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PRINCIPAL INVESTIGATOR: Dr. Peter J. Hotez

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Peter J. Hotez

E-Mail: hotez@bcm.edu

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14. ABSTRACT

Building on extensive preliminary data and an established consortium of academic, government, military, and product development partners, we propose to develop a production process for a novel, bivalent vaccine against leishmaniasis, a serious neglected tropical disease (NTD) of military and civilian personnel now spreading rapidly in conflict zones of the Middle East and Central Asia. The proposed Cutaneous Leishmania Vaccine (CL-Vax) is a bivalent, recombinant protein-based vaccine that will be comprised of a specific Leishmania parasite antigen together with a novel sand fly salivary antigen, co-administered at bedside with an adequate adjuvant. The components of the vaccine will be extensively characterized, and their immunogenicity and efficacy will be confirmed in animal models.

15. SUBJECT TERMS

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1. Introduction

This proposal seeks to develop an effective, safe, and innovative vaccine to combat cutaneous leishmaniasis (CL), a neglected tropical disease caused by protozoan parasites of the genus *Leishmania* and transmitted to humans by the bite of a phlebotomine sand fly. Building on extensive preliminary data, we are developing a production process for a novel, bivalent vaccine against leishmaniasis and test its immunogenicity and efficacy in a mouse model of disease. The Cutaneous Leishmania Vaccine, CL-Vax, is based on recombinant proteins and is comprised of a specific *Leishmania* parasite antigen (LdNH36) together with a novel sand fly salivary antigen (PpSP15), co-administered at bedside with an adequate adjuvant (GLA-SE). Our hypothesis is that vaccination with CL-Vax is much more efficacious in reducing the lesion size caused by the infection and in reducing the parasite count at the infection site, than vaccination with just the *Leishmania vaccine* antigen alone. CL-Vax would induce a robust immune response to two independent antigens that are co-localized at the site of infected bites. Our approach builds on more than a decade of preliminary studies and publications.

2. Keywords

Sand fly; cutaneous leishmaniasis; LdNH36; PpSP15; Process development; neglected tropical diseases; bivalent vaccine; salivary antigen

3. Accomplishments (For activities scheduled for Year 1).

What were the major goals of the project?

Specific Aim 1 To develop a scaled-up process for the production of the sand fly antigen, PpSP15, and to produce PpSP15 and LdNH36 for immunogenicity and efficacy studies.	**Timeline**
Major Task 1, Activity 1.1. Expression and purification of LdNH36-dg2.	Months
Subtask 1: Expression	1
Subtask 2: Purification	2-4
Milestone(s) Achieved: LdNH36 expressed	2
Milestone Achieved: LdNH36 purified, available for Aims 2 and 3	4
Major Task 2, Activity 1.2. Cloning and small-scale expression of PpSP15.	Months
Subtask 1: Cloning, Transformation	1
Subtask 2: Small-scale expression	1-4
Milestone(s) Achieved: PpSP15 is expressed	4
Major Task 3, Activity 1.3. Process development for PpSP15 production.	Months
Subtask 1: Activity 1.3.1. Fermentation.	3-12
Subtask 2: Activity 1.3.2. Purification of PpSP15.	3-12
Subtask 3: Activity 1.3.3. Depyrogenation of PpSP15.	5-12
Milestone(s) Achieved: PpSP15 is expressed at the 5 L scale in a fermenter.	6
Milestone(s) Achieved: Fermentation process is optimized.	12
Milestone(s) Achieved: PpSP15 is available for initial studies under Aim 2 and 3.	6
Major Task 4, Activity 1.4. Assay development for PpSP15.	Months
Subtask 1: Protein characterization and assay development.	1-12
Milestone(s) Achieved: Assays developed for characterization of PpSP15.	12
Specific Aim 2. To test the immunogenicity of PpSP15 and LdNH36 given at various dosages.	
Major Task 7, Activity 2.1: Immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: route and dose ranging evaluation.	Months
Subtask 1: Write animal protocol and submit for review	1
Subtask 2: Test immunogenicity of PpSP15 and LdNH36 antigens given at various doses	6-12
Subtask 3: Test immunogenicity of PpSP15 and LdNH36 antigens given by ID, SQ, IM administration routes	6-12
Milestone(s) Achieved: Local IACUC Approval	3
Milestone(s) Achieved: ACURO Approval	6
Milestone(s) Achieved: Having set the optimal dose and route of administration for PpSP15 and LdNH36 vaccine.	12
Specific Aim 3. To evaluate the efficacy of PpSP15 and LdNH36 as a vaccine in protecting mice	Months

challenged by the natural mode of transmission, L. major infected sand flies.	
Major Task 9, Activity 3.1: Update the insectary facility to have a high capacity Phlebotomus papatasi sand fly colony for transmission experiments.	1-36

What was accomplished under these goals?

Specific Aim 1: To develop a scaled-up process for the production of the sand fly antigen, PpSP15, and to produce PpSP15 and LdNH36 for immunogenicity and efficacy studies.

We have already developed and recently published a low-cost process for the expression and purification for the recombinant LdNH36 antigen from *Escherichia coli* (McAtee *et al.*, 2017)[1]. For this proposal, we sought funding for a 10 L non-GMP production campaign to produce the necessary LdNH36 in quantities sufficient to evaluate its co-administration with PpSP15. In addition, for PpSP15, activities under this objective include the development and optimization of a production process at the 5 L scale, including up to three reproducibility runs. Overall quality management and documentation oversight will be provided to ensure seamless technology transfer. Throughout this objective, we expect to generate the preferred protein production process and formulation to be used in the subsequent aims. Furthermore, the material generated will be used to establish product characteristics and short- and long-term stability profiles.

We have successfully accomplished the following deliverables stated in the original proposal:

- Protein LdNH36 (~100 mg) was produced and provided to our collaborator at USUHS.
- Proof-of-feasibility for expressing PpSP15 in the yeast *Pichia pastoris* was provided.
- A protocol for the production of PpSP15 has been developed. Prior to optimization during months 15-18 of the grant, the current yield is >700 mg per liter of fermentation supernatant. The stated deliverable in the grant of 60 mg PpSP15 per liter of fermentation has thus been exceeded by a factor >10.
- More than 600 mg purified PpSP15 protein were produced per liter of fermentation supernatant, exceeded the deliverable of 20-25 mg by a factor of >24.
- A set of assays suitable for the characterization of PpSP15 have been developed
- Technical reports have been completed for all activities.

Major Task 1, Activity 1.1. Expression and purification of LdNH36

Subtask 1: Expression

LdNH26 was expressed as recently described in our publication, McAtee *et al.*, 2017[1]. Using this approach, a 10 L fermentation of LdNH36 (non-His tagged) in *E. coli* BL21 (DE3) host cells was used to generate biomass for further purification of the target protein. Briefly, the fermentation procedure involved inoculation of one liter of LB media in a 2.5 L Tunair shake flask with glycerol stock and the culture was subsequently used as a seed to inoculate a 10 L working volume fermenter. Following inoculation, the culture was grown in batch mode at pH 7.0 and at 37°C until the OD_{600} of the culture reached ~0.2. At this time, the temperature of the culture was lowered to 30°C and growth continued until the OD_{600} reached 0.5-0.8. Once the target OD was reached, IPTG was added to a final concentration of 1 mM to induce expression of LdNH36. Following overnight induction, the culture was harvested, and biomass was collected by centrifugation. Biomass (122 g/10 L fermentation) was stored at -80°C until downstream purification could be performed.

Subtask 2: Purification

The purification process for LdNH36 was slightly modified compared to the recently published process. Instead of a differential ammonium sulfate precipitation, we incorporated the use of

tangential flow membranes (Millipore Prostak) for clarification and diafiltration of the process stream yielding a material that was greatly enriched. The purification protocol by anion exchange chromatography, size-exclusion chromatography and another ion exchange depyrogenation step is shown below:

Step 1: The fermentation biomass was suspended in lysis buffer at a pellet to buffer ratio of 20% (w/v) with 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA. Pellets were lysed in an Avestin cell homogenizer for 3 passes @ 15,000 PSI. Cell debris was removed by centrifugation @ 16,000 rpm in Oak Ridge tubes using JA-20 rotor in a Beckman Avanti J-26 Centrifuge. The supernatant was filtered through a Millipore Prostack filter with a 3-volume exchange of the lysis buffer. The clarified cell lysate supernatant was then dia-filtered and concentrated with a minimum of 5 volumes of the subsequent capture buffer.

Step 2: The capture of LdNH36E was accomplished by chromatography of the processed lysate supernatant derived from a 60 g cell pellet lysate on a 45 ml GE QXL column (Dimensions 5.0 X 20 cm). Buffer A was 20 mM Tris-HCl, pH 8.0 and Buffer B (elution buffer) was 20 mM Tris-HCl, pH 8.0 1 M NaCl. The flow rate was 5 ml/min and the column was eluted with the following step gradient: 7.5%, 50%, and 100% Buffer B using a minimum of 5 CV per step. This was followed by a 0.1 N NaOH wash step. Fractions were isolated and evaluated by 4-12% NuPAGE Bis-Tris gel electrophoresis in MES running buffer under nonreducing conditions. The chromatogram (**Figures 1, 2**), LDS-NuPAGE and Western Blot (**Figure 3**) results are shown below.

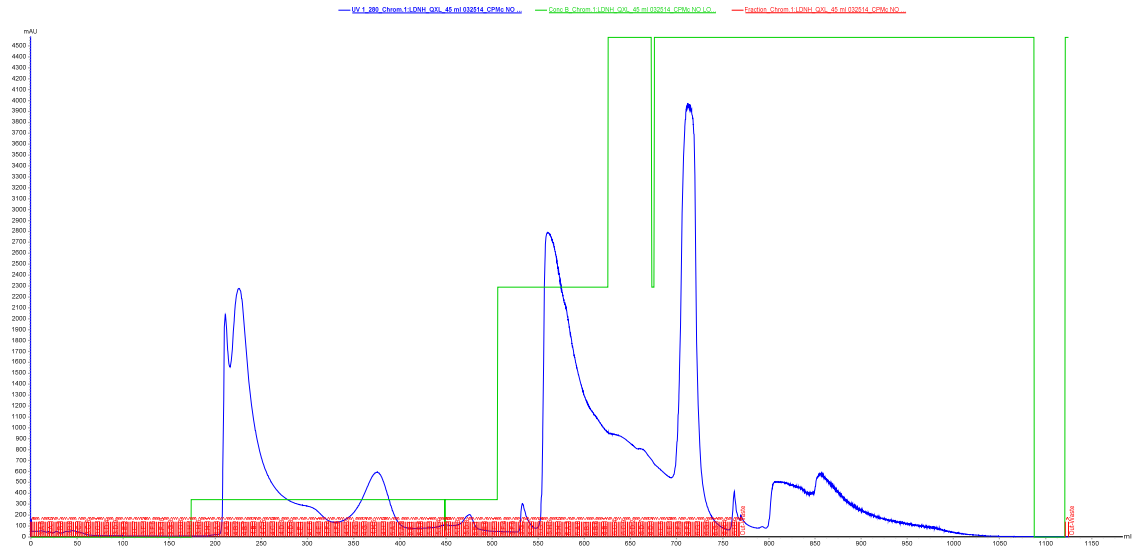

Figure 1. Purification of LdNH36 from *E. coli* using ion-exchange chromatography. Chromatogram of LdNH36 applied to a 45 ml GE QXL column (Dimensions 5.0 X 20 cm).

