Multivalent Fusion Protein Vaccine for Lymphatiac filariasis

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Abstract

Lymphatic filariasis affects approximately 3% of the whole world population. Mass drug administration is currently the major control strategy to eradicate this infection from endemic regions by year 2020. Combination drug treatments are highly efficient in controlling the infection. However, there are no effective vaccines available for human or animal lymphatic filariasis despite the identification of several subunit vaccines. Lymphatic filariasis parasites are multicellular organisms and potentially use multiple mechanisms to survive in the host. Therefore, there is a need to combine two or more vaccine candidate antigens to achieve the desired effect. In this study we combined three well characterized vaccine antigens of *Brugia malayi*, heat shock protein12.6 (HSP12.6), abundant larval transcript-2 (ALT-2) and tetraspanin large extra cellular loop (TSP-LEL) as a multivalent fusion vaccine. Putative immune individuals carry circulating antibodies against all three antigens. Depletion of these antigen specific antibodies from the sera samples removed the ability of the sera to participate in the killing of *B. malayi* L3 in an antibody dependent cellular cytotoxicity (ADCC) mechanism. Vaccination trials in mice with a bivalent [HSP12.6+ALT-2 (HA), HSP12.6+TSP-LEL (HT) or TSP-LEL+ALT-2 (TA)] or trivalent [HSP12.6+ALT-2+TSP-LEL (HAT)] vaccines using DNA, protein or heterologous prime boost regimen showed that trivalent HAT vaccine either as protein alone or as heterologous prime boost vaccine could confer significant protection (95%) against *B. malayi* L3 challenge. Immune correlates of protection suggest a Th1/Th2 bias. These finding suggests that the trivalent HAT fusion protein is a promising prophylactic vaccine against lymphatic filariasis infection in human.

Key Words. *Multivalent vaccine, small Heat Shock Proteins, Abundant Larval transcript-2, Tetraspanin, Antibody Dependent cellular cytotoxicty, Lymphatic Filariasis*

1. INTRODCUTION

Lymphatic filariasis caused by *Brugia malayi, Wuchereria bancrofti* and *Brugia timori* affects more than 120 million people living in 72 different countries [1]. Chronic infections are associated with severe lymphatic pathology characterized by lymph edema and lymphadenitis. Human infection occurs when mosquitoes transmit the third stage larvae (L3) of the parasite. Thus, strategies that can kill L3 can prevent lymphatic filariasis infection in the human. In endemic regions, certain individuals are naturally immune to lymphatic filariasis [2]. These individuals also called endemic normals (en) carry circulating antibodies against several antigens of L3. We recently showed that these circulating antibodies can participate in the killing of L3 in an ADCC mechanism [3, 4]. By screening a phage display cDNA expression library of *B. malayi* L3 with en sera samples, we identified several antigens of L3 as potential vaccine candidates [5-8]. Similarly, our group and others have reported several subunit vaccine candidates with varying degrees of protection in experimental animals [3, 9-14]. Among these, three vaccine antigens; Abundant Larval transcript [ALT-2] [5,9], small Heat Shock Protein 12.6 [HSP12.6] [4] and Tetraspanin Large extracellular loop [TSP-LEL] (unpublished data) were identified as leading vaccine candidates.

L3 stages are multicellular organisms that have evolved multiple mechanisms to evade host immune responses [15,16] for their survival. Thus, it is important to target more than one critical antigens of the parasite to get the desired vaccine-induced protection. This notion was confirmed by our previous studies, where we show that combining two antigens as a multivalent vaccine can synergistically increase the degree of vaccine-induced protection [8,17]. Similar findings were reported by other groups as well [18-20]. Another advantage of using multivalent

vaccine is that it allows generation of multiple peptides with varied MHC restriction. Since human population express a vast repertoire of MHC, a multivalent vaccine can generate a better and broader immune response in a human population than a single subunit vaccine that has limited MHC restriction [21,22]. Therefore, in this study we attempted to combine the three antigens (ALT-2, HSP12.6 and TSP-LEL) of *B. malayi* as a multivalent fusion vaccine and evaluated its vaccine potential *ex vivo* in human and in a mouse model.

2. Materials and methods

2.1. Animals and Parasites

B. malayi L3 were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (University of Georgia, Athens, GA) and Balb/c mice were purchased from Charles River laboratory (Wilmington, MA). Use of animals in this study was approved by the Animal Care Committee of the University of Illinois Rockford.

