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

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RECIPIENT: The Henry M. Jackson Foundation for the Advancement of Military Medicine
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14. ABSTRACT

Background. 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. Leishmaniasis causes a spectrum of diseases that include localized cutaneous leishmaniasis (LCL), and destructive nasal and oropharyngeal lesions of mucosal leishmaniasis (ML). LCL in the New World is most commonly caused by species of the *Viannia* subgenus (*L. braziliensis, L. panamensis, L. guyanensis, L. peruviana*) and to a lesser extent by species of the *Leishmania* subgenus (*L. mexicana*, *L. amazonensis*). Historically, the leishmaniases have had significant impact on military operations. Thousands of cases of visceral and cutaneous leishmaniasis occurred in soldiers in World Wars I and II. 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. Unpublished information indicates that the number of military personnel with cutaneous leishmaniasis could exceed 3,000.

Rationale. A major challenge in the diagnosis of leishmaniasis is that the disease occurs in remote and resource-limited areas of the world with poor or inexistent primary health infrastructure. This also could be true during military field operations and training exercises where sophisticated laboratory equipment and medical personnel are scarce or not available. For CL or ML, scrapings of dermal tissues or punch biopsies of the lesions are necessary and the diagnostic sensitivity by histopathology, microscopy of smears or culture could be unacceptably low (40-70%). The highly sensitive PCR method cannot be implemented in resource-poor settings due to the high costs, personnel training and need of sophisticated equipment. Therefore, novel methods to detect leishmaniasis at the POC are urgently needed. To date, there is no field-standardized molecular method based on 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. Furthermore, RPA does not require refrigeration of reagents and can be adapted easily to lateral flow detection.

Hypothesis: *RPA coupled with a Lateral Flow test strip (RPA-LF) to detect Leishmania DNA will have high sensitivity and specificity to diagnose cutaneous leishmaniasis at the point of care in a field setting.*

Study Design. We propose to utilize for the first time an RPA-based assay coupled with lateral flow (LF) reading to diagnose cutaneous leishmaniasis. We will test novel approaches that could enhance the success of the RPA method in the field, including 1) isolation of DNA from clinical samples using a mini (portable) extractor at the POC or FTA Whatman filter paper specially designed to improve DNA preservation and purification at POC. **Aim 1: To optimize the analytical sensitivity and specificity of the genus- and complex-specific RPA-LF tests using *Leishmania* isolates and clinical samples from collaborating study sites.** We successfully developed *Leishmania spp.* primer sets for RPA that specifically amplified *Leishmania* kinetoplast DNA and were able to detect the equivalent of <10 parasites in spiked clinical specimens. We will compare the analytical sensitivity and specificity of RPA-LF with qPCR using a broad panel of clinical *Leishmania* isolates from the field sites (NAMRU-6 in Peru and NAMRU-3 detachment in Ghana). Clinical validation: A minimum of 20 retrospective convenience samples of clinical specimens known to be parasite positive or negative by PCR sent to UTMB from the field sites will be evaluated by RPA-LF. **Aim 2: To prospectively determine the diagnostic sensitivity and specificity of the RPA-LF test for diagnosis of cutaneous leishmaniasis.** Sub-aim 2.1. New World CL (NAMRU-6): A prospective field trial of the diagnostic test will be conducted in Puerto Maldonado, Madre de Dios, Peru. Based on estimated RPA-LF sensitivity of 95% and specificity of 99% we will enroll 184 positive, parasite confirmed individuals and 42 parasite negative controls to have adequate statistical power. The sensitivity and specificity will be determined using microscopy of dermal samples, and qPCR as the gold standard. Sub-aim 2.2. Old World CL (NAMRU-3): A similar prospective field trial will be conducted through the NAMRU-3 Ghana detachment, at the Noguchi Memorial Institute for Medical Research. Considerable effort will be taken to ensure consistency at the two sites. Patients will be enrolled principally from the villages in the Ho, HoHoe, and Kpando districts of the Volta Region where CL outbreaks due to *L. major* were previously recorded.

The repeatability of the RPA-LF test will be determined in the NAMRU's field sites while the reproducibility will be determined in the central diagnostic lab at UTMB where a subset of samples (10%) of positive and negative individuals will be delivered by the investigators of NAMRU-3 and NAMRU-6.

