

Award Number:
DAMD17-00-C-0030

TITLE:
Leishmania Skin Test

PRINCIPAL INVESTIGATOR:
Nielsen, H.S., Jr., Ph.D.

CONTRACTING ORGANIZATION:
Allermed Laboratories, Inc.
San Diego, CA 92111

REPORT DATE:
March 2010

TYPE OF REPORT:
Final, Phase II Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 01-03-2010		2. REPORT TYPE FINAL, PHASE II ADDENDUM		3. DATES COVERED (From - To) 1 APR 2009 - 28 FEB 2010	
4. TITLE AND SUBTITLE Leishmania Skin Test				5a. CONTRACT NUMBER DAMD17-00-C-0030	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) H. S. Nielsen, Jr.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Allermed Laboratories, Inc. San Diego CA 92111				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical and Materiel Command Fort Detrick MD 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT A phase IIB study was conducted to evaluate the sensitizing properties of LtSTA in naïve adult volunteers. Three doses of the product were administered intradermally at 30 day intervals. The doses administered were 15µg, 30µg and 50µg. Study subjects received the same dose each time the product was injected on the forearm. Positive and negative skin test controls were applied concurrently with LtSTA to insure accurate readings. Positive delayed-type hypersensitivity reactions did not occur on the first and second skin test with the 30µg and 50µg doses. However, a positive skin test was observed in some individuals who received the 30µg and 50µg doses on the third skin test. Positive tests were not observed to the 15ug dose on the third skin test, but fewer subjects participated in the 15µg cohort. Based on the results of a phase II study conducted in 2007 and the phase IIB study conducted in 2009, a dose of 50µg will be used in the design of a phase III clinical trial.					
15. SUBJECT TERMS LtSTA = <i>Leishmania tropica</i> Skin Test Antigen					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 15	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

1. INTRODUCTION.....	4
2. BACKGROUND.....	4
3. BODY OF REPORT	5
3.1 Statement of Work	5
3.2 Key Accomplishments	5
3.3 Reportable Outcomes	6
3.3.1 Phase IIB Sensitization Study Results:.....	6
3.3.2 Dose of LtSTA for Phase III Clinical Trial	6
3.3.2.1 Preparation of the Drug Substance (Promastigote Lysate).....	7
3.3.2.1.1 Media Preparation.....	7
3.3.2.1.2 Scale-up.....	8
3.3.2.1.3 Aeration of Celstirs	8
3.3.2.1.4 Harvest and Wash	8
3.3.2.1.5 Microfluidization and heat treatment.....	8
3.3.2.1.6 Filtration.....	9
3.3.2.1.7 Testing.....	9
3.3.2.2 Preparation of the Drug Product (LtSTA)	9
3.3.2.3. Formulation of Bulk Drug Product.....	11
3.3.2.4. Final container Testing	11
3.3.2.4.1 LtSTA Identity Test.....	12
3.3.2.4.2. LtSTA potency Test.....	12
3.3.3 Cross-Reactivity of LtSTA with <i>Leishmania infantum</i>	13
3.3.4 Intended Use of LtSTA.....	14
4. CONCLUSIONS.....	13
5. REFERENCES.....	14

1. INTRODUCTION

This report covers the research conducted between April 1, 2009 and February 28, 2010 on a Leishmania Skin Test (LtSTA) developed from the promastigotes of *Leishmania tropica*.

During this period a phase IIB study was in progress in which the sensitizing capacity of three doses of the product was tested in *Leishmania* naïve individuals. Other activities included modifications in the manufacture of the product, cross reactivity studies with *L. infantum*, meetings in June and December with representatives of USAMMDA regarding the status of the project and the preparation of summary documents intended to provide the basis for a pre-phase III Type B Meeting with the Food and Drug Administration.

