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

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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.**

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Table of Contents

	Page
1. Introduction……	**1**
2. Keywords……	**1**
3. Accomplishments……..……...	**2**
4. Impact……..……	**4**
5. Changes/Problems..…….……	**5**
6. Products…….……..……	**5**
7. Participants & Other Collaborating Organizations……	**6**
8. Special Reporting Requirements……	**9**
9. Appendices……	**10**

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	100% Lab assays were completed. Clinical samples from the field (NAMRU-6) have been evaluated using a simple extraction method. Results were satisfactory
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%. RSB renewal in progress; IRB (Cairo) pending final 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	90% Training was completed and equipment purchased. Lab construction was completed, and patient samples are obtained on a regular basis. Still no RPA-LF test has been performed on site.
Milestone Achieved: Local IRB and HRPO approved protocols	6	UTMB 100% NAMRU-6 100% NAMRU-3 80%
<u>Aim 2</u>: To prospectively determine the diagnostic sensitivity and specificity of the RPA-lateral flow test for diagnosis of cutaneous leishmaniasis.		
Sub-aim 2.1. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RAP-Lateral Flow vs. standard kDNA PCR at NAMRU-6; Lima and	12-36	UTMB 50% NAMRU-6 50%. Close to 90 clinical samples from

Puerto Maldonado, Madre de Dios, Peru Delivery of subset of positive and negative clinical samples (10%) from NAMRU-6 to UTMB for reproducibility testing		patients suspicious of having cutaneous leishmaniasis were obtained in Madre de Dios, Peru. PCR was carried out at NAMRU-6, Lima. The performance of RPA-LF using these clinical samples was further evaluated in UTMB (see below)
Technical meeting at NAMRU-3, Ghana	14	Not accomplished. A technical meeting will be planned once the field work is implemented.
Sub-aim 2.2. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RAP-Lateral Flow vs. standard PCR at NAMRU-3, Ghana detachment, Noguchi Memorial Institute for Medical Research, Ho Volta region Delivery of subset of positive and negative clinical samples (10%) from NAMRU-3 to UTMB for reproducibility testing	12-36	As mentioned in aim 2.1 the work has reached 50% completion in the NAMRU-6-UTMB component. NAMRU-3 activities have been delayed due to IRB pending approval.
Technical meeting at NAMRU-6, Peru	24	100% A Coordination meeting at NAMRU-6, Peru was carried out in May 2016

Milestone(s) Achieved:

- All the activities between NAMRU-6 and UTMB are in course and the percentage completion of the aims are as planned.
- A one-week workshop on the utilization of RPA-LF diagnostic test was carried out at NAMRU-6.
- We standardized and implemented the protocol in Peru for obtaining and transporting clinical samples of dermal lesions compatible with cutaneous leishmaniasis. The protocol demonstrated to be well suited for further processing by RPA-LF.
- Close to 90 clinical samples from patients suspicious of having cutaneous leishmaniasis were obtained in Madre de Dios and processed by PCR at NAMRU-6, Lima.
- These clinical samples were used at UTMB to evaluate the performance of RPA-LF.

- Subsequent to the first year evaluation of the RPA-LF analytical sensitivity (0.1 parasites/μL) we generated preliminary data on the diagnostic sensitivity of the test (see below).

- Coordinated diagnostic activities carried out at Madre de Dios, NAMRU-6 and UTMB generated encouraging preliminary data on the diagnostic efficacy of the RPA-LF test.

- Evaluation of the first 87 clinical samples showed that the point of care RPA-LF test had a sensitivity of 95.06% while the laboratory-based PCR used as gold standard had a sensitivity of 96.29%. Therefore, these preliminary data indicated that there was only a 1.23% difference in sensitivity. Five of the 87 clinical samples resulted negative for both molecular methods.

- Only 50% of the clinical samples evaluated by different operators (including different staining protocols and microscopes) detected Leishmania amastigotes in lesion smears. This result underscores the low sensitivity of the standard microscopy method.

- An added advantage of RPA-LF was its capacity to detect Leishmania DNA in the presence of bacterial contamination, which contributes to the low sensitivity of the microscopy diagnosis.

- Using the standard microscopy method patients should first be treated for bacterial co-infection to increase diagnostic sensitivity. However, the requirement of 2 or more visits to the clinic would result in a high rate of patient non-compliance. Therefore, attempts are made to diagnose cutaneous leishmaniasis in the co-infected patients which leads to low sensitivity. This problem would be overcome with the implementation of RPA-LF.

