product was diluted 1:25 in the dipstick assay buffer and 30 µL were placed in a 1.5 Eppendorf tube or 96-well microplate. The bottom tip of the lateral flow strip was then immersed in the sample (GenLine HybriDetect, Milenia Biotec, Germany). Parasite amplification was confirmed with the naked eye after 5 minutes by the appearance of the test band in the lower part of the strip. The reaction was validated by the appearance of the control band in the upper part of the strip.

Quantitative PCR. The RPA-LF sensitivity was compared with SYBRgreen® real-time PCR using the primers described by Pita-Pereira *et al*. (9).

***Leishmania* samples.** The analytical evaluations of RPA-LF were carried out using known concentrations of DNA (10ng/µL). We evaluated banked strains of *L. braziliensis* from Brazil (n=15), Colombia (n=5), and Peru (n=13); *L. guyanensis* from Brazil (n=11) and Colombia (n=6); *L. panamensis* from Colombia (n=7), Nicaragua (n=1), and Panama (n=1); *L. lainsoni* from Brazil (n=3) and Peru (n=7); and *L. shawi* (n=2) and *L. naiffi* (n=6) from Brazil. Also, we evaluated DNA purified from lesion biopsies of patients from Peru who were infected with *L. braziliensis* (n=9) and *L. guyanensis* (n=4), as well as non-leishmanial (PCR-negative) skin lesions (n=5).

RESULTS

The RPA-LF amplified *Leishmania* DNA with an analytical sensitivity equivalent to 0.1 parasite per reaction, which corresponded to aCt value of 28 in the real-time PCR used as the gold standard (**Figure 1**). The capacity of RPA-LF to detect the most relevant species of the

subgenus *Viannia* was initially determined by the amplification of a small number of banked strains of *Leishmania Viannia* spp: *L. braziliensis, L. panamensis*, *L. guyanensis, L. lainsoni*, *L. shawi* and *L. naiffi*. The specificity was confirmed by the lack of amplification of *L. donovani*, *L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. major*, *Trypanosoma cruzi* and human DNA (**Figure 2**).

We further evaluated panels of strains from different species within the *Viannia* subgenus isolated in endemic areas of Brazil, Colombia, and Peru. Fifteen out of 15 *L. braziliensis* strains from Brazil, 6/6 strains from Colombia, and 12/12 from Peru, isolated from humans or dogs from different geographical areas, were amplified by RPA-LF **(Table 1)**. The test also demonstrated good sensitivity to detect several *L. guyanensis* strains obtained from endemic regions of Brazil (11/11) and Colombia (6/6) **(Table 1)**. Similarly, *L. panamensis* strains originally isolated from patients of Colombia (7/7), Nicaragua (1/1), and Panama (1/1) were readily amplified by RPA-LF. A small group of *L. Viannia* species known to occasionally infect humans were also evaluated by RPA-LF. Two Brazilian strains of *L. shawi,* a species closely related to *L. guyanensis,* produced strong bands indicating that the primers efficiently amplified this parasite species. However, 5/6 strains of *Leishmania naiffi*, usually found in mammals of the Amazon region and less frequently in other parts of South America, were amplified less efficiently than other *Viannia* species and generated weaker bands **(Table 1)**. In the case of *L. lainsoni*, a parasite found in wild mammals and sporadically infecting humans, RPA-LF produced a weak yet clearly detectable band in 3/3 strains from Brazil and 6/7 from Peru. One *L. naiffi-L. lainsoni* hybrid from Brazil was also detected by RPA/LF. Collectively, these results indicated that the test is capable of detecting all the epidemiologically relevant species of the *Viannia* subgenus. We developed an interactive map that depicts the geographical distribution of *Leishmania* species evaluated by RPA-LF (http://www.scribblemaps.com/maps/view/Leish_Viannia/9-18-15)

Table 1. RPA-LF detection of *Leishmania Viannia* species isolated from different countries in Latin America.