2. BACKGROUND

In a phase II trial conducted in 2007, a 30 µg dose of LtSTA was shown to be safe and effective in detecting cellular hypersensitivity to *L. major* in persons with current or past infection with this parasite. The 30 µg dose elicited a positive DTH response in 100% of persons with active cutaneous leishmaniasis. In persons with cured *L. major* disease, 85% were DTH positive to the 30 µg dose; the remaining 15% of this group who were skin test negative to 30 µg dose were DTH positive to LtSTA when skin tested with 50 µg of the antigen.

Previously published studies reported that repeat skin tests with preparations made from *Leishmania* promastigotes caused positive delayed-type reactions in naïve individuals. These observations demonstrated that antigenic materials obtained from various species of *Leishmania* were immunogenic and could result in the sensitization of persons that had not previously encountered the parasite. Weigle et al.⁽¹⁾ found that antigen containing 10×10^6 /mL of merthiolate -fixed promastigote divided between *L. panamensis* and *L. amazonensis* did not induce DTH reactions in 19 healthy *Leishmania* naïve adults when the antigen was administered two or three times.

Mayrink et al.⁽²⁾ observed that 100 persons who were non-reactive to a skin test consisting of sonicated *Leishmania* promastigotes remained non-reactive to the same antigen when tested 3 months later.

Sutti et al.⁽³⁾ studied the effects of repeat skin tests in healthy volunteers residing in a leishmaniasis non-endemic area. The *Leishmania* antigen used in the study was made from *L. major* containing 5×10^5 promastigotes/0.1 mL. Subjects were given three doses of the antigen at 0, 30 and 90 day time points. Conversion from negative to positive DTH reaction was not observed in the six subjects included in the protocol. These investigators contrasted their findings with those of Nascimento et al.⁽⁴⁾ who demonstrated DTH conversion using repeat doses of a *Leishmania* skin test antigen made from equal parts *L. braziliensis*, *L. amazonensis* and *L. guyanensis* at a protein concentration of 4.0 µg/0.1 mL.

Jose et al.⁽⁵⁾ reported that twelve *Leishmania* naïve volunteers in Group I of their study who were skin tested with antigen containing 25 µg/0.1 mL of *L. amazonensis* protein converted from a DTH negative response to a DTH positive response. Their results were as follows: after

30 days the second skin test was positive in 33% of subjects; after 90 days, the skin test was positive in 67% of subjects.

The extent to which sensitization might occur with the repeat use of LtSTA was a primary concern in the development of this test for use in military personnel serving in *Leishmania* endemic areas, such as Iraq and Afghanistan. For this reason, a study was designed to assess the sensitizing capacity of the two doses of LtSTA that were used in the phase II clinical trial conducted in 2007 in Tunisia. The doses employed in the 2007 study were 30µg and 50µg. A third dose of 15µg was added to the phase IIB study protocol to determine if sensitization was dose dependent.

3. BODY OF REPORT

3.1 Statement of Work

- 3.1.1 Complete a phase IIB Sensitization Study conducted in San Diego, CA.
- 3.1.2 Analyze data collected from the phase IIB Sensitization Study and submit a final study report to USAMMDA, HRPO and FDA.
- 3.1.3 Meet with representative of USAMMDA and discuss the outcome of the clinical studies of LtSTA and plan a phase III clinical trial.
- 3.1.4 Submit documents to FDA supporting a pre-phase III Type B Meeting.
- 3.1.5 Continue work on the Chemistry, Manufacturing and Controls of LtSTA in support of a Biologics License Application.

3.2 Key Accomplishments

- 3.2.1 Phase IIB Sensitization Study was completed on January 13, 2010.
- 3.2.2 Data from phase IIB Sensitization Study were analyzed and a final report submitted to USAMMDA, HRPO and FDA on January 27, 2010
- 3.2.3 Meetings were held with representatives of USAMMDA on June 1, 2009 at Fort Detrick, MD and on December 9, 2009 at Allermid Laboratories, Inc. in San Diego, CA. The current status of product development and the intended use of the product were discussed during these meetings.
- 3.2.4 Documents relating to the current status of product development were submitted to the FDA to the attention of Dr. Joseph Temenak in support of a Pre-phase III Type B Meeting application. Allermid was advised that a written response was being prepared by the FDA regarding the chemistry, manufacturing and controls of the product, as well as product safety and efficacy as reported in the phase I, phase II and phase IIB clinical trials.
- 3.2.5 Work continues on the validation of manufacturing and testing methods for the 50µg dose of LtSTA. At the present time, future clinical trials with LtSTA will be conducted using a 50µg dose of the product.