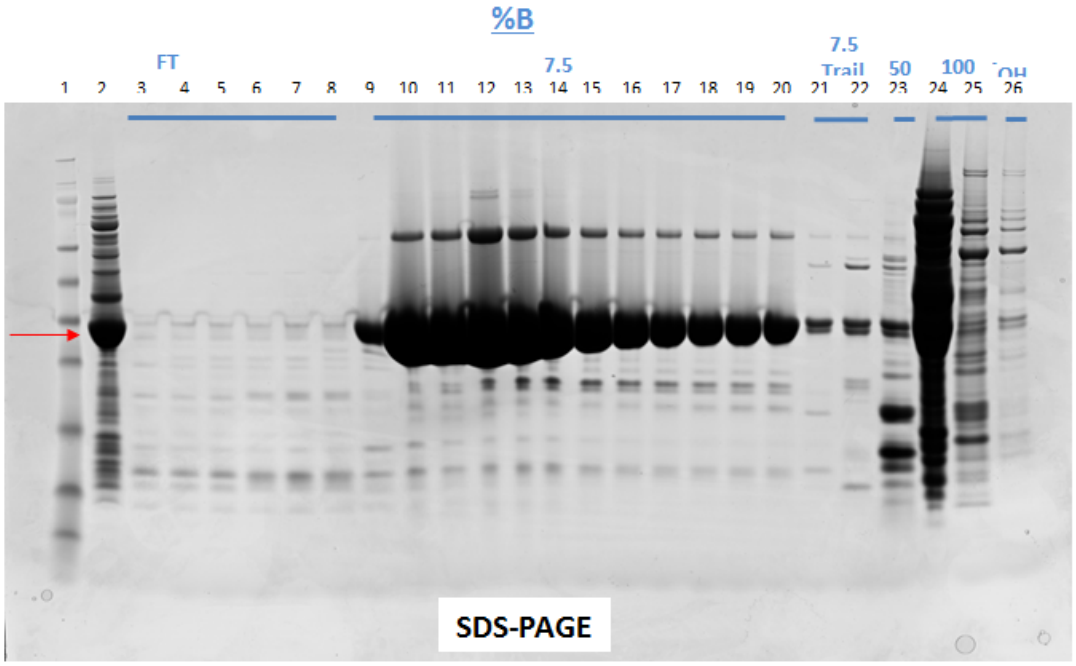

Figure 2. Purification of LdNH36 from *E. coli* using ion-exchange chromatography. The target protein, LdNH36, consistently runs as a doublet band with slightly different profiles on a non-reduced 4-12% BisTris gel. Electrophoresis was carried out in MES running buffer (per Thermo Fisher instructions). The gel was run at 125 Volts for 40 minutes and stained with 0.25% Coomassie Blue R-250, then destained with successive washes of 5% methanol in 7.5% acetic acid.

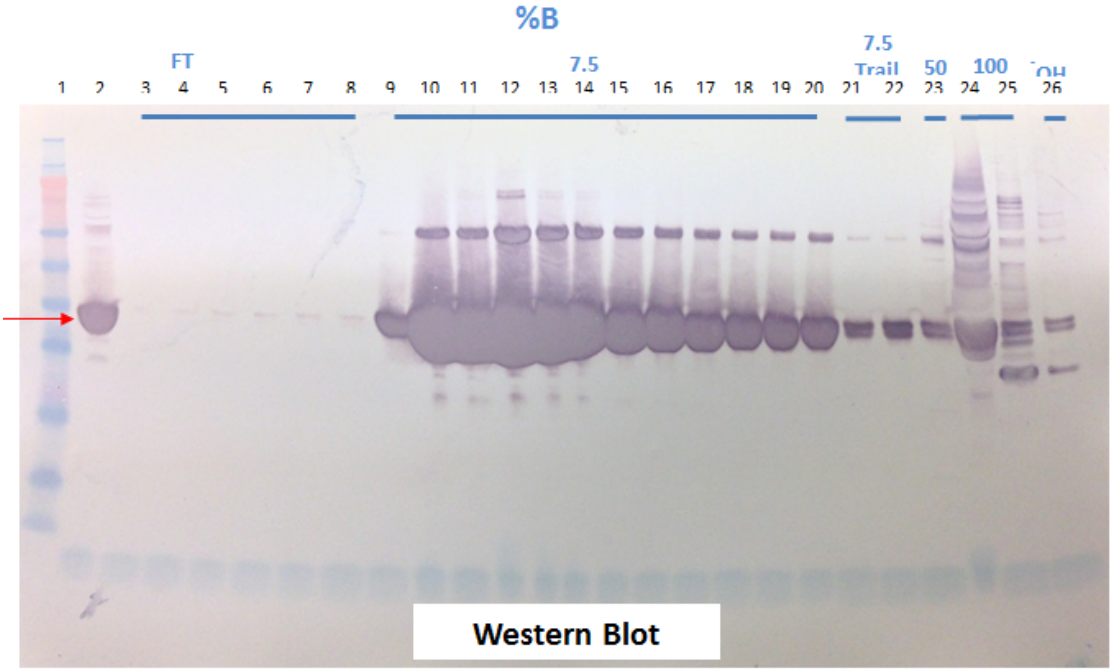

Figure 3 Western Blot analysis of LdNH36. Western blots were developed using anti-LdNH36 in-house generated polyclonal mouse sera. The primary antibody was diluted 1:4,000. The secondary antibody was a goat anti-mouse antibody (IgG) conjugated to alkaline phosphatase. The secondary antibody was diluted 1:1,000. Each incubation was carried out for one hour at RT followed by multiple washings of 1X PBS + 0.05% Tween-20. Initial blocking of the membrane (nitrocellulose) utilized 5% dry milk in wash buffer. Development was accomplished using a BCI/NBT membrane phosphatase substrate solution (KPL Laboratories).

Step 3: Polishing of QXL fractions by Superdex S 200

Fractions from the 7.5% step elution off the QXL column were pooled and concentrated using an Amicon Ultra 15 (Millipore) 10-kDa cutoff spin column. Concentrated material was separated on a 5 x 60 cm Sephacryl S 200 gel filtration column. The chromatogram below (**Figure 4**) is a representative profile of one of the runs. Material recovered from the S-200 column was determined to be > 98 % pure by a variety of analytical techniques including SDS-PAGE, densitometry, HPLC, and mass spectrometry. A shoulder preceded the main peak in the chromatogram profile and was determined to be a multimer of LdNH36 when in the original running buffer (20mM Tris, 100mM sodium chloride at pH 8.0). Additional analysis indicated that the multimers were formed through intermolecular disulfide bond on LdNH36. By adding 1 mg/mL of cysteine to the original running buffer, it successfully lowered the percentage of LdNH36 multimers. However, at pH 8, cysteine formed cystine (cysteine dimer, a less soluble form) and eventually precipitated within two weeks at room temperature, and thus, the protein in such formulation would only be more stable when stored at -80°C. To solve this issue, we further optimized the buffer composition to 50 mM sodium citrate, 100 mM sodium chloride, 0.1 mg/mL cysteine at pH 6.0 to prevent any possible formation of cystine (cysteine dimer).

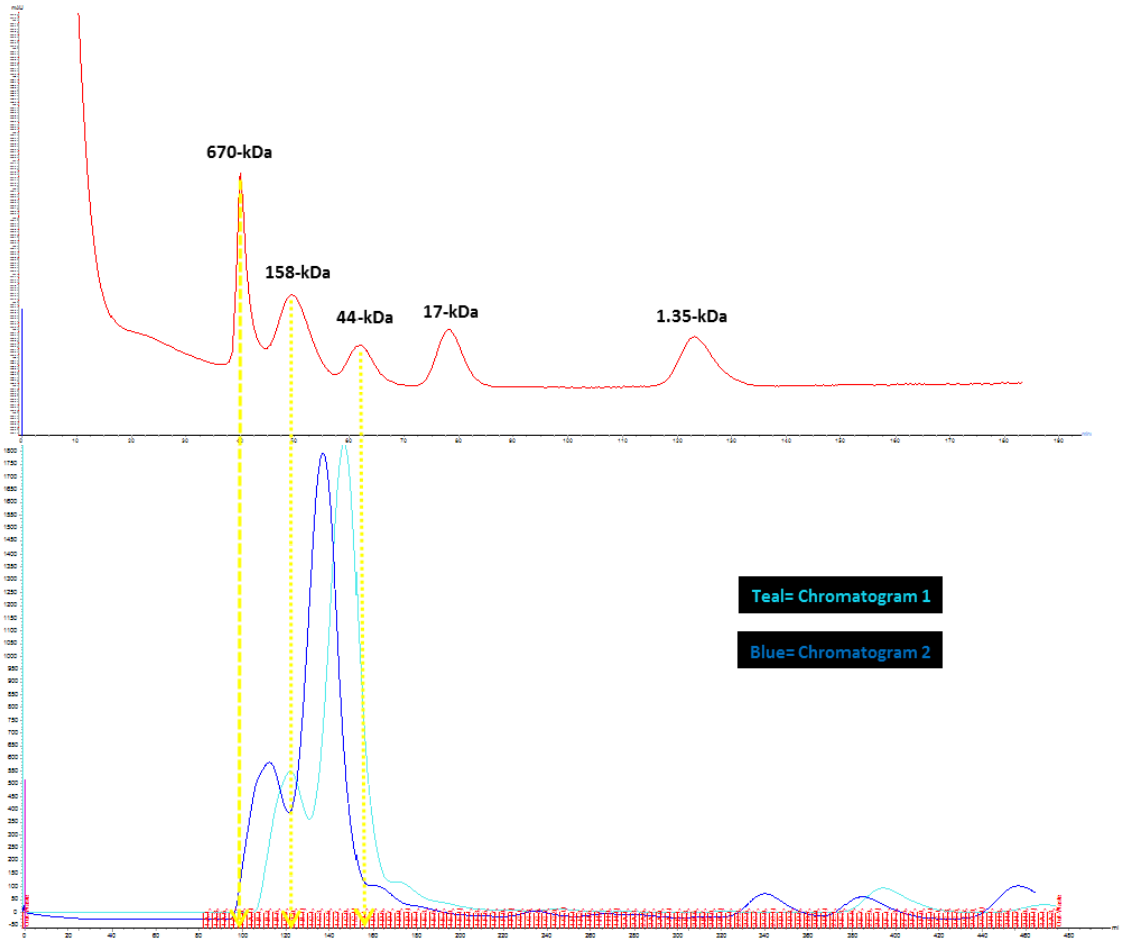

Figure 4. Purification of LdNH36 from *E. coli* using size-exclusion chromatography. Chromatogram: A Sephacryl S 200 column was equilibrated with 50 mM citrate, 100 mM NaCl, 0.1 mg/ml cysteine at pH 6. BioRad molecular weight standards were run to standardize the separation (top) and then LdNH36 was run in parallel (bottom).