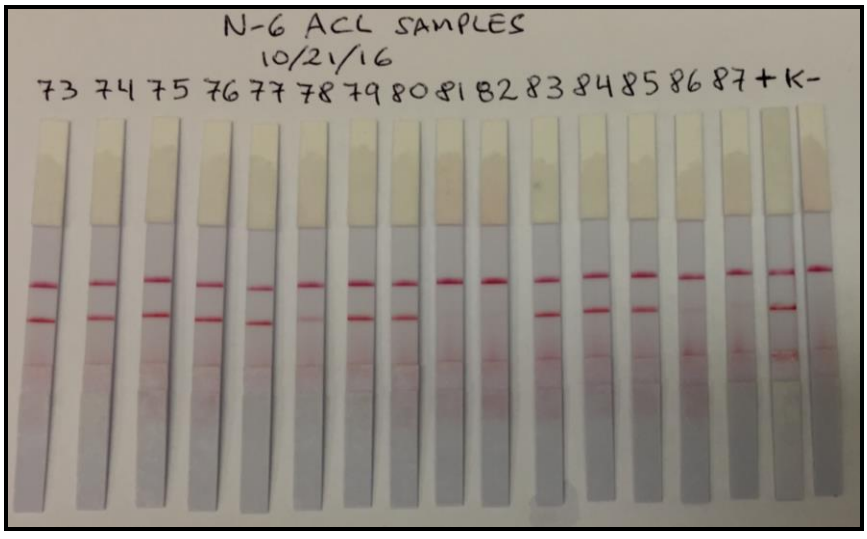

Figure. Representative results of RPA-LF test in clinical samples of patients suspected of having cutaneous leishmaniasis in Peru. Light positive bands on patients 78, 86, and 87 coincide with very weak bands detected in the gel of PCR (gold standard). +: positive control; K-: negative control

Training Activities

A one-week workshop on the utilization of RPA-LF diagnostic test was carried out at NAMRU-6. The principal goal of the workshop was to make research and diagnostic personnel familiar with the molecular tests for cutaneous leishmaniasis. The secondary goal contemplated training on malaria diagnosis using another RPA-LF test developed at UTMB. Both cutaneous leishmaniasis are prevalent diseases in Peru and several countries in the Americas.

A mobile lab was organized in order to have a clean room for preparing the RPA-LF reagents. Also, two separated areas were dedicated to 1) extract the DNA from clinical samples and complete the amplification process and 2) read the amplification products using lateral flow strips.

Participants: personnel from NAMRU-6 Lima, Puerto Maldonado and Cusco, NAMRU-3 Ghana and Cali Colombia participated in this workshop.At the end of the workshop all participants had processed samples and became acquainted with the RPA-LF methods to identify Leishmania and Plasmodium.

Results disseminated to communities

Nothing to report

Plans for the next reporting period

We expect that NAMRU-3 will obtain IRB approval to initiate their corresponding field work.

The collection of clinical samples at NAMRU-6 (Pto. Maldonado) is ongoing and on schedule. We plan to complete the number of patients evaluated by PCR and RPA-LF as proposed in the SOW.

During the last months of the study (2017) personnel from NAMRU-6 will assess the applicability of RPA-LF in the basic lab setting of Madre de Dios. This will determine which procedural details are working or need to be modified to make the tests fully functional under these conditions.

4. IMPACT

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

The RPA-LF is an innovative diagnostic test to detect cutaneous leishmaniasis, which affects populations in tropical or subtropical countries. Its sensitive and specificity and field applicability would simplify and improve diagnosis in civilians and also military personnel deployed in endemic countries.

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 approval for NAMRU-3 (Ghana Detachment) is seriously delayed. It is still in the review stage at the Scientific Review Board, a process that started in August 2016. Once the approval is granted by the SRB it will be forwarded to IRB. Due to this hurdle the field work aim at collecting patient samples could not be initiated. An expedited review process was requested at both the SRB and IRB.

Personnel from NAMRU-3 visited known endemic areas of cutaneous leishmaniasis with the goal of expanding the future area under surveillance (e.g. Hohoe, Kpando and other Districts). Several communities were visited and contacts made with Disease Control Officers and community volunteers. Due to the delay in starting the field studies there was a need to train new staff that will collaborate in the study.

NAMRU-6 has ordered all the necessary reagents to carry out RPA-LF diagnosis, in addition to PCR. On the other hand, NAMRU-3 has started the paperwork to purchase RPA-LF while lateral flow strips will be provided by NAMRU-6 in November.

As mentioned above, implementation of RPA-LF in Puerto Maldonado has been planned

to take place at a later stage of the study. This will allow completing the assessment of the RPA-LF diagnostic efficacy under well-controlled conditions. Then, the final step will involve the performance of the test under stringent field conditions in the basic lab setting of Puerto Maldonado. We expect that a similar schedule of activities will be applied to the NAMRU-3 component.

6. PRODUCTS

Nothing to report

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 (already reported in year 1)

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