Leishmania spp.	Country	Region[1]	WHO code	RPA-LF	Map code[2]
L. braziliensis	**Brazil**	Pará	MHOM/BR/1975/M2903	+	**1**
		Ceará	MHOM/BR/1987/H-210	+	**2**
		Amazonas	MHOM/BR/1988/IM3482	+	**3**
		Ceará	MCAN/BR/1990/C35	+	**4**
		Ceará	MCAN/BR/1991/C51	+	**5**
		Amazonas	MHOM/BR/1994/IM3946	+	**6**
		Espírito Santo	MHOM/BR/1994/HAD-1	+	**7**
		Bahia	MHOM/BR/1996/SBS	+	**8**
		Bahia	MHOM/BR/2001/LTCP13183	+	**9**
		Acre	MHOM/BR/2002/NMT-RBO037	+	**10**
		Bahia	MHOM/BR/2001/NMT-LTCP14369-P	+	**11**
		Rio de Janeiro	MHOM/BR/2008/NC	+	**12**
		Pernambuco	MHOM/BR/2010/MMS	+	**13**
		Santa Catarina	MHOM/BR/2006/LSC128	+	**14**
		Santa Catarina	MHOM/BR/2006/LSC185	+	**15**
	Colombia	Caqueta	MHOM/CO/87/1270	+	**16**

		Nariño	MHOM/CO/85/2388	+	**17**
		Putumayo	MHOM/CO/82/L71	+	**18**
		Caqueta	MHOM/CO/88/1403	+	**19**
		Meta	MHOM/CO/85/1110	+	**20**
		Nariño	MHOM/CO/97/3144	+	**21**
	Peru	Cusco	MHOM/PE/14/LDP-0053	+	**22**
		Loreto	MHOM/PE/14/LDP-0057	+	**23**
		Junín	MHOM/PE/14/LDP-0060	+	**24**
		Junín	MHOM/PE/14/LDP-0065	+	**25**
		Cusco	MHOM/PE/14/LDP-0067	+	**26**
		Junín	MHOM/PE/14/LDP-0073	+	**27**
		Cusco	MHOM/PE/14/LDP-0075	w	**28**
		Madre de Dios	MHOM/PE/13/LDP-2036	+	**29**
		Madre de Dios	MHOM/PE/13/LDP-2039	+	**30**
		Madre de Dios	MHOM/PE/13/LDP-2059	+	**31**
		Madre de Dios	MHOM/PE/14/LDP-2074	+	**32**
		WHO	MHOM/PE/84/LTB300	+	**33**
L. guyanensis	**Brazil**	Amazonas	MHOM/BR/1997/NMT-MAO 210P	+	**34**
		Amazonas	MHOM/BR/1997/NMT-MAO 212P	+	**35**
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**36**

			237P		
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**37**
			246P		
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**38**
			292P		
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**39**
			307P		
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**40**
			317P		
		Amazonas	MHOM/BR/1997/NMT-MAO	+	**41**
			325P		
		Amazonas	MHOM/BR/2007/031-LOP	+	**42**
		Amazonas	MHOM/BR/2007/033-MECM	+	**43**
		WHO	MHOM/BR/75/M4147	+	**44**
	Colombia	Caqueta	MHOM/CO/83/1028	+	**45**
		Putumayo	MHOM/CO/82/L76	+	**46**
		Putumayo	MHOM/CO/82/L75	+	**47**
		Caqueta	MHOM/CO/88/1390	+	**48**
		Tolima	MHOM/CO/2008/A197	+	**49**
		Putumayo	MHOM/CO/83/1011	+	**50**
L. panamensis	**Nicaragua**	Chontales	MHOM/NI/1988/XD45	+	**51**
	Colombia	Putumayo	MHOM/CO/92/1735	+	**52**
		Valle	MHOM/CO/84/1048	+	**53**
		Nariño	MHOM/CO/85/2476	+	**54**
		Nariño	MHOM/CO/85/2472	+	**55**