Step 4: Depyrogenation of final processed material

Post Sephacryl S 200 chromatography, fractions were pooled and subjected to depyrogenation using a 5 ml QXL column equilibrated in the same running buffer as the Sephacryl S 200 fractions. Since endotoxin is highly negatively charged, it bound to the QXL column even with a buffer containing 100 mM sodium chloride while LdNH36 remained unbound and passed through the column directly. The unbound LdNH36 was collected and filtered (0.22 μm filter) as the final product.

Summary of Activity 1.1:

Activity 1.1 is complete. 96.3 mg LdNH36 (94-99% pure) with an endotoxin level of <2.31 EU/mg were provided to USUHS (**Table 1**).

Table 1. LdNH36 produced from *E. coli*

Lot	Purity by SDS-PAGE	Purity by SE-HPLC	Amount sent to UHU (mg)	Endotoxin (EU/mg)	Buffer
LdNH36012318CPMC1	95%	99%	30.3	<1.72	20 mM Tris, 100 mM NaCl, 1 mg/mL cysteine, pH8
N070218CPMC2	94%	97%	50.6	1.19	20 mM Tris, 100 mM NaCl, 1 mg/mL cysteine, pH8
N240418CPMC1	97%	99%	15.4	<2.31	50 mM sodium citrate, 100 mM NaCl, 0.1 mg/mL cysteine, pH 6

Major Task 2, Activity 1.2. Cloning and small-scale expression of PpSP15.

We had originally sought to express PpSP15 in *E. coli*. The high sequence similarity with the better characterized PdSP15[2] suggest *E. coli* would be suitable for expressing PpSP15. However, we noted that expression in *E. coli* led to insoluble product. We therefore switched to the yeast, *Pichia pastoris*, as our lead expression host. Starting with the *in silico* analysis of the protein sequence, and ending with the analysis of the purified protein the process is described in detail below.

Subtask 1: In silico analysis, Cloning, Transformation

PpSP15 (NCBI protein_id ="AAL11047.1", GenBank ID: AF335487.1), is a 15 kDa salivary antigen from *Phlebotomus papatasi* that facilitates the infection of *Leishmania major*. Mice immunized with DNA vaccine of PpSP15 produced protective immunity against infection of *L. major* (Valenzuela JG *et al.*, 2001; Oliveira *et al.*, 2008). However, because DNA vaccines frequently fail in the clinic, whereas there is a robust history of recombinant protein vaccines reaching licensure, the strategic plan of our consortium will pair LDNH36, with PpSP15 expressed as a recombinant protein.