3.3 Reportable Outcomes

3.3.1 Phase IIB Sensitization Study Results:

Sensitization to LtSTA at doses of 15 µg, 30 µg and 50 µg did not occur after two skin tests administered at 30-day intervals. However, conversion from DTH negative to DTH positive after three intracutaneous tests administered at 30-day intervals was observed in one of 10 subjects tested with a 50 µg dose of LtSTA and in one of twenty-three subjects tested with a 30 µg dose of antigen. In eight subjects that received a 15 µg dose of the product, conversion from DTH negative to DTH positive was not observed.

Based on the observation that dose concentrations of 30 µg and 50 µg each elicited positive DTH responses in one individual following the third intracutaneous injection of the antigen, the LtSTA skin test at these doses should not be used more than two times in the same individual. Data obtained from the phase I safety trial in which doses of 20, 40, 80 and 120 µg were tested in *Leishmania* naïve persons confirmed the absence of false positive DTH responses to the product following a single skin test. The data obtained in this investigation show that false positive DTH responses do not occur after two skin tests at dose concentrations of 15, 30 or 50 µg. This finding indicates that it is possible to use a LtSTA skin test two times in the same individual without concern of sensitization from a previous skin test.

Adverse events that occurred in study participants were largely expected events that are known to occur following the administration of a skin test antigen. Local AE included itching, swelling and pain at the site of the skin test. These events were mostly mild and occurred within the first 48 hours after skin testing. Systemic events included body ache, weakness, dizziness and nausea. The cause of these events is not known, since a skin test with a positive DTH control, placebo and saline were administered concurrently with LtSTA. Unlike local AE where the reaction site can be identified with a specific article, it was not possible to identify the article responsible for a systemic AE. One unexplained adverse event occurred that involved an increase in protein and erythrocytes in the end-of-study urine specimen of one male subject.

All laboratory data were reviewed by the principal investigator. Laboratory tests were repeated if out-of-range results were considered clinically significant. Trace (T) values were not considered to be clinically significant in pre and post study evaluations. Subjects in whom pre-study values were normal and post-study values were above normal were asked to repeat the laboratory test. However, not all subjects were compliant in following the investigator's recommendation to have a repeat test performed.

3.3.2 Dose of LtSTA for Phase III Clinical Trial

Based on the outcome of the phase IIB trial, manufacturing, testing and validation work on LtSTA were revised to support a 50 µg dose of LtSTA. This dose was selected for continued study in a phase III trial, because it showed a greater degree of sensitivity

in detecting prior exposure to *L. major* in the phase II trial, even though it appeared to be slightly more sensitizing in naïve persons than the 30µg dose in the phase IIB trial.

Refinements in the manufacture and quality testing of 50µg LtSTA are being made in accordance with current Good Manufacturing Practices. Developmental procedures relating to increasing the yield of source material and the stability, identity and potency of the final product are being addressed and validated. The current materials of manufacture and testing for the 50µg dose are summarized below.

3.3.2.1 Preparation of the Drug Substance (Promastigote Lysate)

The drug substance is a heat-treated, soluble lysate of the promastigote phase of Strain WR#1063:C1A. The lysate is prepared through a process of microfluidization of the whole promastigote cells, followed by heating at 60°C for 30 minutes, a series of centrifugations, and sterile filtration to produce the drug substance. The drug substance is prepared with the raw materials listed in Table 2 according to the procedures described below and outlined in Figure 2.