2.2. Collection of human blood samples

Blood samples were collected after proper consent from endemic normal (en) subjects residing in and around Sevagram Maharashtra, India. en subjects are asymptomatic, non-microfilaraemic individuals with no circulating parasite antigens in their blood [3-5]. Sera samples from healthy non-endemic normal (nen) subjects were collected at Rockford, IL after proper consent. Use of human subjects in this study was approved by the Institutional Review Board (IRB) of the University of Illinois, College of Medicine at Rockford and the IRB committee of Mahatma Gandhi Institute of Medical Sciences, Sevagram, Maharashtra, India.

2.3. Construction of multivalent DNA vaccine

DNA sequences were codon optimized (Genscript, Piscataway, NJ) for optimal expression in mammalian cells. To prepare multivalent construct, *Bmhsp12.6* and *Bmalt-2* genes were cloned into *pVAX™200-DEST* vector as described earlier [4]. No stop codons were added to these constructs for continuous transcription of both the genes. *Bmtsp-LEL* DNA was then cloned into *pVAXBmhsp12.6+Bmalt-2* plasmid using forward primer 5′-CGC**GAATTC**ACCATGGTCCTGGAG-3′ containing EcoRI restriction site and the reverse primer 5′- GC**TCTAGA**TCAGTCCTTCTGGCTAG-3′ containing XbaI restriction site and stop codon. Bivalent DNA constructs *Bmhsp12.6+Bmalt-2* (*ha*), *Bmhsp12.6+Bmtsp-LEL* (*ht*) and *Bmtsp-LEL+Bmalt-2* (*ta*) were prepared using respective primers.

2.4. Construction and expression of rBmHAT multivalent fusion protein

pRSETA *Bmhsp12.6+Bmalt-2+Bmtsp-LEL* (rBmHAT) was constructed in the same manner as above. The primer sequences were *BmHSP12.6* (forward primer 5′-CG**GGATCC**ATGGAA GAAAAGGTAGTG-3′ and reverse primer 5′-CCC**TCGAGT**GCTTTCTTTTTGGCAGC-3′). *BmALT-2* (forward primer 5′-CCC**TCGAGA**TGAATAAACTTTTAATAGCAT-3′ and reverse primer 5′-GGG**TACCCG**CGCATTGCCAACCC-3′). *Bmtsp-LEL* (forward primer 5′-GGG**GTACCC**CGGCAAGGATCAATTTAAAA-3′ and reverse primer 5′-CG**GAATTC**TCA ATCTTTTTGAGATGAAT-3’). Bivalent constructs (HA, HT and TA) were also cloned individually into pRSETA vectors using appropriate primer pairs. Recombinant fusion proteins

were expressed in *E. coli* BL21 (pLysS), purified and endotoxin removed by Pierce High Capacity Endotoxin removal resin column (Thermo Fisher Scientific, Rockford, IL).

2.5. Immunization of animals

Six weeks old Balb/C mice were randomly divided into 8 groups with 10 mice per group. Each set of mice were immunized at 15 days interval with two doses of 100 μg of endotoxin free DNA (intradermally) followed by two doses of 15μg of protein (subcutaneously) suspended in Imject® Alum (Thermo Fisher Scientific). Vaccination protocol is schematically presented in Supplement Fig. 1A. The following groups were used (a) HSP12.6, (b) ALT-2, (c) TSP-LEL, (d) HSP12.6+ALT-2 (HA), (e) HSP12.6+TSP-LEL (HT), (f) TSP-LEL+ALT-2 (TA), (g) HSP12.6+ALT-2+TSP-LEL (HAT) and (h) Controls that received *pVAX* vector and alum. The experiments were repeated three times with all the groups

A second set of experiment was performed with the trivalent HAT vaccine. Groups of 10 mice were divided into 6 groups and immunized at two weeks intervals with (a) 100 μg of endotoxin free codon optimized *hat* DNA suspended in PBS, (b) 15μg of rBmHAT proteins in Imject® Alum, and (c) Heterologous prime boost vaccination (primed with two injections of *hat* DNA at 2 weeks interval, and boosted with 2 injections of rBmHAT proteins also given at two weeks interval). Vaccination protocol is schematically presented in Supplement Fig. 1B. There were three control groups (d) *pVAX DNA*, (e) Alum and (f) *pVAX vector* plus Imject® Alum. The experiments were repeated three times with all the groups.