The cDNA encoding for PpSP15 (selected from previous vaccination studies) is as follows.

```
>AF335487.1 Phlebotomus papatasi 15 kDa salivary protein precursor (sp15)
GAGTTTTAATTACGACCATGAAGTACTTAGGACTTGCTTTAATTTCCGCAGTGTTCTTAATTGGAACCTGCCAAGCTGAAA
ATCCATCAAAGAAGTGCGAGGAAAAATTTAAGAATGATGCTTCGAAAATGGCTTGCATTCCCCATTGCAAATATCAGTATT
ACGGATTTGTAGCTATGGATAATAACATCGCTAAACCAGAGATTAGAACATTTTCTAATGTTCTAATCAAATATAATGTTG
TGGACAAAAGCCTGAAAGCAGACATTAGGAAAATTATGCACGAATGTGCTAAAAAAGTTAAGAAACAAGCTAGAGAAGACT
CTCATTGGTTGAATTGTCGTACAACTATTAATTATTATAGATGTATTTTGACCGACAAACGAATTGGACCTCAAAGATTTG
ACAGAGCCATTCAAGAATATGATAAAACAATCAATATATAACCACAAAATTTATACTTAAATAAAGCTTGAAAGCACTGAA
GTTGCTTTCCCAAAAAAAAAAAAAAAAAAAAAAAA

>AAL11047.1 15 kDa salivary protein precursor [Phlebotomus papatasi]
MKYLGLALISAVFLIGTCQAENPSKKCEEKFKNDASKMACIPHCKYQYYGFVAMDNNIAKPEIRTFSNVLIKYNVVDKSLK
ADIRKIMHECAKKVKKQAREDSHWLNCRTTINYYRCILTDKRIGPQRFDRAIQEYDKTINI
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Figure 5: DNA (AF335487.1) and amino acid (AAL11047.1) sequences of PpSP15 in FASTA format. In DNA sequence, start codon (ATG) and stop codon (TAA) are highlighted in red.

Protein sequence analysis
Signal Peptide:

The PpSP15 amino acid sequence was submitted to "SignalP 4.1 Server" (http://www.cbs.dtu.dk/services/SignalP/) for signal peptide prediction. The server predicted that the amino acids 1 to 20 constitute the signal peptide (**Figure 6**).

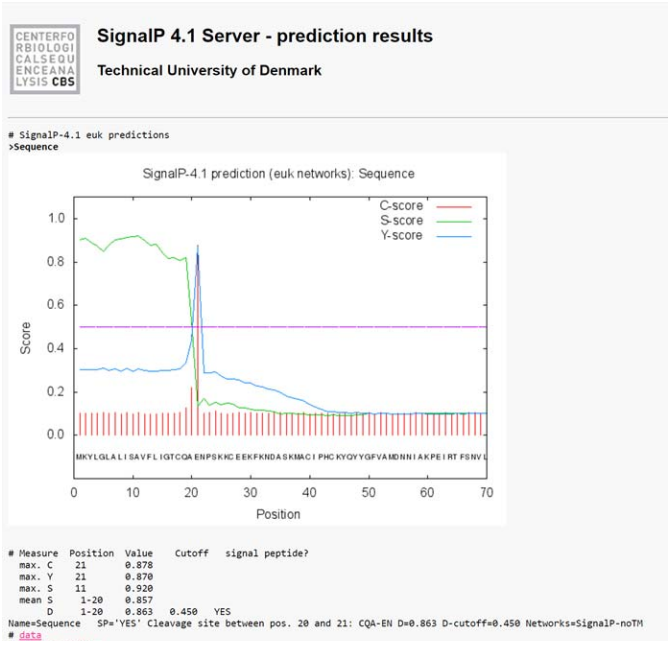


Figure 6: Signal peptide prediction of PpSP15 by SignalP-4.1.

N-glycosylation:

There is a consensus sequence for N-glycosylation: AsnXxxSer/Thr/Cys, where Xxx can be any amino acid except proline (Medzihradszky KF 2008). In the PpSP15 amino acid sequence, there are nine Asn residues, but only Asn22 is followed by Ser24. However, the amino acid in between Asn22 and Ser24 is Pro23, therefore Asn22 is most likely not be glycosylated.

Disulfide bridge:

PpSP15 (from *P. papatasi*) contains seven Cys residues; the crystal structure of the homologous PdSP15 (PDB 4OZD, from *P. duboscqi*) showed that there are 3 disulfide bridges, with an additional Cys residue located near the N-terminus. From the sequence alignment between PdSP15 and PpSP15, all Cys residues are conserved (**Figure 7**). Hence, PpSP15 protein should have three disulfide bridges.

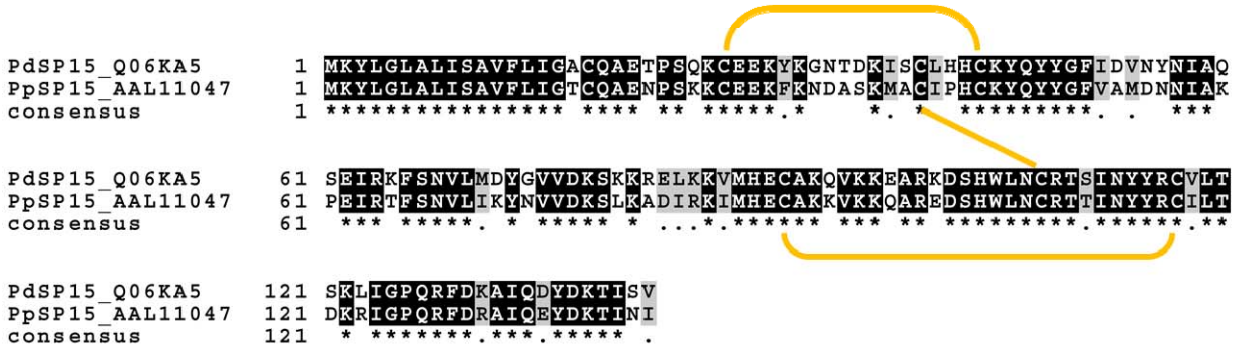


Figure 7: Amino acid sequence alignment of PdSP15 and PpSP15. Yellow lines are disulfide bridges from PdSP15 crystal structure.

DNA synthesis

The coding DNA sequences for PpSP15 with and without a hexahistidine tag were codon optimized based on yeast usage preference (**Figure 8**) and then synthesized and cloned into pPICZαA vector by GenScript, namely pPICZaA_PpSP15 and pPICZaA_PpASP15His.

>PpSP15 (yeast codon optimized)
GAATTCGAAAATCCATCTAAGAAATGTGAAGAAAAGTTTAAAAACGATGCTTCAAAGATGGCATGTATCCCACATTGTAAG
TACCAATACTACGGTTTCGTTGCTATGGATAACAACATCGCAAAACCAGAAATCAGAACTTTTTCTAACGTTTTGATTAAA
TACAATGTTGTTGATAAATCATTAAAAGCTGATATTAGAAAGATCATGCATGAATGTGCTAAGAAAGTTAAGAAACAAGCA
AGAGAAGATTCTCATTGGTTGAATTGTAGAACTACAATTAATTACTACAGATGTATCTTGACTGATAAGAGAATCGGTCCA
CAAAGATTCGATAGAGCAATTCAAGAATATGATAAAACAATTAATATTTAATCTAGA

>PpSP15His (yeast codon optimized)
GAATTCGAAAATCCATCTAAGAAATGTGAAGAAAAGTTTAAAAACGATGCTTCAAAGATGGCATGTATCCCACATTGTAAG
TACCAATACTACGGTTTCGTTGCTATGGATAACAACATCGCAAAACCAGAAATCAGAACTTTTTCTAACGTTTTGATTAAA
TACAATGTTGTTGATAAATCATTAAAAGCTGATATTAGAAAGATCATGCATGAATGTGCTAAGAAAGTTAAGAAACAAGCA
AGAGAAGATTCTCATTGGTTGAATTGTAGAACTACAATTAATTACTACAGATGTATCTTGACTGATAAGAGAATCGGTCCA
CAAAGATTCGATAGAGCAATTCAAGAATATGATAAAACAATTAATATTCATCATCATCATCATCATTAATCTAGA

Figure 8: The DNA sequence of PpSP15 and PpSP15His, GAATTC = EcoRI, TCTAGA = XbaI, underlined sequence at the 3' is sequence for his-tag.

Expression vector

The 3.6 kb expression vector pPICZαA (**Figure 9)** was used to express and to secrete recombinant proteins in *Pichia pastoris*. Recombinant proteins are expressed as fusions to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. The vector allows high-level, methanol inducible expression of the gene of interest in Pichia and can be used in any Pichia strain including X-33,

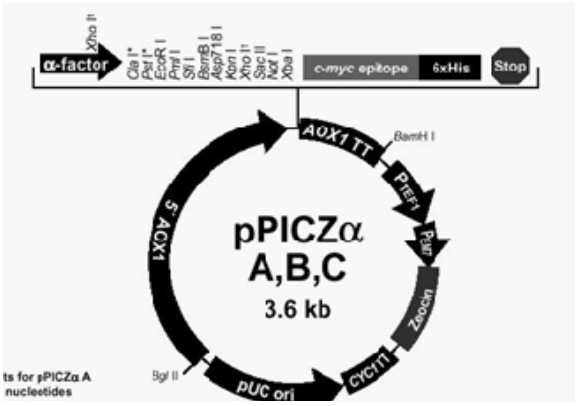


Figure 9: The map of pPICZαA, a plasmid used to clone and express PpSP15 in *Pichia pastoris*.

Expression yeast strain

Pichia pastoris X-33 (Invitrogen, Cat# K1740-01) was used to express pPICZaA_PpSP15 and pPICZaA_PpSP15His. *P. pastoris* X-33 is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O2, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia.* The detailed information of *Pichia pastoris* expression vector and the yeast strain is available from the manufacturer: EasySelect™ Pichia Expression Kit, Rev. Date 18 June 2010, Manual part no. 25-0172 (Invitrogen)

Transformation of recombinant plasmid DNA into yeast competent cells

Plasmid pPICZaA_PpSP15 and pPICZaA_PpSP15His were transformed by heat/shock method into *Escherichia coli* DH5α competent cells (Invitrogen, Cat# 1197872) and plated on LB plate supplemented with kanamycin (50 μg/mL). Later, the *E. coli* colonies were inoculated in to LB broth (kanamycin 50 μg/mL) and plasmid DNAs were extracted with QIAprep Spin miniprep kit (QIAGEN, cat# 27106) from *E. coli* cell culture for subsequent experiment.

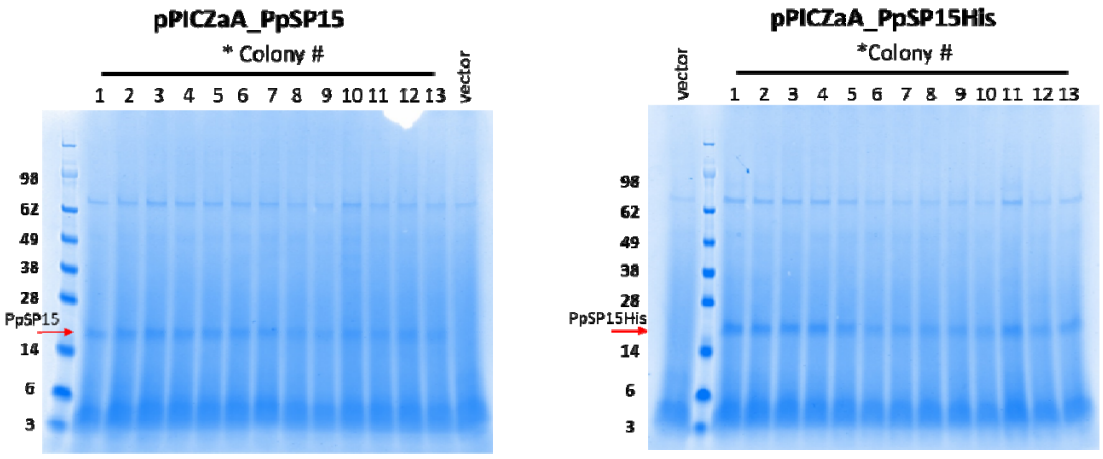
The recombinant plasmids pPICZaA_PpSP15 and pPICZaA_PpSP15His were transformed into *P. pastoris* X-33 as described in the Invitrogen EasySelect™ Pichia Expression Kit Manual. Briefly, the recombinant plasmid DNAs were linearized with SacI for both plasmids. The linearized plasmid DNAs were transformed into *P. pastoris* X-33 using electroporation. The transformants were selected on YPD plates either with 100 μg/ml or 500 μg/ml of Zeocin. After being incubated at 30°C for three days, colonies from each transformation were selected for small-scale (10 mL) expression using BMGY/BMMY media, induction with 0.5% methanol/day at 30°C for three days for recombinant protein expression.

Subtask 2: Small-scale expression

Identification of the clone with highest expression level

The expressed recombinant PpSP15 and PpSP15His were identified with SDS-PAGE. Expression of PpSP15His was confirmed by Western blotting with anti-His antibodies. The clone with the highest expression was chosen from each transformation for making research seed stocks.

Thirteen colonies from each transformation were picked to inoculated 10 mL of media with 0.5% methanol induction as described at section 5.5. On SDS-PAGE analysis, the expression of PpSP15 and PpSP15His was visible (**Figure 10**). From the gel, colonies that were selected from the 500 μg/mL Zeocin plate (colony number 1 to 5) gave higher expression levels than those selected from the 100 μg/mL plates (colony number 6-13). The expressed PpSP15His was further confirmed through Western Blot analysis with anti-His antibodies (**Figure 11**).



(a) (b)

Figure 10: SDS-PAGE analysis of small-scale expression of selected colonies from (a) pPICZaA_PpSP15 (b) pPICZaA_PpSP15His. Colony # 1-5 were selected from 500 µg/mL Zeocin plate, while colony # 6-13 were selected from 100 µg/mL Zeocin plate.

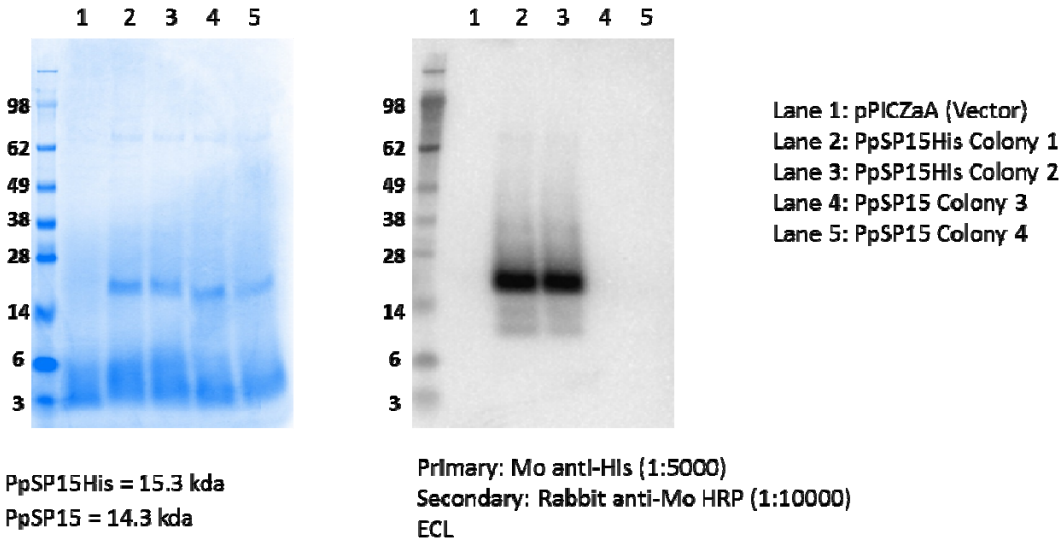


Figure 11: Western blot analysis of PpSP15 expressing clones using anti-his antibodies, confirming the expression of PpSP15His.

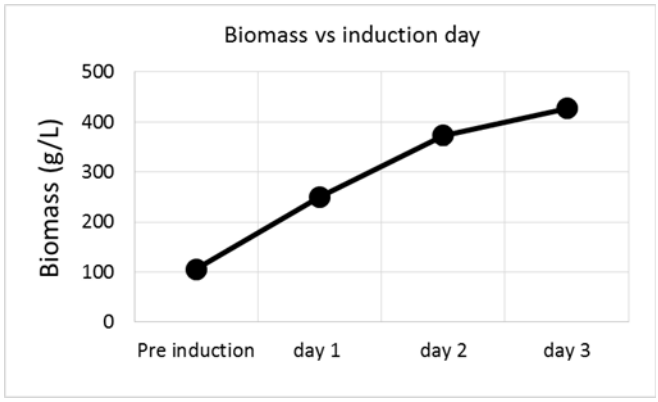
Major Task 3, Activity 1.3. Process development for PpSP15 production.

Subtask 1: Activity 1.3.1. Fermentation.

Briefly, the fermentation of the tag-free PpSP15 clone (Run ID: PDF091817_PpSP15, Lab book CAS1102-04) was expanded overnight in batch mode at 30°C and then upon glycerol depletion, expression was induced with methanol. After that, methanol was ramped from 1-12 ml/L/hr. over an 8 hour period followed by maintenance at 12 ml/L/hr. until the end of the run. During the first hour of the induction phase, the temperature reduced from 30°C to 26°C. Total induction time will be approximately 72 hours. Fermentation supernatant was collected by centrifugation at 12,227 x g at 4^0C for 45 minutes and filtered with 0.22 µm PES filter. The fermentation supernatant (4.8 L) was kept at -80^0C.

In an initial experiment, wet biomass was monitored throughput the fermentation process and reached 410 g/L (**Figure 12a**). A representative SDS-PAGE showing protein quality throughout the fermentation is shown in **Figure 12b**.

(a)



(b)

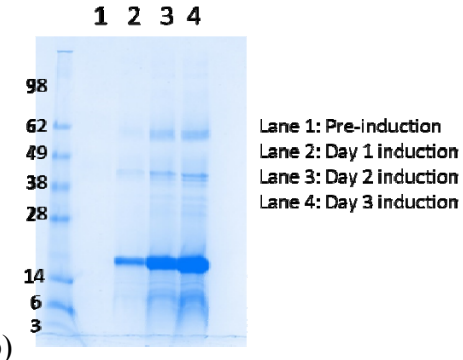


Figure 12. (a) Biomass accumulation during fermentation of PpSP15. (b) SDS-PAGE time point analysis.

Subtask 2: Activity 1.3.2. Purification of PpSP15.

Purification of recombinant PpSP15 and PpSP15His

The respective colonies of PpSP15 and PpSP15His with the highest expression levels were grown in 2 L shake flasks with 200 mL BMGY/BMMY medium. After 3-day induction, the culture media were harvested by centrifugation (4000 x g, 30min) and filtered.

To purify PpSP15, culture media of *P. pastoris* that expressed PpSP15 was harvested and dialyzed with 50 mM Phosphate Buffer pH 6.0 using a dialysis membrane with a cut-off value of 3,000 Da. After overnight dialysis, the sample was loaded onto a SP HP (3 mL, GE) column at a flow rate of 2 mL/min. The column was washed with 50 mM Phosphate Buffer pH 6.0 for 10 column volumes (CVs) and eluted with 10 CVs (7 CVs of gradient elution from 0% to 70% elution buffer, 3 CVs of step elution using 100% elution buffer) elution buffer containing 50 mM Phosphate Buffer pH 6.0, 1 M NaCl (**Figure 13a**). Elution fractions collected in 2 ml per fraction. After analysis using SDS-PAGE (**Figure 13b**), the peak fractions containing PpSP15 protein were pooled and buffer exchanged to 1X phosphate buffer solution (pH 7.4). Later, the protein was concentrated using VivaSpin (MWCO 6,000-8,000) and stored at -80°C prior to the next experiment.