Table 2: Materials Used to Manufacture the Drug Substance

Component	Grade	Source	Catalog Number
Schneider's Insect Medium	N/A	Sigma	S9895
Sodium Chloride	USP	Mallinckrodt	7713
Potassium Chloride	USP	Spectrum	P0170
Sodium Phosphate, Anhydrous, Dibasic	USP	Spectrum	S0138
Potassium Phosphate, Monobasic	ACS	Spectrum	P1380
Polysorbate 80	NF	Spectrum	P0138
Hydrochloric Acid	ACS	Spectrum	H0135
Deionized Water	N/A	Allermed	N/A

3.3.2.1.1 Media Preparation

Schneider's Insect Medium (Sigma-Aldrich, Inc., product number S9895) containing Fetal Bovine Serum (FBS) is the culture medium that is used to propagate *L. tropica* promastigotes. The FBS is γ -irradiated manufactured by SAFC Biosciences. A comparability protocol performed at Allermed confirmed that parasites grown in γ -irradiated FBS are comparable to parasites grown in heat-inactivated FBS. Antibiotics are not used in culture media.

Schneider's Insect Medium base is prepared with deionized (DI) water and FBS at the appropriate concentration. Phosphate buffered saline is added to prepare Half-Schneider's Medium. The medium is filter sterilized into sterile bottles or large culture vessels through a 0.2 µm filter. Containers are labeled with the media name, lot number, expiration date and the initials of the person responsible for preparation. Media is tested for sterility and stored under quarantine at 1 - 5°C until released for use.

3.3.2.1.2 Scale-up

All *L. tropica* cultures are grown at $25.0 \pm 1.5^\circ\text{C}$ in an environmental chamber. All manipulations involving live parasites are performed aseptically under BSL2 conditions in a Class 100 Biological Safety Cabinet located within a Class 10,000 cleanroom. For initiation of production cultures, a single WCB vial is removed from cryostorage, thawed, and reactivated. All cultures are microscopically observed for contamination at the time they are subcultured. Subcultures and production batch cultures are inoculated with $\sim 4 \times 10^6$ parasites/mL.

Cultures are grown in T-25 flasks in Schneider's containing 10% FBS and subsequently transferred to T-150 and T-300 flasks containing the same medium. The T-25 culture is expanded to a T-150 flask and then into three T-300 flasks. The production-size culture is grown in 3 L Celstir suspension bottles (Celstir, Wheaton cat#356887) containing Half-Schneider's with 5% FBS. Four Celstir suspension bottles containing 1.5 L of media each are inoculated with the parasites from the combined T-300 cultures. After inoculation, the Celstirs are transferred to the $25^\circ\text{C} \pm 1.5^\circ\text{C}$ incubator and placed on a magnetic stir plate.

3.3.2.1.3 Aeration of Celstirs

Tubing is connected to the inlet of a sterile filter that is used to supply medical-grade compressed air into the Celstirs. The flow rate is approximately one bubble per second per Celstir. Production batch cultures are microscopically observed on the day of harvest for promastigote morphology and microbial contamination. A cell count is taken at this time. A culture must be in the stationary phase of development (determined by microscopic examination) and meet morphology and purity requirements before it is harvested and retained for further processing.

3.3.2.1.4 Harvest and Wash

Cultures are harvested by centrifugation at $7,000\times g$ for 15 minutes. The supernatant is discarded and the pelleted cells are resuspended in 0.85% saline (SAL) and centrifuged at $7000\times g$ for 15 minutes. This procedure is repeated five times to remove media components. The total volume of SAL used is 1.5 times the original combined culture volume. Samples are taken prior to the last centrifugation step to determine yield (cell concentration), bioburden and morphology. The final centrifugation run is performed using a conical shaped 250mL tube which is centrifuged at $6,000\times g$ for 30 minutes. The supernatant is removed and the pellet (cell paste) is stored at -80°C .