2.6. Evaluation of antibody responses in the sera of human and mice

Levels of anti-BmHSP12.6, anti-BmALT-2 and anti-BmTSP-LEL IgG antibodies were determined in the sera of human subjects (en and nen) and mice (immunized or control) using an indirect ELISA as described previously [3] Antibody specificity was confirmed after separating the recombinant proteins on a 12% SDS-PAGE gel, transblotting to nitrocellulose sheet and probing with respective antibodies [3].

2.7. Antibody-dependant cellular cytotoxicity (ADCC) assay

An *in vitro* ADCC assay was performed as described previously [3,4] to determine if serum antibodies can participate in the killing of L3. Briefly, ten (10) L3 of *B. malayi* were incubated with 50 µl of pooled (n=10) sera samples from en subjects and 2 x 10^5 PBMC from healthy volunteers in a 96 well culture plate at 37°C and 5% CO_2 for 48hrs. In the mouse studies, 10 L3 were incubated with 2 x 10^5 peritoneal cells (PEC) from normal Balb/C mice and 50 µl of pooled (n=10) sera samples from immunized mice. Larval viability was determined under a light microscope as described previously [3]. Parasites that were clear and straight with no movements were counted as non-viable; if they still showed no movement and remained straight for the next 8 hours at 37°C, they were counted as dead. The live larvae remained active. ADCC was estimated as percent larval death, calculated using the formula: number of dead larvae x 100 ÷ total number of larvae used for ADCC.

In another study, we depleted total IgG, anti-BmHAT, anti-BmHSP12.6, anti-BmALT-2 or anti-BmTSP-LEL antibodies from mouse and human sera samples [4]. Briefly, 200 µl of pooled sera

samples were incubated overnight at 4°C with rBmHSP12.6, rBmALT-2 and/or rBmTSP-LEL that were coupled to IMAC resin and flow through was collected. Total IgG was removed by incubating sera samples with Protein A gel beads (Thermo Fisher Scientific). Depletion of respective antibodies in the sera samples was confirmed by an indirect ELISA as described previously [3]. The antibody depleted sera was then used in ADCC assays.

2.8. Challenge studies in mice using micropore chamber technique

Vaccine-induced protection was determined by implanting a challenge dose of twenty (20) L3 into the peritoneal cavity of immunized mouse in a micropore chamber as described previously [4,17,23]. 48 h after implantation, contents of each chamber were examined microscopically for larval death. Percentage protection was calculated using the formula; number of dead parasites / number of recovered parasites x 100.

2.9. Splenocyte proliferation:

2 x 10^5 single cell suspension of spleen cells were stimulated with 1 µg of recombinant proteins (rHSP12.6, rALT-2 or rTSP-LEL) or 1 µg of ConA for 72 hours at 37°C in 5% CO2. Cells cultured in media served as controls. Stimulation indices (S.I.) were calculated as a measure of cell proliferation [3]. For cytokine analysis, splenocytes were stimulated with 1 µg of rBmHAT for 72 hours at 37°C. Secreted levels of IFN-γ, IL-4, IL-10 and IL-2 were determined in the culture supernatants using cytokine ELISA kits (Thermo Fisher Scientific). Each sample was analyzed in triplicate wells and the experiment was repeated three times.

2.10. Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) was used to analyze the data. Comparisons between two individual data points were made using a Student's t-test. For multiple comparisons, one way ANOVA was used along with the Tukey-Kramer and/or Dunnet's post-test wherever appropriate. For cytokine analysis two-way ANOVA was used with Bonferroni post test. A probability (p) value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. en subjects carry high titer of IgG antibodies against all three vaccine antigens.

Putatively immune en individuals carry significantly ($P<0.001$) high titers of anti-BmHSP12.6, anti-BmALT-2 and anti-BmTSP-LEL IgG antibodies in their sera compared to nen subjects (Fig. 1) A Student's t-test was performed to compare the values between en and nen subjects. No significant reactivity was observed when anti-HAT antibodies were depleted from en sera samples (Supplement Fig. 2A).