Training: The project will provide training to field and laboratory personnel, as well as military personnel temporarily stationed in the field of endemic areas to ensure effective deployment of the POC test.

15. SUBJECT TERMS

Cutaneous leishmaniasis-diagnosis-point of care-DNA amplification-field applicable

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	15	**19b. TELEPHONE NUMBER** *(include area code)*
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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, Afghanistan, and Kuwait, 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.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The overarching goal of this project is to test and evaluate a novel Leishmaniasis diagnostic (the RPA-LF) against different parasite species. Completing this study will provide an essential product for the warfighter. Our role in this project is to identify and isolate *Leishmania* samples in South America and to test the RPA-LF with retrospective (year 1) and prospective samples (year 2 & year 3).

What was accomplished under these goals?

During the third year of the project, we reach our enrolment target of 226 participants with clinical suspicion of leishmaniasis from the Peruvian amazon. Collected specimens were used for *Leishmania* detection by kinetoplast-based PCR, RPA-LF assay and species identification by Nested Real Time PCR.

RPA-LF was performed on all collected samples resulting in 137 positives and 89 negatives. In parallel, kinetoplast-DNA PCR was performed on the same dataset resulting in 204 positives and 22 negatives. Real time PCR detected 133 *Leishmania braziliensis* and 2 *L. Lainsoni* whereas the remaining specimens did not yield detectable products.

NAMRU-3 Ghana Detachment accomplishments:
During the first year of the project, retrospective *Leishmania enrietti* parasites isolated in Ghana were blotted on filter paper and shipped to UTMB for testing with the RPA-LF assay in October 2015. These tests demonstrated the proof-of-concept that the RPA-LF primers are capable of detecting *Leishmania enrietti* parasites cultured from CL specimen collected in Ghana.
A total 26 suspected cutaneous leishmaniasis samples were collected from the Volta Region during two enrollment trips, the first on 27th July 2018 and the second from September 12-13, 2018. Out of these samples, 24 were placed in culture, with no growth and 25 were blotted on FTA cards for DNA extraction and molecular testing at the Noguchi Memorial Institute for Medical Research. Two sets of PCRs were performed to detect genetic material from leishmania parasites; minicircle kinetoplast-DNA specific PCRs as well as PCRs that detect the RPS7 intergenic sequence of the leishmania parasite. All the PCRs were negative, except for the positive controls.
25 lesion blots were shipped to the UTMB Texas on Wednesday, 21st November 2018 for confirmatory testing.

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 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 L.enrietti retrospective Isolates were* provided from Ghana
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-3 100% All approvals completed
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	100% Training completed and equipment purchased.
Milestone Achieved: Local IRB and HRPO approved protocols	6	UTMB 100% NAMRU-6 100% NAMRU-3 100%
Milestone(s) Achieved: Coordination meeting completed Approvals of IRBs in place to initiate field studies in human populations RPA-LF test fully adapted for field application on-site molecular diagnosis of cutaneous leishmaniasis in Madre de Dios	12	See specific items described above in the table
Aim 2: To prospectively determine the diagnostic sensitivity and specificity of the RPA-LF for diagnosis of cutaneous leishmaniasis.		
Sub-aim 2.1. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RPA-LF vs. standard kDNA PCR at NAMRU-6; Lima Peru. Delivery of subset of positive and negative clinical samples (10%) from NAMRU-6 to UTMB for reproducibility testing	12 – 36 50	226 samples have been sent to UTMB during the reporting period. 25 samples were shipped to UTMB from Ghana in November 2018
Technical meeting at NAMRU-3, Ghana	14	The technical meeting did not take place