3.3.2.1.5 Microfluidization and heat treatment

Promastigote Disruption and Centrifugation

Promastigotes are disrupted and homogenized by microfluidization. The pelleted cells are suspended in 0.85% saline containing 0.0001% polysorbate 80, NF (SALT) and processed through a Microfluidics microfluidizer model 110Y at a pressure of $\sim 19,000$

psi for 10 passes. Process pressure is monitored and the product path is cooled with an ice bath. Upon completion of the microfluidization process the lysate is centrifuged at 18,500xg for 30 minutes at 8°C. The pellet is discarded and the supernatant retained for further processing.

Heat Treatment, Cooling and Centrifugation

The microfluidized lysate is placed in a 60°C water bath for approximately 20 minutes until it reaches 60°C. It is held at 60°C for 30 minutes, and then transferred to an ice bath for approximately 5 minutes until it reaches 10°C. The lysate is centrifuged for 30 minutes at 18,500xg at 8°C to remove any non-soluble materials resulting from the heating process. The supernatant is saved and the pellet is discarded. The total process takes less than 1 hour.

3.3.2.1.6 Filtration

The supernatant from the centrifugation is filtered through a sterile 0.2 µm Sartorius cellulose acetate capsule filter (part# 18052D) into a sterile, depyrogenated Pyrex® bottle. This material is the **Drug Substance**.

3.3.2.1.7 Testing

The drug substance is sampled for protein content (Ninhydrin), SDS-PAGE and non-viability testing. See Table 3 below:

Table 3: Drug Substance Specifications

Test	Method	Specification
SDS-PAGE	SOP 917-101	Characteristic Bands
Protein (Ninhydrin)	SOP 916-001	*1.5mg/mL – 4.0mg/mL
Non-Viability	SOP 918-005	Non-viable

*The protein content is used as a guide to determine the dilution necessary to achieve a final protein concentration.

3.3.2.2 Preparation of the Drug Product (LtSTA)

The drug product is prepared by diluting the drug substance to a pre-determined Ninhydrin protein concentration in buffered, stabilized and preserved diluent. The final product is referred to as *Leishmania tropica* Skin Test Antigen (LtSTA). Figure 3 is a schematic diagram of the Drug Product manufacturing process.

Table 4 contains a list of all components used to manufacture LtSTA.

Table 4: Components Used to Formulate Drug Product

Component	Grade	Source	Catalog Number	Purpose
Sodium Phosphate, Monobasic, Anhydrous	USP	Spectrum	S0187-10	pH Stabilizer
Sodium Phosphate, Dibasic, Anhydrous	USP	Spectrum	S0138-10	pH Stabilizer
Sodium Chloride	USP	Mallinckrodt	7713	Isotonicity
Polysorbate 80	NF	Spectrum	P0138	Protein Stabilizer
Glycerin, 99.5% (synthetic)	USP	Spectrum	G1015	Protein Stabilizer
Liquefied Phenol	USP	Spectrum	PH125	Preservative
Water for Injection	USP	Allermed Laboratories	N/A	Diluent

Two diluents are manufactured to dilute the drug substance to skin test strength. Both diluents contain phenol as the preservative. These phosphate buffered saline diluents are referred to as 2X Phosphate Diluent and 1X Phosphate Diluent. The 2X Phosphate Diluent is used to expand the volume of the Drug Substance by combining the two in equal parts. The 1X Phosphate Diluent is used to dilute the formulated material to the desired protein concentration. (**Attachment L**).

Table 5: 2X Phosphate Diluent Formulation

Component	Quantity/Liter	Concentration
Sodium Chloride, USP	8.5 grams	0.85%
Sodium Phosphate Monobasic, Anhydrous, USP	2.22 grams	50 mM Phosphate
Sodium Phosphate, Dibasic, Anhydrous, USP	4.48 grams	
Glycerin, USP	20 mL	2.00%
Phenol, USP	8.8 mL	0.80%
Water for Injection, USP	Q.S. to 1.0 Liter	N/A

Table 6: 1X Phosphate Diluent Formulation

Component	Quantity/Liter	Concentration
2X Phosphate Diluent	500 mL	1X (50% of 2X)
0.85% Saline (SAL)	500 mL	0.85%

Ten percent (10%) Polysorbate 80 solution (w/v) is added to the formulated product to a final concentration of 0.01%.