3.2. Antibodies in the sera of en subjects can participate in the killing of L3

Pooled sera samples from en subjects participated in the killing of *B. malayi* L3 (66%) when used in the ADCC assay (Table 1). However, when total IgG was depleted, the L3 killing ability of en sera was reduced to 6% (significant $P<0.001$), suggesting a key role for IgG antibodies in the L3 killing. Depletion of anti-BmHSP12.6, anti-BmALT-2 or anti-BmTSP-LEL antibodies from en sera also resulted in significant ($P<0.001$) reduction of L3 killing activity (41%, 29% and 21% killing respectively). When two of the antibodies (anti-HA, anti-HT, anti-TA) were

depleted, the L3 killing ability of en sera samples were substantially compromised (5%, 10%, 26% respectively) (significant $P<0.001$). When all three antibodies (anti-BmHAT) were depleted, the L3 killing ability of en sera dropped down to 5% confirming a central role for the three antibodies in the killing of L3. These findings suggested that all three candidate antigens have potential to be vaccine candidates against lymphatic filariasis in the human. Comparison of ADCC mediated killing between en sera and anti BmHAT antibodies depleted sera was performed by one way ANOVA along with Dunnett's post statistics test.

3.3. Each component antigen in the multivalent fusion construct is immunogenic.

A hybrid DNA vaccine in *pVAX 1* vector (850 bp) was constructed (Supplement Fig. 3) and fusion protein prepared in pRSETA vector using all three antigens. The trivalent rBmHAT had a molecular size of ~40 kDa (Supplement Fig. 4A). Endotoxin levels in the recombinant protein preparations were <10 EU. Immunoblot analysis confirmed that all three antigens are expressed in the multivalent construct and are immunologically reactive (Supplemental Fig. 4B).

3.4. Mice immunized with the trivalent vaccine develop high titer of antibodies against all three antigens

Immunization of mice with the trivalent DNA or protein vaccine (*pVAX-hat* and/or rBmHAT protein) elicited higher titers of IgG antibodies (1:30000) compared to those vaccinated with bivalent or monovalent constructs (Fig. 2). Mice vaccinated with the trivalent heterologous prime (DNA) boost (protein) vaccine or protein alone vaccination showed the highest titer ($P<0.05$) of IgG antibodies compared to DNA alone vaccinated group (Fig. 2C). Comparison of antibody

titer against each antigen within the individual vaccine constructs was performed by one way ANOVA with Tukey-Kramer's post test.

3.5. Antibodies in the sera of vaccinated mice can kill L3 in an ADCC mechanism.

We then evaluated if the vaccine-induced antibodies can kill L3 in an ADCC assay [23-25]. Our results show that sera from mice immunized with HAT or HA vaccine constructs exhibited maximum larval killing effect (approximately 90%) ($P<0.001$) (Table-2). Bivalent vaccine constructs of HT and TA also gave significant protection of 82% & 87% respectively compared to control animals ($P<0.001$). When antibodies against rBmHSP12.6, rBmALT-2 and rBmTSP-LEL were depleted from the en sera samples, L3 killing was significantly ($p<0.001$) compromised (6% killing). Depletion of antibodies in the sera samples were confirmed using an ELISA (Supplement Fig. 2B). Statistical comparison between the immunized mice sera and control sera was performed by one way ANOVA along with Dunnett's post test.

3.6. Immunization of mice with the trivalent vaccine confers significant protection against challenge infection.

Vaccine-induced protection in mice was confirmed using an *in vivo* micropore challenge experiment. Mice immunized with HAT as heterologus DNA prime/ protein boost vaccination regimen gave 94% protection (significant $P<0.001$) compared to control group of animals. Comparable levels of protection (significant $P<0.001$) were also obtained when mice were immunized with the bivalent vaccines HA (90%), TA (80%) and HT (82%) (Table 2). However, trivalent (rBmHAT) vaccination when given simply as a protein vaccination alone gave 95%

protection (Fig. 3), which was highly significant ($P<0.001$) compared to the control groups (6%). Comparison between vaccinated groups and control animals were statistically analyzed by one way ANOVA along with Dunnett's post test.

3.7. Immune correlates of vaccine-induced protection in mice

Spleen cells from vaccinated animals proliferated significantly when incubated with each of the three component antigens (rBmHSP 12.6, rBmALT-2 or rBmTSP) in the recombinant fusion vaccine (rHAT) (Fig. 4A). Since there was significant antigen-specific proliferation, we analyzed culture supernatants to determine the cytokine profile of the antigen-specific cells (Fig. 4B). Significantly higher levels of IFN-γ, IL-4, IL-10 and IL-2 ($P<0.001$) were present in the culture supernatant of cells from all vaccinated animals (Fig. 4B) compared to controls. These findings suggest that antigen-specific cells were generated in vaccinated animals. Levels of IFN-γ was 100 fold higher compared to controls, whereas, levels of IL-4, IL-2 and IL-10 were only ~25 fold high. Multiple comparisons of secreted cytokines in the vaccinated groups with the control cells were performed by two-way ANOVA along with Bonferroni post test.