Sub-aim 2.2. To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RPA-LF 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	46-48	25 participants were enrolled with samples collected during the reporting period
Technical meeting at NAMRU-6, Peru		100% 01 training Workshop in RPA-LF developed on May 02-06, 2016
Sub-aim 2.3. To determine differences in RPA-Lateral Flow sensitivity and specificity between NAMRU-6 and NAMRU-3.	45	100% Compared against kDNA, RPA sensitivity and specificity performed at NAMRU-6 is 70.54% (95%CI: 61-78%) and 100% (95%CI: 83-100%), respectively. This has not been completed at NAMRU-3.
Aim 3: To prospectively determine associations between the sensitivity and specificity of RPA the Leishmania species.		
Sub-Aim 3.1: To identify the infecting Leishmania species of all isolates collected in NAMRU-6 for this project by a FRET-based Real Time PCR.	12-44	100% Real Time PCR was performed on all collected samples.
Sub-Aim 3.2: To identify if there is any association between the infecting Leishmania species and RPA-Lateral Flow sensitivity and specificity	12-44	100%
Milestone(s) Achieved: Primary milestone: Validated RPA-Lateral Flow test for Point of Care utilization Secondary milestones: Updated epidemiological assessment of cutaneous leishmaniasis in the endemic areas of Peru and Ghana	48	0% All samples need to be collected in order to perform the statistical analysis

What opportunities for training and professional development has the project provided?

- During the second year of the project, both, Luis Angel Hurtado and Rocio Santos, NAMRU-6 research scientists, were trained in 1. Isolation of DNA from cutaneous leishmaniasis samples of peruvian patient collected by FTA Whatman filter paper protocol, 2. Perform identification of *Leishmania* at genus level by kDNA-PCR, and 3. Determination of *Leishmania* species by Nested Real Time PCR FRET probes-based.
- A training Workshop in RPA-LF took place during May 2-6, 2016.
  The participants were:
  - Dr. Bruno Travi, UTMB (trainer)
  - Dr. Maxy De Los Santos, NAMRU-6
  - Blgist. Rocio Santos, NAMRU-6
  - Blgist. Luis A. Hurtado, NAMRU-6
  - Ms. Naiki Puplampu, NAMRU-3
  - Dr. Diana Carolina Gallego, CIDEIM, Colombia
  - Blgist. Jose Luis Malaga, UPCH-UTMB

  This 05-days workshop consisted of 02 days of theory lectures, and 03 days of laboratory work, were 13 samples collected in Madre de Dios were analyzed.
- During the third year of the project, Dr. De Los Santos, Blgist. Rocio Santos and Blgist. Luis A. Hurtado received training on Leishmaniasis microscopy. This training is key since microscopy is still considered the gold standard for diagnosis and microscopy results could farther be used to assess the potential of RPA as a better alternative.

How were the results disseminated to communities of interest?

During the first year of the project, Dr. Bruno Travi and Dr. Christian Baldeviano presented progress on the project at the Military Health System Research Symposium (MHSRS) in Fort Lauderdale during August 17-20, 2015. No additional dissemination activities have been held during the following two years, as we preferred to collect all data for a final publication.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Testing the RPA-LF on retrospective Leishmania samples isolated in Peru during year one confirmed the performance of the RPA-LF. Preliminary results on 74 samples that were sent to the Travi lab for analysis during this project period. One-hundred percent of the tested samples were confirmed positive

for Leishmaniasis, demonstrating that the assay has high sensitivity to detect parasite from field isolates. Importantly, these results suggest that the RPA-LF could be used to detect Leishmaniasis from US military personnel operating in South America.

What was the impact on other disciplines?

The diagnostic method- isothermal amplification of DNA has impacted the field of molecular biology in austere environments. While the reaction mechanism is used in this project to detect cutaneous leishmaniasis, a parasite that has plagued US soldiers during the war on terror, the technology can be applied to detect a plethora of other pathogens. These pathogens include, but are not limited to: Malaria, HIV, and pox viruses.

What was the impact on technology transfer?

Testing the RPA-LF on retrospective cutaneous Leishmaniasis samples provides an additional proof of concept and only makes the RPA-LF a more enabling technology. We anticipate that the third year of the study (testing prospective samples) might attract commercial partners to help us develop this product further.

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including: transfer of results to entities in government or industry; instances where the research has led to the initiation of a start-up company; or adoption of new practices.

Not applicable at this time. However, we anticipate that completing our testing of all prospective samples will allow to attract commercial partners.

What was the impact on society beyond science and technology?