Table 7: 10% Polysorbate 80 Solution

Component	Quantity/Liter	Concentration
Polysorbate 80, NF (Tween 80)	100 mL	10% w/v
Water for Injection, USP	900 mL	N/A

3.3.2.3. Formulation of Bulk Drug Product

A calculation is performed to determine the correct volume of 1X phosphate diluent necessary to achieve a target protein concentration in the Drug Product. The final formulation contains 0.85% sodium chloride, 0.4% phenol, 0.01% (w/v) polysorbate 80, 1% (v/v) glycerin, in a 25 mM phosphate buffer.

The formulated bulk is mixed and sampled for protein and phenol prior to sterile filtration. Adjustments to protein and phenol may be made one time with 1X phosphate diluent. Bioburden is tested after any adjustment is made and prior to sterile filtration.

The bulk product is aseptically filtered through a sterile cellulose acetate 0.2 µm filter (Sartorius #18052D) into a sterile, depyrogenated glass container. A bubble-point test is performed on the filter after use to confirm that the filter was integral. The bulk product is tested for sterility.

The released sterile bulk drug product is aseptically filled at a volume of 1.3 mL into 2 mL sterile, depyrogenated Type I glass tubing vials (Comar 24-0065-001) using platinum-cured silicone tubing and a Wheaton peristaltic pump. Filling takes place in a Class 100 laminar flow hood within a Class 10,000 cleanroom. Depyrogenated 13 mm West V-35 4405/50 gray stoppers are inserted in to the vials and sealed with West 13 mm aluminum seals crimped onto the vial neck.

3.3.2.4. Final container Testing

Quality control tests (see Table 8) are performed on the final product. If all final container testing shows that the drug product is within specifications, it is released for inspection and labeling. The product is stored at 2 - 8°C.

Table 8: Final Container Testing Release Criteria.

Assay	Method	Acceptance Limits
Final Sterility	SOP 918-003	<i>Sterile</i>
General Safety	SOP 908-000	<i>Pass</i>
pH	SOP 405-100	<i>6.5 – 7.5</i>
Visual	SOP 651-000	<i>Clear, No Defects</i>
BSA Content	SOP 931-200	<i><500ng/mL</i>
Phenol	SOP 930-000	<i>0.34 – 0.46%</i>
Sodium Chloride	SOP 969-000	<i>0.80 – 0.95%</i>
Phosphate	SOP 966-100	<i>2.07 – 2.67 mg/mL</i>
Glycerin	SOP 914-000	<i>0.85% - 1.10%</i>
SDS-PAGE	SOP 917-101	<i>Bands at 8, 20, 25, 51 and 70 KD</i>
Protein	SOP 916-001	<i>TBE*</i>
Identity by ELISA	SOP 944-102	<i>Pass</i>
Potency	DP 0018	<i>Mean induration @ 24 hrs. 6-12mm</i>

3.3.2.4.1 LtSTA Identity Test

The LtSTA Identity test is performed using an indirect ELISA procedure with rabbit anti-*Leishmania tropica* serum as the primary antibody and goat anti-rabbit IgG HRP, as the secondary antibody. This reaction is visualized with ABTS, and read spectrophotometrically at 405nm. The positive *Leishmania* control and LtSTA lots produced a dark color in comparison to the negative controls. The background controls showed no sign of color development.