The culture media of *P. pastoris* that expressed PpSP15His was loaded onto a HisTrap FF 16/10 (20 mL) (GE) at a flow rate of 5 mL/min. The column was washed with Tris-HCl 50 mM, NaCl 0.5 M (pH 8.0) for 10 column volumes (CVs) and eluted with 20 CVs (15 CVs of gradient elution from 0% to 70% elution buffer, 5 CVs step elution with 100% elution buffer) elution buffer containing Tris-HCl 50 mM, NaCl 0.5 M, Imidazole 0.5 M. Elution fractions collected in 4 ml per fraction. After analysis using SDS-PAGE, the peak fractions containing PpSP15His protein were pooled and buffer

exchanged to 1X phosphate buffer solution (pH 7.4). Later, the protein was concentrated using VivaSpin (MWCO 3,000) and stored at -80°C prior to the next experiment.

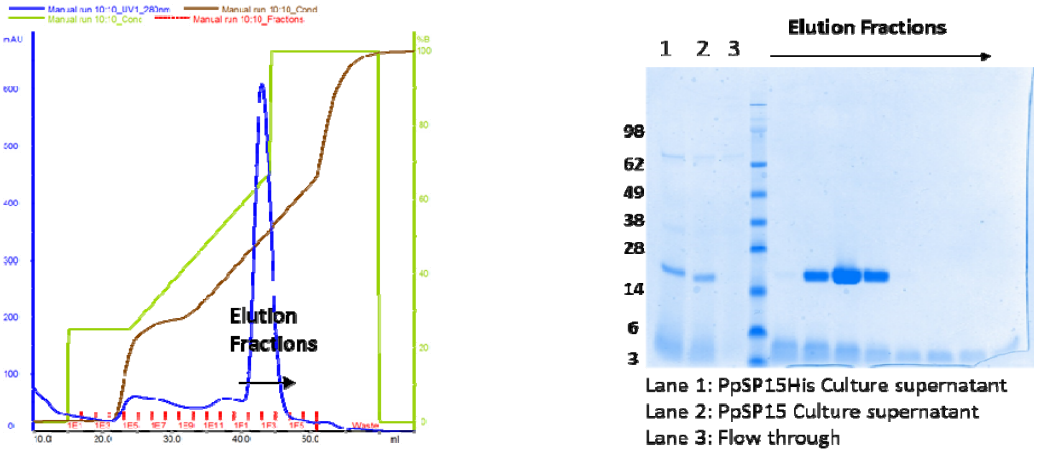


Figure 13 (a) Purification chromatogram of recombinant protein PpSP15 with HiTrap SP HP, the protein was eluted by salt gradient. (b) SDS-PAGE gel analysis showed the recombinant protein PpPS15 has been purified from culture supernatant.

Generation of research seed stocks

The clones with highest expression from each transformation identified in the small-scale (10 mL) expression as described at section 5.6 were chosen for making the research seed stocks. Each chosen clone was picked from the corresponding transformation plate to streak on a new selecting plate (YPD with 100 µg/ml of Zeocin) to grow at 30°C for 72 hours to make single colonies. One single colony from each plate was used to inoculate 25 mL of YPD medium made with plant derived phytone. After overnight growth at 30°C, plant-derived glycerol was added to the culture at final concentration of 20%. After being well-mixed, the glycerol-culture mixture was aliquoted into cryovials at 1 mL and stored at -80°C as the research seed stocks. There are no animal derived products used in the generated research seed stocks.

Subtask 3: Activity 1.3.3. Depyrogenation of PpSP15.

Since the protein expressed well in *P. pastoris*, there was no need to remove endotoxin.

Summary:

Table 2 summarizes the current Mass Balance for the purification of PpSP15 from a 10 L fermentation of *P. pastoris*. Further optimization of the process is ongoing in preparation of the reproducibility runs scheduled for months 15-18.

Table 2: Purification of PpSP15 from *P. pastoris*

Sample	#Yield (mg/L FS)	PpSP15 Concentration (mg/mL)	Recovery (%)	*Purity (%)
1. Fermentation Supernatant	779	0.78	100	44.7
2. TFF Permeate (diafiltration)	15	0.01	2	-
3. TFF final product	738	2.42	95	53.7

4. IEX pool fractions	675	6.94	87	96.5
5. HIC pool fraction + dialysis to PBS	617	2.73	79	97.6

Major Task 4, Activity 1.4. Assay development for PpSP15.

Subtask 1: Protein characterization and assay development.

We have characterized the yeast expressed PpSP15 by SDS-PAGE with Coomassie blue staining (**Figure 14a**) and silver staining. The results show that the protein's size is 15 kDa and its purity is approximately 95%. It is noted that the small-molecular-weight impurities (~ 1-2% of total intensity) observed under non-reduced condition could be reduced into one protein band at around 5 kDa. Both protein bands at 15 kDa and 5 kDa were further analyzed by mass spectrometry, and the results indicated that the protein at 15 kDa was PpSP15 while the protein at 5 kDa was a mixture of ~50% of PpSP15 and 50% of host cell protein. Western blot probed with in-house mouse anti-PpSP15 serum only showed a single band at 15 kDa, but not at 5 kDa, which suggests the degraded 5 kDa PpSP15 might not be antigenic (**Figure 14b**). Size analysis by dynamic light scattering (DLS) showed that purified PpSP15 protein is monodispersed with a size of 17 kDa (**Figure 14c**), which is comparable with the theoretical size and the results from SDS-PAGE.

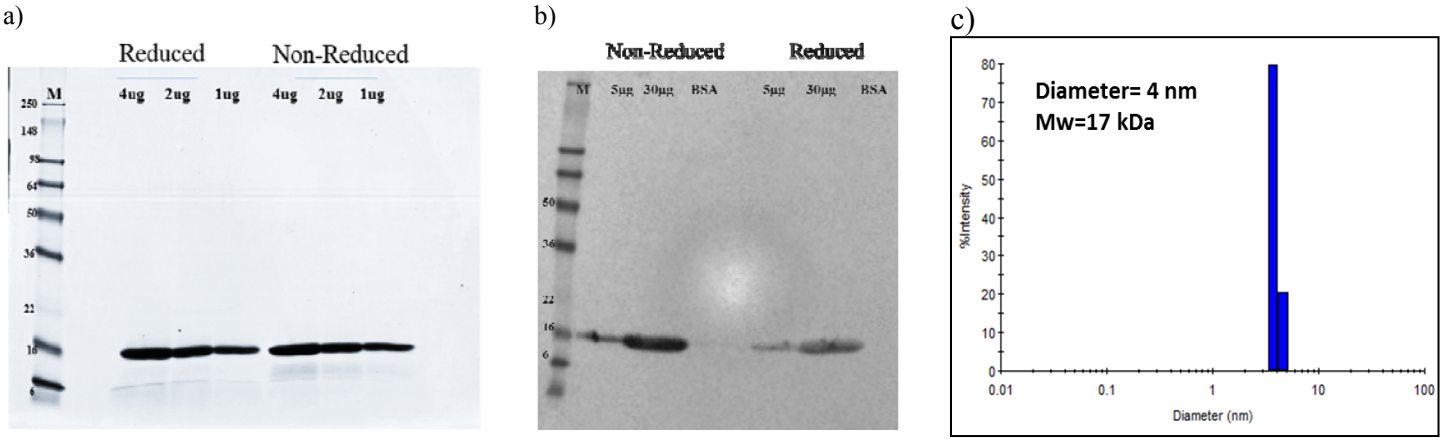


Figure 14. Identity and purity analysis of PpSP15 by (a) SDS-PAGE with Coomassie blue staining and (b) western blot. (c) Size analysis by dynamic light scattering.

We have also developed an RP-HPLC for PpSP15 assay using a C8 column. When PpSP15 is intact, it elutes at 13-15 minutes with a purity of 99.2%. When PpSP15 was forced to aggregate by formaldehyde, a broad peak followed by a shoulder eluted from minutes 12-23 was observed, and the purity decreased accordingly with the increasing level of aggregation (**Figure 15**). However, it would be preferable if distinct peaks of aggregates from the intact protein could be resolved. Thus, we may seek alternative types of columns (i.e., SE-HPLC and/or IEX-HPLC) for the analysis of such protein and finalize the assay prior to the stability testing.

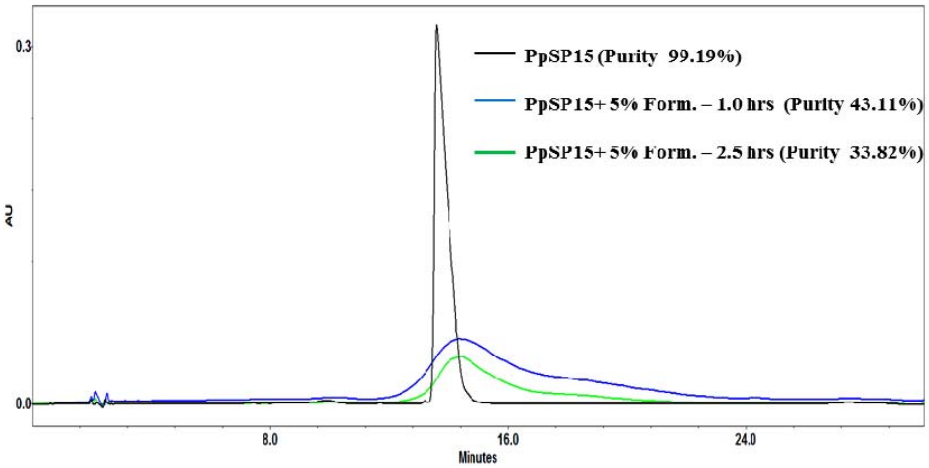


Figure 15. Purity analysis and aggregation study of PpSP15 by RP-HPLC.

Specific Aim 2: To test the immunogenicity of PpSP15 and LdNH36 given at various dosages.

Major Task 7, Activity 2.1: Immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: route and dose ranging evaluation.

USUHS will perform a series of murine immunogenicity experiments using a combination of the recombinant LdNH36 and PpSP15, with and without the Toll-like receptor 4 (TLR-4) agonist and clinically-tested adjuvant, GLA-SE. We plan to comprehensively analyze the biological response to the combined antigens and each antigen alone, *in vitro* and *in vivo*. Dose ranging experiments of up to five dosages will be performed, administered subcutaneously, intradermally or intramuscularly and boosted at monthly intervals for a series of three immunizations per animal. Antibody responses to both antigens will be measured by an enzyme-linked immunosorbent assay (ELISA) over the course of the experiment. In addition, at 3-4 weeks after the final (3rd) immunization interferon gamma (ELISPOT) and cytokine profiles will be assessed by a cytokine binding assay or Luminex technology. The purpose of this aim will be to select the dose and route of administration that elicits the best humoral and cell mediated immune response and to determine if GLA-SE adjuvant improves immunogenicity.

For the first-year reporting period we completed Major Task 7, the immunological assessment of C57Bl/6 mice vaccinated with recombinant PdSP15 and LdNH36 vaccine: evaluating route and dose. This included writing and obtaining approval of a new animal protocol MED 17-018 entitled "Dose ranging and immunogenicity studies of CL-vax (PpSP15/LdNH36)" as well as completing an annual progress report. Additionally, we tested the immunogenicity of PpSP15 and LdNH36 antigens given at various doses and combinations by various administration routes (intradermal, subcutaneously and intramuscularly). Our objective was to set the optimal dose and route of administration for CL-vax, combined PpSP15 and LdNH36 vaccine, by testing the humoral response against antigen, adjuvant, and the cell mediated immune responses in the spleen.
In the first set of experiments aiming to determine the best dose and route for vaccination with the combined vaccination of LdNH36 and PpSP15, we immunized C57Bl/6 mice following this scheme:

Table 2 *Study group design for determining the best dose and route for vaccination with LdNH36 and PpSP15.*

Antigen		Intramuscular	Intradermal	Subcutaneous
LdNH36	**PpSP15**			
5 μg	500 ng	5 mice	5 mice	5 mice
	5 μg	5 mice	5 mice	5 mice
	Prime boost	5 mice	5 mice	5 mice
20 μg	500 ng	5 mice	5 mice	5 mice
	5 μg	5 mice	5 mice	5 mice
	Prime boost	5 mice	5 mice	5 mice
	Prime boost 5	5 mice	5 mice	5 mice
Controls				
20 μg NH36		5 mice	5 mice	5 mice
500 ng PpSP15		5 mice	5 mice	5 mice
PBS		5 mice	5 mice	5 mice

Immunizations were performed 3 times, at one-month interval. The injected antigen preparations were well tolerated by the mice. No adverse effects were seen except dermatitis developed in 7 out of the 150 mice (per Dr. Mullins C57BL/6 known to develop dermatitis) which were treated with bleach bath or bacitracin. Mice were bled by tail nick pre-vaccine and then 3 weeks after each immunization

to assess the humoral response to both antigens. Two weeks after the last immunization, mice were euthanized, bled by cardiac puncture, and spleen harvested.

We first checked if mice developed any immune response against the administered antigens. We showed that a high level of anti LdNH36 or anti PpSP15 total IgG was found with almost all the preparations given SQ or ID. The highest IgG levels were found with these combinations: 5 μg LdNH36/5 μg PpSP15 SQ or ID, 5 μg LdNH36/0.5 μg PpSP15 SQ or ID and Prime boost 5 μg LdNH36/0.5 μg PpSP15 SQ. In contrast, IM immunization was not associated with elevated levels of IgG. We also determined the levels of Ig2a and IgG1 in the sera at the terminal bleed. Our results show that similar levels of Ig2a and IgG1 were triggered by the various vaccine preparations.

As protection from *Leishmania* related diseases is associated with cellular immune response, we assessed the response in splenocytes after stimulation with LdNH36 and PpSP15. We first checked IFNγ secretion after 72 h stimulation with both antigens using ELISA. The combination giving the highest IFNγ results were in the SQ arm: 5 μg LdNH36/5 μg PpSP15, Prime boost 5 μg LdNH36/0.5 μg PpSP15 and Prime boost 20 μg LdNH36/0.5 μg PpSP15. For the ID route, immunizations with 5 μg LdNH36/5 μg PpSP15 or Prime boost 20 μg LdNH36/5 μg PpSP15 were associated with the highest levels of IFNγ secretion. Culture supernatants from the 5 listed vaccine conditions were analyzed by Luminex™ using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex™ Panel. In order to define the best combinations that will move forward to define CL-Vax, we determined the highest ratios of IFNγ/IL-10 as a predictor of vaccine success. 3 antigen doses/routes met our selection criteria and were chosen to be tested in association with GLA-SE as shown in **Table 3**.

Table 3 *Study group design for determining the effect of GLA-SE adjuvant on the immune response in mice vaccinated with LdNH36 and PpSP15.*

	+ GLA-SE	-GLA-SE
5ug LdNH36/5ug PpSp15 SQ	5 mice	5 mice
5ug LdNH36/5ugPpSp15 ID	5 mice	5 mice
Prime 5ug LdNH36/0.5 ug PpSp15 SQ	5 mice	5 mice
Controls		
PBS ID	5 mice	
PBS SQ	5 mice	
GLA-SE ID	5 mice	
GLA-SE SQ	5 mice	