4. Discussion

In this study we attempted to combine TSP-LEL with two of the well established antigens of *B. malayi*, (ALT-2 and HSP12.6) to produce a trivalent vaccine. Vaccination trials in a mouse model show that combining the three antigens as a fusion protein or as a hybrid DNA can synergistically increase the level of protection conferred against a challenge infection.

Natural immunity is reported in human against lymphatic filariasis [2-5,10,24,26]. These putatively immune individuals (en) carry high titer of antibodies against all three antigens (BmALT-2, BmHSP12.6 and BmTSP-LEL). Previous studies show that the circulating antibodies in en subjects have the ability to kill *B. malayi* L3 through an ADCC mechanism [23-25]. When we depleted the antigen specific antibodies, the en sera failed to participate in the killing of L3 suggesting that the three antigens can be potentially developed as vaccine candidates against human lymphatic filariasis. This formed the basis for developing the multivalent vaccine in this study.

The vaccine antigens were identified in our laboratory by screening a phage display cDNA expression library of *B. malayi* with antibodies from en sera [5]. ALT-2 is highly conserved among filarial parasites and is shown to be important in the establishment of infection [10]. Vaccination trials in mice, jirds and mastomys models showed that rBmALT-2 could confer protection ranging from 73% to 76% [5,8-10,27]. A recent study showed that lipidation of BmALT-2 significantly increased its vaccine potential and is effective without an adjuvant [28]. Similarly, HSP12.6 is also an important vaccine candidate that can act as a carrier and as a natural adjuvant for vaccines [29-31]. Combining HSP12.6 and ALT-2 as a multivalent formulation synergistically improved the vaccine-induced protection in mice and jirds [17]. A similar finding was observed in the present study as well. Combining HSP12.6 with ALT-2 as a bivalent vaccine gave >90% protection in mice.

We previously reported cloning of BmTSP-LEL [7]. TSP-2 is a potential vaccine candidate for schistosomiasis [32] and BmTSP-LEL is homologous to the schistosome TSP-2 [7]. We found that en subjects carry antibodies against TSP-LEL. Therefore, in this study we evaluated if adding TSP-LEL to the bivalent HA vaccine can increase the level of protection. Our results show that the trivalent vaccine is much superior to the bivalent vaccines. However, we found that there was no added advantage to priming with *pVAX-hat* DNA vaccine. rHAT protein by itself was highly effective as a prophylactic vaccine in the mouse model. Similar results were reported following vaccination with BmALT-2 [9] and with Sseat-6 [33] against *S. stercoralis*, where protein vaccination gave slightly better protection than DNA prime/protein boost or DNA vaccine regimens. We did not measure the levels of anti-HAT IgE antibodies in the sera of en human subjects. Since IgE antibodies could lead to deleterious allergic effects including anaphylactic shock it is important to determine if the vaccine antigens elicit IgE antibodies in the human before advancing the vaccine further. Evaluation of the immune correlates of protection showed that both Th1 and Th2 type immune responses were elicited in HAT vaccinated animals. A role for Th1 and Th2 responses in the protection against *B. malayi* has been reported by other groups as well [28,34].

In conclusion, we report here preparation of a trivalent hybrid vaccine against lymphatic filariasis. When tested in a mouse model, the trivalent vaccine gave significant protection against challenge infections. Vaccination with fusion protein (rBmHAT) alone was highly effective in generating protective antibodies compared to DNA vaccination or a heterologous prime boost vaccination. Putative immune individuals carry antibodies to all three antigens in HAT and these

antibodies were shown to participate in the killing of *B. malayi* L3 in an ADCC assay. These findings combined with our vaccine studies in the mouse model suggest that rBmHAT is a promising prophylactic vaccine against lymphatic filariasis in human. Additional trials are needed to understand the mechanisms of this vaccine-induced immunity.

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Figure captions.