Acceptance Criteria

1. The average absorbance (OD) values for negative controls shall be ≤ 0.300 .
2. The average absorbance values for *Leishmania tropica* Reference Standard (LRS) and LtSTA lots (test samples) shall be $\geq 2x$ (the highest control absorbance value among background and negative controls).
3. The average absorbance of the background controls shall be ≤ 0.100 .
4. All assays performed shall meet the acceptance criteria.

3.3.2.4.2 LtSTA potency Test

LtSTA is tested in parallel with the *Leishmania tropica* Reference Standard (LRS) to estimate the potency of a LtSTA lot with respect to the reference LRS. The assigned potency of LRS is 50 μ g per 0.1mL.

Four dilutions of LRS and a LtSTA lot are prepared. Starting with the undiluted strength (1:1), three five-fold serial dilutions of 1:5, 1:25 and 1:125 are made as follows:

- Dilution 1 = 1:1 (undiluted strength of the lot)
- Dilution 2 = 1:5 (one fifth the strength of the lot)
- Dilution 3 = 1:25 (one twenty-fifth of the strength of the lot)
- Dilution 4 = 1:125 (one hundred twenty fifth the strength of the lot)

Determining Reactivity

This reaction for each test site (on each animal) is determined by averaging the logest orthogonal axis measurements.

Evaluation of Results:

1. For a test to be valid, the following conditions must be met:
 - a. The p-value for slope significance for LRS and Test Lot must be < 0.1 .
 - b. The p-value for equality of slopes must be > 0.01 .
2. If a test does not meet the validity criteria, the test is invalid and must be repeated.

3.3.3 Cross-Reactivity of LtSTA with *Leishmania infantum*

Because the future use of LtSTA may involve persons infected with *L. infantum*, cross reactivity studies were conducted with *L. infantum* to determine if LtSTA could elicit a positive DTH response in guinea pigs that had been sensitized to *L. infantum*. A similar study was conducted in guinea pigs sensitized to *L. major* prior to conducting the phase II clinical trial involving *L. major* sensitized human volunteers. As with *L. major*, LtSTA proved to be efficacious in detecting delayed -type hypersensitivity to *L. infantum* using this animal model. A summary of the work is reported below:

Two isolates of *Leishmania infantum* were received from Walter Reed Research Institute with the following information:

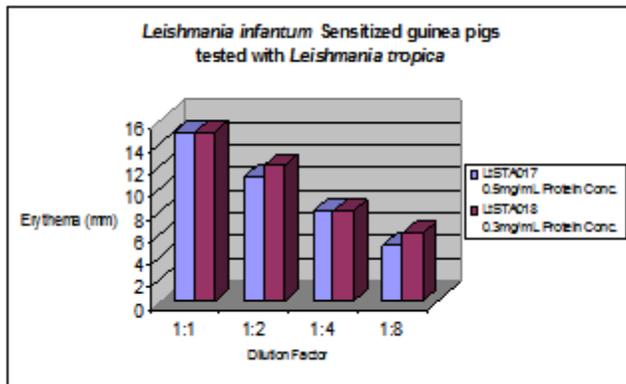
WR2709 – *L. infantum* from Afghanistan, cultured in Schneider's media with 25% FBS

WR2808 – *L. infantum* from Turkey, cultured in Schneider's media with 25% FBS.

The two isolates were cultured, harvested, and stored at -80°C for further processing. A cell concentration was determined by Spectrophotometer using OD_{600} . The material was then taken out of the freezer and placed in a water bath until thawed then placed in a container filled with liquid nitrogen until frozen. This Freeze/Thaw process was performed for 12 cycles. A sample was taken for viability testing. An additional sample was withdrawn for protein concentration. The results of this testing showed that the protein concentration was approximately 1.0 mg/mL and the material non-viable and suitable for use.

Isolate WR2709 (from Afghanistan) was then used to sensitize hairless guinea pigs. The sensitization procedure was performed by mixing Freund's adjuvant (complete) and the F/T antigen until an emulsion was formed.