In summary, we found the combination of parasite and vector sand fly antigens to be immunogenic and were able to select 3 conditions with the best performance. Interestingly, subcutaneous and intradermal administration were associated with higher levels of IFNγ and antigen specific IgG than intramuscular administration. There were some indications of mixed Th1/Th2 responses with the CL-vax vaccine, so we anticipate driving a more Th1 predominant response using the adjuvant GLA-SE in the next experiment.

Specific Aim 3. To evaluate the efficacy of PpSP15 and LdNH36 as a vaccine in protecting mice challenged by the natural mode of transmission, *L. major* infected sand flies.

The Vector Molecular Biology Section (VMBS) at The National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH) will collaborate with USUHS to ensure technical consistency is maintained throughout the project. Animals at NIAID will be immunized with both antigens (produced in Aim 1) using the best dose and route of immunization determined in Aim 2. The VMBS will setup an insectary dedicated to the growth and maintenance of the sand fly *P. papatasi*, a natural vector of *L. major*, a model previously used at a low scale at the VMBS. The VMBS will also update the infrastructure necessary for the infection of *P. papatasi* and maintain a virulent parasite stock for the natural challenge of the vaccinated mice. Three to four weeks after the last immunization, control and vaccinated mice will be challenged with *L. major*-infected sand flies. Animals will be followed-up for cutaneous lesion development at the site of bites and analyzed at specific time points to determine the parasite burden and the immunological parameters related to development of protective immunity or disease.

Major Task 9, Activity 3.1: Update the insectary facility to have a high capacity Phlebotomus papatasi *sand fly colony for transmission experiments.*

We secured a second sand fly insectary chamber to grow the sand fly *Phlebotomus papatasi.* We further secured four insectary incubators to grow the sand fly larvae in the new insectary. Moreover, we hired personnel to be dedicated to grow sand flies in the LMVR/NIAID insectary.
With those factors in place, we have now started to expand the sand fly, *P. papatasi,* colony. We started with less than 100 sand flies per week and are now producing about 1,500 sand flies per week.

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

Nothing to Report

How were the results disseminated to communities of interest?

Beyond the scientific papers, during this past year our group published several key advocacy documents in order to raise the profile of cutaneous leishmaniasis, and highlight both its hidden disease burden due to scarring, social stigma, and mental health and how and why this disease is emerging in global areas of conflict and political instability and why this disease is of military importance:

Hotez PJ. The rise of leishmaniasis in the twenty-first century. Trans R Soc Trop Med Hyg. 2018 Sep 1;112(9):421-422. doi: 10.1093/trstmh/try075. No abstract available.

Bailey F, Mondragon-Shem K, Hotez P, Ruiz-Postigo JA, Al-Salem W, Acosta-Serrano Á, Molyneux DH. A new perspective on cutaneous leishmaniasis-Implications for global prevalence and burden of disease estimates.
PLoS Negl Trop Dis. 2017 Aug 10;11(8):e0005739. doi: 10.1371/journal.pntd.0005739. eCollection 2017 Aug. No abstract available.

Press outreach. We conducted several interviews for leading websites and newspapers regarding our leishmaniasis project:

http://outbreaknewstoday.com/pakistan-hundreds-leishmaniasis-cases-reported-mohmand-agency-87439/

https://www.wired.com/story/hajj-who-early-disease-warning-system/

https://actualite.housseniawriting.com/sante/maladies-tropicales/2017/10/27/pourquoi-des-maladies-facilement-guerissables-sont-elles-toujours-presentes/24607/

ASM Podcast, "Neglected tropical diseases and vaccine advocacy with Peter Hotez" (December 8, 2017, Julie Wolff)

Public lectures. Dr. Hotez, the Principal Investigator, spoke extensively about leishmaniasis vaccine efforts:

1. September 14, 2018 Harris County Health, 2018 One Health Conference (Houston, TX), "Vaccines, Autism, and Blue Marble Health"
2. September 12, 2018 University of Oklahoma, College of Public Health, Epidemiology and Biostatistics Student Association (Oklahoma City, OK), "Vaccines"
3. September 11, 2018 University of Oklahoma, College of Arts and Sciences (Norman, OK), "Vaccines"
4. September 11, 2018 University of Oklahoma, College of Arts and Sciences (Norman, OK), "Shifting paradigms in global health"
5. August 28, 2018 Cook Children's Medical Center, Pediatric Grand Rounds (Fort Worth, TX), "The rise of neglected and emerging infections in Texas and globally"
6. July 17, 2018 Curious 2018 Future Insight Conference Keynote (Darmstadt, Germany), "The neglected diseases: Will a new world order reverse global gains"
7. July 11, 2018 University of Michigan School of Public Health, 53rd Summer Epidemiology Session, Alfred S. Evans Memorial Lecture (Ann Arbor, MI), "Neglected tropical disease and the antipoverty vaccines"
8. July 9, 2018 Baylor College of Medicine, SMART Program (Houston, TX), "Vaccines"
9. July 6, 2018 Colombian Pediatric Society Annual Meeting Keynote (Cartagena, Colombia),

Vacunas "Antipobreza"

10. July 6, 2018 Colombian Pediatric Society Annual Meeting Keynote (Cartagena, Colombia) "Las vacunas no causaron el autism de Rachel"
11. June 29, 2018 AAAS Science Diplomacy Webinar (Washington DC) "Vaccine Diplomacy"
12. June 22, 2018 UT Health Northeast Grand Rounds, and Conference on Emerging Issues in Occupational and Environmental Health (Tyler, TX), "Emerging infectious diseases"
13. June 16, 2018 Academie Royale de Medecine de Belgique, Séance dediee aux maladies tropicales negligees (Brussels, Belgium), "Neglected tropical diseases in the time of blue marble health and the anthropocene"
14. June 13, 2018 APIC, Association for Professionals in Infection Control and Epidemiology, Plenary Lecture (Minneapolis MN), "Neglected Tropical Diseases and the Antipoverty Vaccines"
15. June 1, 2018 Pediatric Grand Rounds, Department of Pediatrics, Stanford University School of Medicine, and Lucille Packard Children's Hospital (Stanford University), "Vaccines, autism, and blue marble health"
16. May 30, 2018 Epidemiology of Infectious Diseases Course, Stanford University School of Medicine (Stanford, CA), "The rise of neglected tropical diseases 'the NTDs"
17. May 21, 2018 SciTS (Science of Team Science) Conference 2018 (Galveston, TX), Keynote Address, "Vaccines, autism, and blue marble health"
18. May 4, 2018 Baylor College of Medicine, Alumni Reunion (Houston, TX), "Vaccines, autism and blue marble health"
19. May 3, 2018 Henry Ford Medical Center, Medical Grand Rounds (Detroit, MI), "The rise of the poverty related neglected diseases, the #NTDs"
20. April 27, 2018 University o Arizona, Science Health and Engineering Policy Seminar Series (Tucson, AZ), "Vaccine diplomacy"
21. April 25, 2018 Baylor College of Medicine, Lunch and Learn (Houston, TX), "Vaccines and autism"
22. April 19, 2018 Harvard TH Chan School of Public Health, ID Epidemiology Seminar Series (Boston, MA), "The rise of poverty-related neglected tropical diseases"
23. April 18, 2018 University of Massachusetts Amherst, College of Natural Sciences, Annual Undergraduate Life Science Research Symposium (Amherst MA), "The rise of poverty-related neglected tropical diseases"
24. April 16, 2018 Baylor College of Medicine, Biomedical Research Awareness Day Lecture (Houston, TX), "Vaccines and Autism"
25. April 11, 2018 University of Texas Austin, Departments of Philosophy and Biology (Austin, TX), "Vaccines, Autism, and Blue Marble Health"
26. April 5, 2018 World Vaccine Congress (Washington DC), Plenary Lecture: "Will a new 2018 global leadership commit to NTDs"
27. April 3, 2018 World Vaccine Congress (Washington DC), Plenary Panel: "Responding to the next emerging diseases globally"
28. March 28, 2018 University of Oklahoma, Provost's Seminar (Norman, OK), "The rise of the poverty-related neglected diseases – the NTDs"
29. March 23, 2018 Texas A&M University, Brockman Scholars Programme (College Station, TX), "The rise in poverty-related neglected diseases"
30. March 14, 2018 Research!America Advocacy Awards Dinner (Wash DC), Acceptance remarks for Sustained Leadership Award
31. March 8, 2018 Scowcroft Institute of International Affairs, Texas A&M University (College Station, TX), "Vaccines, autism, and blue marble health"
32. March 7, 2018 World Affairs Council Houston (Houston, TX), Panel on the "Future of Global Health"
33. March 6, 2018 Texas Medical Center TMC Policy Institute (Houston, TX), Panel on Public Health
34. February 28, 2018 Texas Children's Hospital, Baylor College of Medicine, Obstetrics-

Gynecology Grand Rounds (Houston, TX), "The NTDs – the most common afflictions of girls and women in poverty"

35. February 26, 2018 Rice University, Class on Public Science Communications (Houston, TX), "Vaccines and Autism"
36. February 19, 2018 Duke University, Victor L. Dzau Lecture in Global Health (Durham, NC), "Vaccines autism and blue marble health"
37. February 9, 2018 Wellcome Sanger Institute, EMBL-EBI, Cambridge University, Distinguished Lecture Series (Hinxton, UK), "The Rise of Poverty-Related Neglected Diseases - The "NTDs"
38. February 8, 2018 King's College, Cambridge University (Cambridge, UK), Global Health Seminar, "Access to medicines panel"
39. February 6, 2018 America House, US Embassy, Consulate in East Jerusalem (Jerusalem, Israel), "Vaccine development for regional and infectious diseases"
40. January 25, 2018 Foreign Policy Association, Great Decisions Series (Houston, TX), "Vaccines, autism, and blue marble health"
41. January 22, 2018 Eisai, Ltd (Tokyo, Japan), "NTDs V.2.0"
42. January 10, 2018 Eli Lilly and Company (Indianapolis, IN), Grand Rounds Series, "The rise of poverty-related neglected diseases – the NTDs"
43. January 9, 2018 Marian University (Indianapolis, IN), 2017-18 Global Studies Speaker Series, "The rise of poverty-related neglected diseases – the NTDs"
44. December 12, 2017 24th Annual Frontiers of Vaccinology Lecture, University of Maryland Baltimore, Center for Vaccine Development (Baltimore, MD), "Vaccines, Autism, and Blue Marble Health"