		Cauca	MHOM/CO/86/1180	+	**56**
		Narino	MHOM/CO/83/2017	+	**57**
		Cauca	MHOM/CO/95/1989	+	**58**
	Panama	WHO	MHOM/PA/71/LS94	+	**59**
L. lainsoni	**Brazil**	Pará	MHOM/BR/1981/M6426	+	**60**
		Rondônia	MCOE/BR/1983/IM1367	+	**61**
		Pará	MCUN/BR/1983/IM1721	+	**62**
	Peru	Amazonas	MHOM/PE/14/LDP-0061	w	**63**
		Loreto	MHOM/PE/13/LDP-1021	+	**64**
		Madre de Dios	MHOM/PE/15/LDP-2138	+	**65**
		Madre de Dios	MHOM/PE/15/LDP-2169	w	**66**
		Madre de Dios	MHOM/PE/15/LDP-2236	+	**67**
		Madre de Dios	MHOM/PE/15/LDP-2242	-	**68**
		Huanuco	MHOM/PE/88/BAB1730	+	**69**
L. shawi	**Brazil**	Pará	IWHI/BR/1985/IM2322	+	**70**
		Pará	MCEB/BR/1984/M8408	+	**71**
L. naiffi	**Brazil**	Pará	ISQU/BR/1985/IM2264	+	**72**
		Pará	MDAS/BR/1987/IM3280	-	**73**
		Pará	MDAS/BR/1979/M5533	+	**74**

	Amazonas	MHOM/BR/1991/IM3740	+	**75**
	Pará	MHOM/BR/2011/S50	+	**76**
	Pará	MHOM/BR/2011/58-AMS	w	**77**
L. naiffi/ lainsoni	Acre	MHOM/BR/2002/NMT-RBO004	+	**78**

119

120 + indicates positive reading; W = indicates weak band

121 [1]Region: Brazil= State; Colombia= Department; Peru= Region

122 [2]Link to the interactive map: http://www.scribblemaps.com/maps/view/Leish_Viannia/9-18-15

123

124 In a small number of clinical samples we found that RPA-LF has excellent agreement with PCR
125 as determined in DNA samples from patients of Peru infected with *L. braziliensis* or *L.*
126 *guyanensis* **(Table 2)**. All 9 of the samples from clinical lesions due to *L. (V.) braziliensis*, and all
127 4 of the samples from clinical lesions due to L. (*V.) guyanensis* were positive by RPA-LF. The
128 samples from negative controls were uniformly negative by RPA-LF. The high sensitivity and
129 specificity identified with these limited number of samples warrants large-scale field testing to
130 determine the diagnostic sensitivity of the RPA-LF.

131 **Table 2. Agreement between RPA-LF and PCR to amplify *Leishmania Viannia* DNA**
132 **purified from lesions of cutaneous leishmaniasis patients from Peru.**

***Leishmania* spp.**	**Country**	**Region**	**WHO Code**	**qPCR**	**RPA-LF**	**Map Code[1]**

L. braziliensis	**Peru**	Ucayali	MHOM/PE/2012/LDP-0005-Bx	*L.b.*	+	**79**
		Cusco	MHOM/PE/2012/LDP-0011-Bx	*L.b.*	+	**80**
		Junín	MHOM/PE/2012/LDP-0012-Bx	*L.b.*	+	**81**
		Madre de Dios	MHOM/PE/2013/LDP-0034-Bx	*L.b.*	+	**82**
		Junín	MHOM/PE/2014/LDP-0052-Bx	*L.b.*	+	**83**
		Junín	MHOM/PE/2014/LDP-0052-FP	*L.b.*	+	**84**
		Loreto	MHOM/PE/2014/LDP-0057-Bx	*L.b.*	+	**85**
		Loreto	MHOM/PE/2014/LDP-0057-FP	*L.b.*	+	**86**
		Loreto	MHOM/PE/2014/LDP-0057-L	*L.b.*	+	**87**
L. guyanensis		Huánuco	MHOM/PE/2012/LDP-0007-Bx	*L.g.*	+	**88**
		San Martin	MHOM/PE/2012/LDP-0014-Bx	*L.g.*	+	**89**
		San Martin	MHOM/PE/2013/LDP-0041-Bx	*L.g.*	+	**90**
		San Martin	MHOM/PE/2013/LDP-0041-FP	*L.g.*	+	**91**
Negative Controls		San Martin	MHOM/PE/2012/LDP-0017-Bx	-	-	
		Junín	MHOM/PE/2012/LDP-0030-Bx	-	-	
		Ucayali	MHOM/PE/2013/LDP-0042-Bx	-	-	
		Cusco	MHOM/PE/2013/LDP-0043-Bx	-	-	
		Iquitos	MHOM/PE/2015/LDP-0083-Bx	-	-	