Cutaneous Leishmaniasis infects approximately 1.2 million people worldwide each year. Infections leave patients disfigured. Rapid identification of infections in soldiers that travel to Leishmania endemic areas would reduce treatment delays and reduce the risk of unnecessary disfigurement during deployment. Employment of a rapid diagnostic for cutaneous leishmaniasis could lead to more effective mass-treatment campaigns in countries that struggle with the parasite (endemically). Because the RPA-LF assay lacks a requirement for a cold chain, the system is a candidate for use in austere settings where access to refrigeration systems is lacking. Rapid identification and treatment of leishmaniasis in these austere, endemic settings could reduce lost work hours and potentially increase productivity (Gross domestic product etc.) for nations where the parasite is endemic.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

- LCDR. Danett Bishop replaced Carmen Lucas as PI of protocol NMRCD.2007.0018 which covers this project.

Actual or anticipated problems or delays and actions or plans to resolve them

- None

Plans to solve the issues:

- None

Changes that had a significant impact on expenditures

- Completion of minor electrical and plumbing upgrades to use equipment and reagents in NAMRU-6, Puerto Maldonado.
- Delay in purchase of reagents and equipment.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

N/A- none.

Significant changes in use or care of human subjects

N/A- none.

Significant changes in use or care of vertebrate animals

N/A- none.

Significant changes in use of biohazards and/or select agents

N/A-none.

6. PRODUCTS

(1) Lay Press: *Nothing to Report*
(2) Peer-Reviewed Scientific Journals: *Nothing to Report*
(3) Invited Articles: *Nothing to Report*

(4) Abstracts: *Nothing to Report*
(5) Books or other non-periodical, one-time publications: *Nothing to Report*
(6) Other publications, conference papers, and presentations: *Nothing to Report*
(7) Website(s) or other Internet site(s): *Nothing to Report*
(8) Inventions, patent applications, and/or licenses: *Nothing to Report*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Personnel	Role	Nearest Person Month
LCDR. Danett Bishop, PhD	PI (Peru site)	1
Contribution: Coordinated overall research effort for project in Peru. Served as PI of the IRB protocol under which this project falls. Recruited appropriate staff to work at the Puerto Maldonado research facility. Planned research project and project modifications with project lead (Dr. Bruno Travi).		
CDR Andrew Letizia	PI (Ghana site)	1
Contribution: Functioned as the lead PI for the project in Ghana. Assisted in writing the SRB, IRB, and DoN HRP approved protocol. Supervised principal contractor (Noguchi Memorial Institute for Medical Reserach).		
Carmen Lucas, MSc	Co-Investigator	1
Contribution: Coordinated transport of samples from Peru to UTMB and amended IRB protocols for use in this project.		
Hugo Valdivia, PhD	Co-Investigator	1
Contribution: Provide support on then amendment of the IRB protocol under which this project falls. Supervise execution of molecular work at NAMRU-6 Lima.		
Maxy B. De los Santos, PhD	Co-investigator	2
Contribution: Responsible for executing molecular work in NAMRU-6		
LCDR Nehkonti Adams	Past-PI (Ghana site)	1
Contribution: Functioned as the lead PI for the project in Ghana while stationed in Ghana. Supervised principal contractor (Noguchi Institute). Her efforts resulted in host country approval of the project.		

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period
Nothing to Report
(**note**: LCDR Sarah-Blythe Ballard was replaced by LCDR. Danett Bishop as partnering PI)

8. **SPECIAL REPORTING REQUIREMENTS**

- Collaborative Awards

This is a collaborative award working with the University of Texas Medical Branch, PI: Bruno L. Travi (PR130282).

- Quad Charts

See appendix.

9. **APPENDICES**

A Novel Field-Deployable Point-of-Care Diagnostic Test for Cutaneous Leishmaniasis
PR130282P1
W81XWH-14-2-0196