The *L. infantum* sensitized guinea pigs were given a two week rest period before performing a cross-reactivity study using *Leishmania tropica* skin test antigen (product). Two separate lots with protein concentrations of 0.5mg/mL and 0.3mg/mL were diluted using four 2-fold dilutions and placing them side by side on the guinea pigs. The results clearly demonstrated strong cross-reactivity as shown below:



3.3.4 Intended Use of LtSTA

From a strategic standpoint, a *Leishmania* skin test antigen can be used to identify U.S. service members who have been infected with the parasite during deployment to an endemic area. The test can be used in the same manner that the tuberculin skin test is used to identify persons exposed to *Mycobacterium tuberculosis*. Specifically a *Leishmania* skin test can be effectively used to: (1) confirm the diagnosis of cutaneous leishmaniasis in the field, so treatment can be started without transporting the infected person to a military hospital, (2) test other personnel in military units in which active cases have occurred and (3) test military personnel leaving an endemic service area, such as Afghanistan, for latent infection caused by *L.infantum*, the agent of visceral leishmaniasis (This organism can remain viable and potentially infective for months/years without causing overt disease until the infected individual becomes immune-suppressed due to other disabling conditions.).

Within the present geopolitical climate, it is possible that U.S. military personnel will see service in *Leishmania* endemic regions. Based on the known incidence of cutaneous leishmaniasis in Iraq, Afghanistan, Syria, and Iran, the possibility of active military, reservists, National Guard and U.S. civilians who serve as auxiliary personnel becoming exposed to *Leishmania* parasites must be considered. The availability of a *Leishmania* skin test antigen which is licensed by the Food and Drug Administration would be a valuable tool to protect the health and welfare of these individuals.

Physicians in the United States must be alert to the possibility of *Leishmania* infection in travelers returning from endemic areas. Fifty to one-hundred cases of cutaneous leishmaniasis are diagnosed each year. As civilian travel increases the incidence of the disease can be expected to increase. In addition, non-military support personnel returning from combat areas are at risk of latent infection with *Leishmania* becoming clinically active and misdiagnosed.

4. CONCLUSIONS

Work performed between April 1, 2009 and February 28, 2010 provided data that were needed to design a phase III clinical trial using a dose of LtSTA that will provide acceptable sensitivity and specificity for a skin test antigen.

At a concentration of 50µg/0.1mL, LtSTA may be administered two times to *Leishmania* naïve individuals without causing false positive results.

Future lots of LtSTA will be manufactured and tested at a concentration of 50µg/0.1mL and process validation and stability testing will be performed with product made at this concentration.

5. REFERENCES

1. Weigle, K.A., Valderrama, L., Arias, A.L., Santrich, C. & Saravia, N.G. (1991). Leishmanin skin test standardization and evaluation of safety, dose, storage, longevity or reaction and sensitization. *American Journal Trop Med Hyg*, 44, 260-271.
2. Mayrink, W., Da Costa, C.A., Magalhaes, P.A., Melo, N.M., Oliveira Lima, A., Michallick, M.S., Williams, P. (1979). A field trial of a vaccine against American dermal leishmaniasis. *Trans R Soc Trop Med Hyg* 73:385-387.
3. Satti, I., Hassan, A., Khalil, A.G., Akuffo, H. (2002). The effect of repeated leishmanin skin testing on the immune response to *Leishmania* antigen in healthy volunteers. . *Trans R Soc Trop Med Hyg* 96:565-567.
4. Nascimento, M.D., Alcantara-Neves, N.M., Muniz, M.E., Nunes, S.F., Paranhos, M. & Pontes de Carvalho, L.C. (1993). Induction and modulation of the immune response to *Leishmania* by Montenegro's skin test. . *Trans R Soc Trop Med Hyg* 87:91-93.
5. Jose, F.F., da Silva, I.M., Araujo, M.I. Almeida, R.P., Bacellar, O., Carvalho, E.M. (2001). Evaluation of the sensitization power of Montenegro skin test. *Rev Soc Bras Med Trop* 34:537-542.