133

134 + indicates positive reading; *L.b.*= *L. braziliensis* as determined by qPCR; *L.g.*= *L. guyanensis*
135 as determined by qPCR.
136 [1]Link to the interactive map: http://www.scribblemaps.com/maps/view/Leish_Viannia/9-18-15
137

138 **DISCUSSION**

139 We developed a field-applicable molecular diagnostic test that distinguishes between the
140 subgenera *Viannia* and *Leishmania* by selectively detecting strains of the *Viannia* subgenus.
141 Our primers and probes were designed to target the kinetoplast DNA minicircles due to the high
142 copy number (≈ 10,000) of this circular network of genomic mitochondrial DNA (10). This
143 remarkable number of copies provides a comparative advantage over other parasite targets with
144 regard to test sensitivity. We targeted the *Viannia* subgenus because it encompasses the most
145 relevant species causing cutaneous leishmaniasis in Latin America. The evaluation of the RPA-
146 LF test included strains from Brazil, Colombia, and Peru, in which the recently reported
147 incidence was 26,008, 17,420, and 6,405 cases/year, respectively (1). The number of patients
148 requiring diagnosis in these countries could be even greater since it was estimated that under-
149 reporting varied between 2.8 and 4.6 fold (1).

150 The discrimination between *Viannia* and *Leishmania* subgenera is clinically relevant because in
151 Latin America these infections may be treated differently (11). Also, infection with *L. braziliensis*,
152 *L. panamensis*, and less frequently *L. guyanensis* require prolonged patient follow up due to the
153 risk of mucosal metastasis after apparent successful treatment (12, 13).*Leishmania (V.) shawi*
154 was readily detected by the RPA-LF test. Early studies suggested that *L. shawi* was not
155 frequently reported in humans and seemed to be of low prevalence in nature (14, 15). However,
156 more recent studies in Northeastern Brazil found that 6.5% (5/77) of isolates were identified as
157 *L. shawi* and that some of them could be considered hybrids with *L. braziliensis* (16).The RPA-

158 LF was less efficient at amplifying *L. naiffi*, a species found in armadillos and occasionally
159 infecting humans in different countries of South America (17, 18). Therefore, further test
160 optimization would be necessary for epidemiological studies aimed at this particular species.

161 During the development phase, we detected variability in distinct batches of the lateral flow
162 strips (Milenia Biotec, Germany) regarding increased background that led to the appearance of
163 faint test bands in the negative controls. The problem was resolved by using higher dilutions of
164 the amplification product (1:100-1:200). Each laboratory should standardize and select the
165 lateral flow strips that best suits its needs. There are different commercial options of
166 immunochromatographic strips for lateral flow reading. They are offered in containers with
167 multiple strips (Milenia, Biotec), individual cards (Abingdon Health, UK), or cassettes (UStar,
168 China) that are putatively less prone to contamination.