Figure1. Total levels of IgG antibodies against **A**) rBmHSP12.6 **B)** rBmALT-2 or **C)** rBmTSP-LEL in the sera of endemic normal individuals (en) (n=50) and non-endemic normal individuals (nen) (n=10). Each point represents an individual human sample. The horizontal lines in each group represent the geometric mean for the group. en subjects showing significant (***P<0.001) levels of IgG antibodies compared to nen individuals (Student's t-test).

Figure2. Total IgG antibody titer in mice: A) Mice immunized with the monovalent vaccines HSP12.6, ALT-2 or TSP-LEL by DNA prime and protein boost vaccination approach **B)** Mice immunized with the bivalent vaccine constructs HSP12.6+ALT-2 (HA), TSP-LEL+ALT-2 (TA), HSP12.6+TSP-LEL (HT) by DNA prime and protein boost vaccination approach **C)** Mice immunized with trivalent vaccine constructs HSP12.6+ALT-2+TSP-LEL as DNA vaccine (*hat*), protein vaccine (rBmHAT) or DNA prime/protein boost vaccine (HAT). Antibody titers were assessed as the highest serum dilution giving an absorbance (0.15) that is higher than the control sera. Mice immunized with multivalent rBmHAT protein or *hat* DNA prime/ protein boost vaccine showed significantly high titer of IgG antibodies compared to *hat* DNA alone vaccinated animals. Each bar represents the Mean ± S.D. Significant **(P<0.05) titer of IgG antibodies against respective specific antigen compared to the other antigens within the individual vaccine constructs (One way ANOVA along with Tukey-Kramer post statistics test was used). Data is from one of three similar experiments showing comparable results.

Figure3. *In situ* micropore chamber analysis: Mice were immunized at two weeks intervals with 100 µg of endotoxin free codon optimized *hat* DNA vaccine suspended in PBS and/or 15µg of rBmHAT proteins in alum. Mice were immunized with *hat* DNA and rBmHAT proteins at two weeks interval for DNA prime/protein boost vaccination. One month after the last immunization, 20 *B. malayi* L3 placed in a micropore chamber were surgically implanted into the peritoneal cavity of each mouse. 48 hrs later, chambers were removed aseptically and larval death was determined. Each bar represents the Mean ± SD, N=5. Data is from one of three similar experiments showing comparable results. Significant larval death *** ($P<0.001$) occurred in vaccinated animals compared to control group of mice. Data analyzed by one way ANOVA along with Dunnett's post test.

Figure4. Immune correlates of vaccine-induced protection in the mice immunized with *hat* DNA vaccine, rBmHAT protein vaccine, Heterologus *hat* DNA prime/ rBmHAT protein boost vaccine or pVAX/alum control mice. **A. Splenocytes proliferation assay.** Splenocytes were stimulated with respective proteins or Con A. The data presented is mean stimulation index (S.I.) ± SD. N=10 mice. Significantly high S.I. ($P<0.001$) was present for cells from vaccinated animals compared to cells from pVAX/alum control mice. Data analyzed by two way ANOVA with Bonferonni test. **B.** Cytokines secreted in the culture supernatants of rBmHAT stimulated spleen cells were analyzed by ELISA. IFN-γ levels were high in the culture supernatants of all immunized groups. Levels of IL-2, IL-4 and IL-10 secretions were also high in the immunized groups. The data presented is normalized with values from unstimulated control cultures. Cytokine levels in vaccinated animals were significantly high ($P<0.001$) compared to the control

groups analyzed by two way ANOVA with Bonferonni test. Data is from one of three similar experiments showing comparable results.

Tables

Table 1

***In vitro* Antibody dependent cellular cytotoxicty assay (ADCC), to evaluate the antibodies mediated *B. malayi* L3 killing in the putative immune individuals.**

Sera group	**% Larval Death (Mean±S.D)**
Neat sera (endemic normals)	65.67± 5.87
Antibodies depleted from endemic normal sera	
Total IgG	5.95±2.98 ***
Anti-BmHSP12.6	41.45± 4.23 ***
Anti-BmTSP	21.59±4.82 ***
Anti-BmALT-2	29.86± 10.80 ***
Anti-BmHSP12.6, anti-BmALT -2	5.55±7.85 ***
Anti-BmHSP12.6, anti-BmTSP	10±3.87 ***
Anti-BmTSP, anti-BmALT-2	26±2.52 ***
Anti-BmHSP12.6, anti-BmALT-2, anti-BmTSP	5.55± 3.98 ***

50 µl of pooled human sera (n=10) samples were incubated with 2 x 10^5 normal PBMC and 10 *B. malayi* L3 at 37°C for 48 hrs. Following incubation percent larval death was calculated. Values represent Mean± SD of three wells. Significant reduction in larval death ***($P<0.001$) compared to neat en sera analyzed by one way anova along with Dunnett's post test. Data is from one of three similar experiments showing comparable results.