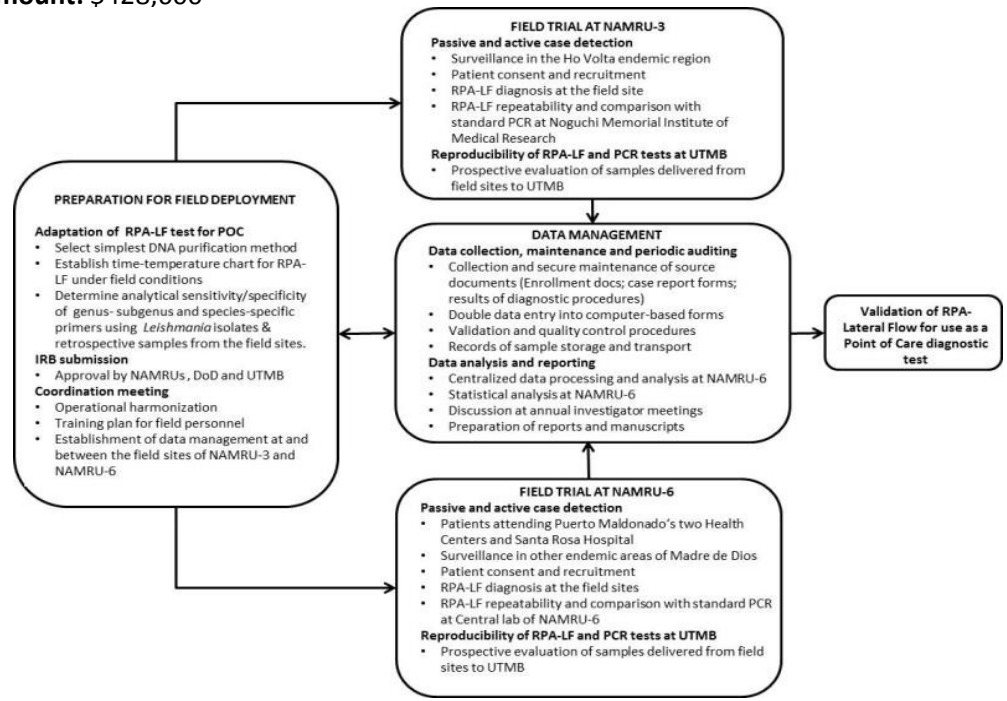
PI: Danett K. Bishop **Org:** The Henry M Jackson Foundation **Award Amount:** $428,600

Study/Product 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.
Aim 3: To prospectively determine associations between the sensitivity and specificity of RPA and the *Leishmania* species.

Approach

Sub-Aim 1.1: To identify temperature constraints for optimal test funcion 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/species-specific primer-probe sets can achieve the same analytical sensitivity/specificity as the genus specific primer-probe set using *Leishmania* isolates and clinical specimens. **Sub-aim 2.1.** To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RPA-Lateral Flow vs. standard kDNA PCR at NAMRU-6. **Sub-aim 2.2.** To recruit suspicious cutaneous leishmaniasis patients and evaluate the sensitivity/specificity of RPA-Lateral Flow vs. standard PCR at NAMRU-3, Ghana detachment. **Sub-aim 2.3**: To determine differences in RPA-Lateral Flow sensitivity/specificity between NAMRU-6 and NAMRU-3. **Sub-Aim 3.1**: To identify the infecting *Leishmania* species of all isolates collected in NAMRU-6 for this project by a FRET-based Real Time PCR. **Sub-Aim 3.2**: To identify if there is any association between the infecting Leishmania species and RAP-Lateral Flow sensitivity and specificity.



Timeline and Cost

Aims	2015	2016	2017	2018
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 .				
Aim 3: To prospectively determine associations between the sensitivity and specificity of RPA and the Leishmania species.				
Actual Expenses ($)	21,487	83,409	159,155	92,490

Updated: (Oct. 30, 2018)

Month 6: Local IRB and HRPO approved protocols. Coordination meeting: **Completed.**
Month 12: Approvals of IRBs in place to initiate field studies in human populations in place. **NAMRU-6 approval obtained;** RPA-Lateral Flow test fully adapted for field application
YEARS 2-3: Month 14: Technical meeting at NAMRU-3-Ghana (**Not executed**)
Month 18: Training workshop held at NAMRU-6, Peru (**Completed on May 2016**)
Month 24: Overall study enrollment: 100% completion. In addition, 50% of samples sent to UTMB for reproducibility testing: **Completed**
Month 36: Overall study enrollment: 100%, progress all collected samples sent to UTMB for reproducibility testing. kDNA-PCR and real time PCR performed in all samples specimens. **Completed on Sept 22-2018**

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**).