169 Scrapings or brushings of cutaneous lesions absorbed in filter paper were shown to be
170 amenable to molecular diagnosis using PCR (19). We have already shown that RPA-LF could
171 use this preservation-transportation method to amplify *Leishmania* DNA from the blood of dogs
172 infected with *L. chagasi* (8).The test was capable of amplifying DNA equivalent to 0.1 parasites
173 in the reaction mix, which was comparable to the detection limit of our qPCR. Preliminary results
174 using a small number of samples from lesions suggested that RPA-LF can efficiently detect
175 parasite DNA in the presence of host DNA with high sensitivity and specificity. Nevertheless,
176 the diagnostic sensitivity will have to be evaluated under field conditions in a larger number of
177 patients. It is well established that parasite burdens tend to be highly variable and that parasites
178 are more difficult to detect in chronic lesions (4). Therefore, it will be particularly important to
179 evaluate the diagnostic sensitivity of the RPA-LF in chronic lesions with >3 months of evolution.

180 A significant advantage of the RPA-LF is that samples can be rapidly processed, without the
181 need of sophisticated equipment, outside of a traditional laboratory (e.g. at a house, school, or

community center). RPA-LF is a less complex test than other isothermal amplification methods. RPA-LF results would be available in approximately one hour and the patients could initiate treatment if tested positive. Compared to a PCR reference test, this approach should enable earlier initiation of treatment, significantly increasing compliance and treatment efficacy. The need for delivering samples to a central reference lab, that leads to delayed therapeutic decisions and increased risk of patient loss, would be avoided. Importantly, the implementation of the field-applicable RPA-LF could replace or repurpose the need for experienced microscopists (and microscopes). It will improve the efficiency to diagnose leishmaniasis of short evolution time and, more importantly, in chronic lesions with parasite burdens below the microscopy threshold. The RPA-LF test may well fill the need for a field-applicable test, which is critical to cutaneous and mucosal leishmaniasis management.

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Competing interests

No competing interests are reported.

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284

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Abstract of manuscript submitted for publication

Cutaneous and mucosal leishmaniasis is widely distributed in Central and South America. *Leishmania of the Viannia* subgenus are the most frequent species infecting humans. *L. (V.) braziliensis, L. (V.) panamensis* are also responsible for metastatic mucosal leishmaniasis. Conventional or real time PCR is a more sensitive diagnostic test than microscopy, but the cost and requirement for infrastructure and trained personnel makes it impractical in most endemic regions. Primary health systems need a sensitive and specific point of care (POC) diagnostic tool. We developed a novel POC molecular diagnostic test for cutaneous leishmaniasis caused by *Leishmania (Viannia)* spp. Parasite DNA was amplified using isothermal Recombinase Polymerase Amplification (RPA) with primers and probes that targeted the kinetoplast DNA. The amplification product was detected by naked eye with a lateral flow (LF) immunochromatographic strip. The RPA-LF had an analytical sensitivity equivalent to 0.1 parasites per reaction. The test amplified the principal *L. Viannia* species from multiple countries: *L. (V.) braziliensis* (n=33), *L. (V.) guyanensis* (n=17), *L. (V.) panamensis* (n=9). The less common *L. (V.) lainsoni*, *L. (V.) shawi*, and *L. (V.) naiffi* were also amplified. No amplification was observed in parasites of the *L. (Leishmania)* subgenus. In a small number of clinical samples (n=13) we found 100% agreement between PCR and RPA-LF. The high analytical sensitivity and clinical validation indicate the test could improve the efficiency of diagnosis, especially in chronic lesions with submicroscopic parasite burdens. Field implementation of the RPA-LF test could contribute to management and control of cutaneous and mucosal leishmaniasis.