45. November 30, 2017 World Vaccine & Immunotherapy West Coast Congress (San Diego, CA) "Blue marble health: an innovative plan to fight diseases of the poor amid wealth" keynote
46. November 17, 2017 BaylorScott&White Health, 24th Annual Jesse D Ibarra Jr MD Lectureship in International Health (Temple, TX), "The neglected diseases and the antipoverty vaccines"
47. November 16, 2017 Fifth International Symposium on Thymosins in Health and Disease (Washington DC) Abraham White Lifetime Public Service Awardee address" Vaccines & Immunotherapeutics in a Rising Tide of American Anti-Science"
48. October 17, 2017 Scowcroft Institute of International Affairs, Bush School of Government and Public Policy, Texas A&M University, Pandemic Summit (College Station, TX) "Neglected diseases in a new world order"
49. October 11, 2017 Millipore-Sigma Scientific Symposium (Burlington, MA), "Vaccines: Innovative Approaches Towards Treating Neglected Diseases"
50. October 7, 2017 International Society of Vaccines (ISV) Annual Meeting, Institut Pasteur (Paris, France), "The Antipoverty Vaccines"
51. September 30, 2017 TEDx Sugarland (Sugarland, TX), "Blue marble health: an innovative plan to fight diseases of poverty amid wealth"
52. September 30, 2017 One Health, 10th Annual One Health Conference, Harris County Public Health and Environmental Services (HCPHES) (Houston, TX), "What about those neglected zoonotic diseases?"
53. September 29, 2017 Yale University School of Management, Executive MBA (New Haven, CT), "Vaccines autism, and blue marble health"
54. September 20, 2017 Baylor College of Medicine, Med-Peds Grand Rounds (Houston, TX), "Vaccines, autism, and blue marble health"
55. September 19, 2017 Texas Children's Hospital, Global Health Seminar Series (Houston TX), "Vaccines, autism, and blue marble health"
56. September 18, 2017 UT Health, Bioterrorism and Emerging Infections Public Health Preparedness and Response Lecture Series (Houston, TX) "Neglected diseases: will a new world order reverse global gains?" September 14, 2017 AAAS Center for Science Diplomacy (Washington DC), "Vaccines, Autism, and Blue Marble Health"

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

For activities scheduled for Major Tasks 1-4, as described above, we remain on schedule to achieve the milestones set in the original statement of work. Major Task 5 (reproducibility) is scheduled to begin in or before Month 15. Major Task 6 (formulation and stability studies) will follow, as scheduled. With these activities we will have a) provided proteins to our partners for immunogenicity and efficacy studies, and b) developed a production process for both vaccine antigens

We will complete Major Task 8, Activity 2.2 as scheduled, the immunological assessment of C57Bl/6 mice vaccinated with recombinant PpSP15 and LdNH36: testing the role of GLA-SE adjuvant. The USU deliverable will be to select optimized CL-vax doses and route of administration and test the effect upon immunogenicity by adding GLA-SE adjuvant.

Under Major Task 9, we plan to continue expanding the sand fly colony to be ready for transmission experiments. We will perform small scale infections in sand fly to test if sand flies are infectious and ready for transmission.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**
  - We are advancing the concept that it is feasible to develop a recombinant vaccine to prevent cutaneous leishmaniasis, one of the leading neglected tropical disease of military importance
- **What was the impact on other disciplines?**
  - We are advancing the concept that simultaneously targeting both the infectious organism + its vector is a promising vaccine strategy to combat vector-borne infectious diseases.
- **What was the impact on technology transfer?**
  - We are optimizing processes for the express purpose of technology transfer of two recombinant protein antigens. These processes appear to be robust and express the proteins at high yield. Our purification processes produce proteins at high levels of purity.
- **What was the impact on society beyond science and technology?**
  - The major impact is that we are developing an innovative vaccine for a serious infection emerging in areas of conflict and political instability. We hope that our leishmaniasis vaccine will not only have an important military use, but also will find use in preventing this disease among highly vulnerable populations, including refugee populations.

5. Changes/Problems

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them.
In May 2018, a mouse parvovirus outbreak occurred in the room where the mice were housed that coincided when USUHS was harvesting their experimental samples from the immunogenicity study. The experimental mice tested negative for parvovirus. However, some investigators petitioned to keep their infected animals in the USUHS vivarium until Oct-Nov 2018. Because of the potential profound immune effects a parvovirus infection could have on the vaccine study, after discussion the

LTC Foster, Head of Laboratory Medicine USUHS and Dr. Cox, Chair IACUC USUHS, decided that mice for studies under this grant project would be moved to another facility until the USU vivarium became parvovirus free. Our grant collaborators at NIAID/LMVR agreed to allow the USHUS team to use the NIAID Twinbrook vivarium; tissues/samples will be returned to USUHS and subjected to immunological assessment as per approved protocol. NIAID has amended their animal protocol to allow Dr. Ines Elakhal Naouar to vaccinate, monitor, bleed and necropsy mice at the NIAID facility, including modifying to allow the intradermal inoculation of vaccine in two areas of the skin of the back (required due to vaccine concentration and volume required). This change of vivaria has resulted in approximately an 8-10 week delay in initiating experiment 2. However, we do not consider this to be significant since it will not affect the anticipated overall SOW. We anticipate that future studies (a repeat GLA-SE and CL vax experiment) will be conducted back at USU vivarium in the Spring of 2019.

Changes that had a significant impact on expenditures. In the past year our federal employee research biologist, Nancy Koles, retired and unexpectedly our Dean has not permitted us to fill that vacant GS position. This has resulted in a loss of 15% personnel effort at our site. We are asking to increase Dr. Elakhal Naouar from 50% to 60% time in year 2. Additionally, NIAID asked for extended immunogenicity profiling in excess of what had been planned, including multiple incubation timepoints and use of Luminex cytokine profiling. This has a significant budget impact.

Significant changes in use or care of human subjects, vertebrate animals, biohazard

In the past year our federal employee research biologist, Nancy Koles, retired and unexpectedly our Dean has not permitted us to fill that vacant GS position. This has resulted in a loss of 15% personnel effort at our site. We are asking to increase Dr. Elakhal Naouar from 50% to 60% time in year 2. Additionally, NIAID asked for extended immunogenicity profiling in excess of what had been planned, including multiple incubation timepoints and use of Luminex cytokine profiling. This has a significant budget impact.

At USUHS there were two changes to the use of vertebrate animals during this reporting period.
USUHS Applicable Institutional Animal Care and Use Committee approval dates are as follows:
Initial approval: 19 September 2017
Major Modification 1 approval: 23 Jan 2018. Changed vaccine volume to 50±10 µl for intradermal route administration, applied in two areas to skin of back. Also removed Nancy Koles.
Minor Modification 1 approval: 23 Jan 2018. 1 mg/ml cysteine added to vaccine buffer to decrease the aggregation problem identified

Significant changes in use or care of human subjects

Not applicable

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. Products

LdNH36 protein

PpSP15 protein

7. Participants & Other Collaborating Organizations

Uniformed Services University of the Health Sciences	
Name:	*Ines Elakhal Naouar*

Project Role:	*Associate Investigator, Junior Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Elakhal Naouar has performed all the mouse vaccination and immunogenicity experiments in Task 7.*
Funding Support:	
Name:	*Naomi Aronson*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Aronson supervised the mouse work, participated in all consortium calls, completed most of the regulatory documents and subaward, agreement, paperwork allowing this project to proceed*
Funding Support:	
Name:	*Saule Nurmukhambetova*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Ms. Nurmukhambetova assisted Dr. Elakhal Naouar with mouse work and the processing of tissue samples.*
Funding Support:	
NIH	
Name:	*Jesus G. Valenzuela*
Project Role:	*Investigator*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-5589-9450*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Valenzuela has performed work in conditioning an insectary room to grow sand flies for vaccine challenge study.*
Funding Support:	
Baylor College of Medicine	
Name:	*Peter J. Hotez*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0001-8770-1042*
Nearest person month worked:	*12*

Contribution to Project:	*Dr. Hotez has directed the team at BCM and has guided the experimental design and reviewed the data analysis.*
Funding Support:	
Name:	*Maria Elena Bottazzi*
Project Role:	*Co-Director Vaccine Center*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-8429-0476*
Nearest person month worked:	*7.2*
Contribution to Project:	*Dr. Bottazzi supervised the upstream and downstream process development teams, as well as the quality control unit at the vaccine center. She also reviewed all technical reports.*
Funding Support:	
Name:	*Coreen Beaumier*
Project Role:	*Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-7095-6214*
Nearest person month worked:	*3.6*
Contribution to Project:	*Dr. Beaumier served as the project manager of this project.*
Funding Support:	
Name:	*Jeroen Pollet*