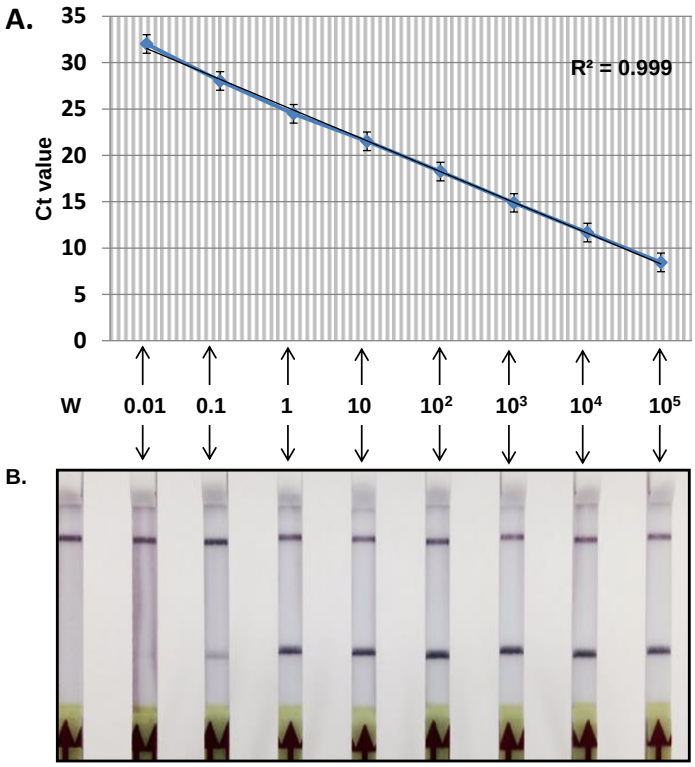

Figure 1. Sensitivity of RPA-LF to detect *L. viannia spp.* compared with real-time PCR (the current gold standard). Ten-fold serial dilutions of parasite DNA, extracted with Qiagen® DNeasy blood and tissue kit, were amplified by qPCR (SYBRgreen) (A) or RPA-LF (B). W=water. The control band is the upper band, while the test band is the lower band.

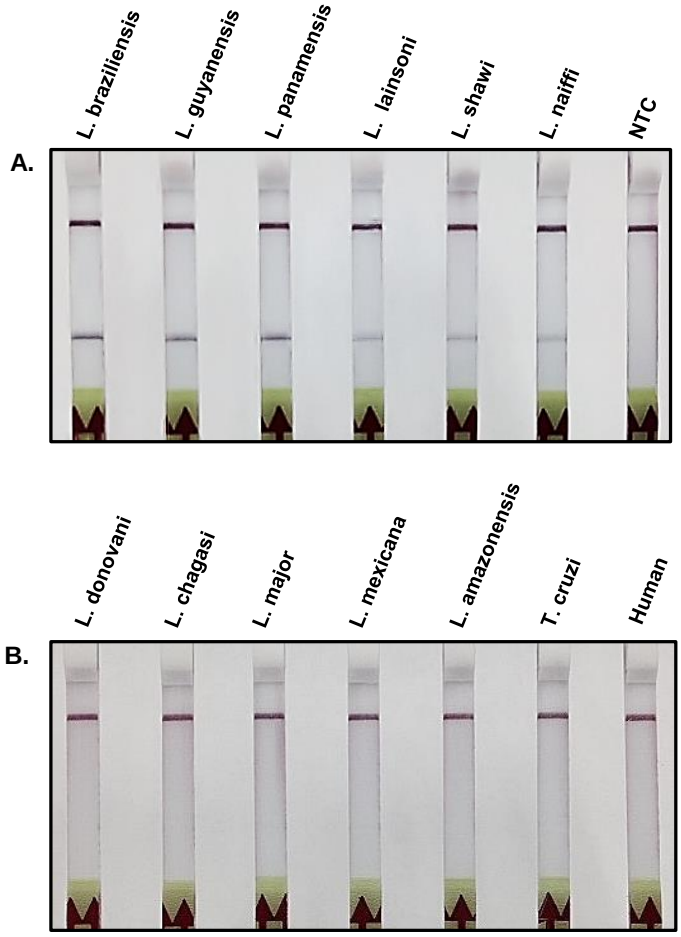

Figure 2. Specificity of RPA-LF to amplify species of the *Viannia* subgenus. A) The most relevant *L. Viannia* species (*L. braziliensis, L. guyanensis, L. panamensis*) produced stronger bands in the lateral flow strip than other less common species of this subgenus. B) Species of the *Leishmania* subgenus, *Trypanosoma cruzi*, and human DNA were not amplified by the RPA-LF test. NTC= no template control.