Project Role:	*Director of Formulation*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0003-1420-4015*
Nearest person month worked:	*3.6*
Contribution to Project:	*Dr. Pollet was involved in assay development and stability assessment of the vaccine antigen candidates*
Funding Support:	
Name:	*Bin Zhan*
Project Role:	*Director, Molecular Biology and Antigen Discovery*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-6884-9452*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Zhan was responsible for designing the PpSP15 construct and for establishing a small-scale expression system.*
Funding Support:	
Name:	*Ulrich Strych*
Project Role:	*Director, Reporting*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0001-9455-7683*

Nearest person month worked:	*3.6*
Contribution to Project:	*Project coordination, Scientific review of all experimental designs at BCM, Composition of annual report*
Funding Support:	
Name:	*C. Patrick McAtee*
Project Role:	*Director, Upstream Development*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*11.52*
Contribution to Project:	*Dr. McAtee conducted the antigen purification.*
Funding Support:	
Name:	*Chris Seid*
Project Role:	*Director, Downstream processing*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6.96*
Contribution to Project:	*Dr. Seid oversaw the fermentation of both vaccine antigens.*
Funding Support:	
Name:	*Zhuyun Liu*
Project Role:	*Director, Downstream processing*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.68*
Contribution to Project:	*With Dr. Peak, Ms. Liu took over Dr. Seid's responsibilities after his departure from the vaccine center.*
Funding Support:	
Name:	*Nyon Mun Peak*
Project Role:	*Postdoctoral Researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*10.8*
Contribution to Project:	*With Ms. Liu, Dr. Peak took over Dr. Seid's responsibilities after his departure from the vaccine center.*
Funding Support:	
Name:	*Wen-Hsiang Chen*
Project Role:	*Director, Quality Control*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*7.2*
Contribution to Project:	*Dr. Chen oversaw all quality control for PpSP15 and LdNH36. He supervised Dr. Biter*
Funding Support:	
Name:	*Amadeo Biter*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	13.44
Contribution to Project:	*Dr. Biter worked on assay development for the vaccine antigens, as well as on stability and compatibility studies.*
Funding Support:	
Name:	*Portia Gillespie*
Project Role:	*Laboratory manager*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3.6*
Contribution to Project:	*Laboratory management*
Funding Support:	
Name:	*Diane Nino*
Project Role:	*Project Manager*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*7.2*
Contribution to Project:	*Project coordination in support of Dr. Beaumier*
Funding Support:	
Name:	*Junfei Wei*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*33.96*
Contribution to Project:	*Dr. Wei worked under supervision of Dr. Zhan on the engineering of the PpSP15 constructs, and their testing at the small-scale level.*
Funding Support:	
Name:	*An Nguyen*

Project Role:	*Senior Project Coordinator, Quality Assurance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*31.56*
Contribution to Project:	*Ms. Nguyen provided quality assurance services during the performance of the process development and quality control testing activities*
Funding Support:	
Name:	*Catherine Smith*
Project Role:	*Senior Project Coordinator, Quality Assurance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*9.6*
Contribution to Project:	*Ms. Smith provided quality assurance services during the performance of the process development and quality control testing activities*
Funding Support:	
Name:	*Shannon McKim*
Project Role:	*Project Coordinator, Quality Assurance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*9.6*
Contribution to Project:	*Ms. McKim took over Ms. Smith responsibilities after Ms. Smith left her position.*
Funding Support:	

Has there been a change in the active other support of PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Aronson has completed two grants, the Surveillance of latent visceral leishmaniasis among Operation Iraqi Freedom deployers, 10% effort and TB drug resistance surveillance in Sub-Saharan Africa and Afghanistan using acid fast stained smears (glass microscopy slides), 15% effort.

Changes for Peter Hotez:

- Title of the project: Chagas Disease Vaccine Development Program – End date extended to 12/31/2018
- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – End date extended to 3/31/2019; Effort at 1.5%
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – End Date extended to 12/31/2018; Effort changed to 1%
- Title of the project: West Nile Virus vaccine development – End date extended to 12/31/2018
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 5%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 8.33%

- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Effort at 10%

Changes for Coreen Beaumier:

- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: West Nile Virus vaccine development – End date extended to 12/31/2018; Effort changed to 3%
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – End Date extended to 12/31/2018; No Effort on Grant
- Title of the project: The Slim Initiative for the Development of the firs Human Chagas Disease vaccine – Effort changed to 7.5%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018; Effort changed to 5%
- Title of the project: Chikungunya Recombinant Subunit Vaccine – End Date extended to 12/31/2018; Effort changed to 5%
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 5%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 2.5%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia – New addition to active support; Effort at 10%

Changes for Maria Elena Bottazzi:

- Title of the project: Chagas Disease Vaccine Development Program – End date extended to 12/31/2018;
- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: Development of a novel adjuvant for vaccine sparring – End date extended to 12/31/2018
- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – End date extended to 3/31/2019; Effort changed to 2%
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – End Date extended to 12/31/2018; Effort changed to 1%
- Title of the project: West Nile Virus vaccine development – End date extended to 12/31/2018
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018
- Title of the project: The development of a recombinant vaccine against Human Onchocerciasis – Effort changed to 5%
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 5%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 5%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Effort at 10%

Changes for Jeroen Pollet:

- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: Developing and Testing a novel, low-cost, effective HOOKworm VACcine to Control Human Hookworm Infection in endemic countries – End date extended to 3/31/2019; Effort changed to 0.5%
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – End date extended to 12/31/2018; Effort reduced to 0%
- Title of the project: Development of a novel adjuvant for vaccine sparring – Effort reduced to 0%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018; Effort changed to 15%

- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 10%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 2.5%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Effort at 10%

Changes for Ulrich Strych:

- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: U.S.-Malaysian Neglected Tropical Disease Initiative – End Date extended to 12/31/2018; Effort changed to 5%
- Title of the project: The Slim Initiative for the Development of the firs Human Chagas Disease vaccine – Effort Changed to 7.5%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018; Effort changed to 5%
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 5%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 2.5%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Effort at 10%

Changes for Bin Zhan:

- Title of the project: RBD Recombinant Protein-based SARS Vaccine for Biodefence – Ended 7/31/2017
- Title of the project: Development of a novel adjuvant for vaccine sparring – End date extended to 12/31/2018; Effort changed to 5%
- Title of the project: Chagas Vaccine Initiative: Accelerated Development of the First Therapeutic Vaccine for Chagas Disease – End Date extended to 12/31/2018; Effort changed to 5%
- Title of the project: The development of a recombinant vaccine against Human Onchocerciasis – No Change
- Title of the project: Network systems biology to identify and test novel multivalent mRNA lipopolyplex vaccines against Chagas disease – New addition to active support; Effort at 5%
- Title of the project: A vaccine to prevent leishmaniasis – New addition to active support; Effort at 5%
- Title of the project: Combatting Next Generation Lethal Epidemics in Saudi Arabia - New addition to active support; Effort at 10%
- Title of the project: Development of a vaccine against Dirofilaria - New addition to active support; Effort at 5%

8. Special Reporting Requirements

Not applicable

9. Appendices

Nothing to report

References:

1. McAtee CP, Seid CA, Hammond M, Hudspeth E, Keegan BP, Liu Z, Wei J, Zhan B, Arjona-Sabido R, Cruz-Chan V, Dumonteil E, Hotez PJ, Bottazzi ME. Expression, purification, immunogenicity and protective efficacy of a recombinant nucleoside hydrolase from Leishmania donovani, a vaccine candidate for preventing cutaneous leishmaniasis. Protein expression and purification. 2017;130:129-36. doi: 10.1016/j.pep.2016.10.008. PubMed PMID: 27773761.
2. Oliveira F, Rowton E, Aslan H, Gomes R, Castrovinci PA, Alvarenga PH, Abdeladhim M, Teixeira C, Meneses C, Kleeman LT, Guimaraes-Costa AB, Rowland TE, Gilmore D, Doumbia S, Reed SG, Lawyer PG, Andersen JF, Kamhawi S, Valenzuela JG. A sand fly salivary protein vaccine shows

efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates. Sci Transl Med. 2015;7(290):290ra90. doi: 10.1126/scitranslmed.aaa3043. PubMed PMID: 26041707.