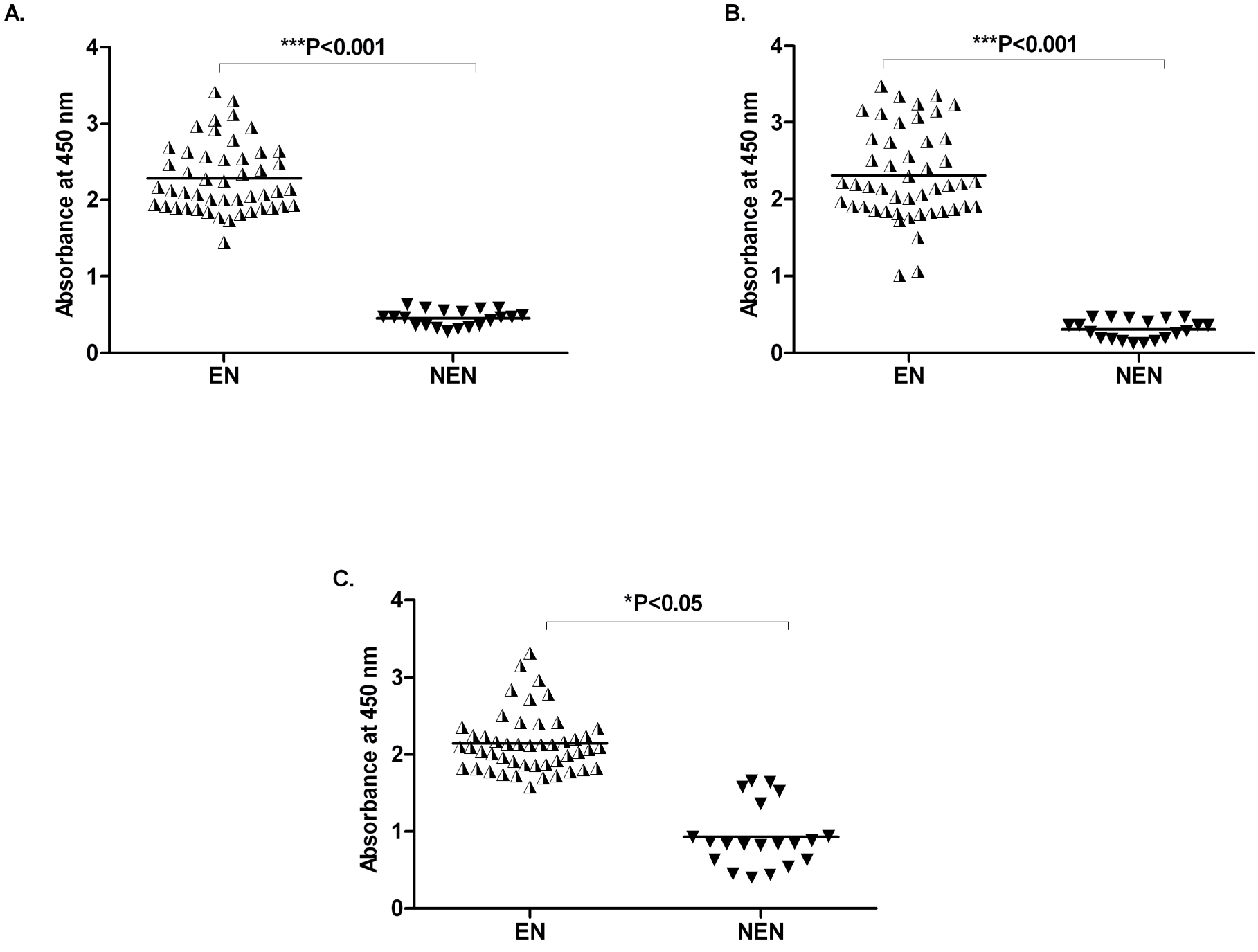
Table 2.

Percentage of *B. malayi* L3 death was determined in the mice immunized with the vaccine constructs using heterologus DNA prime/ protein vaccination regimen.

Groups	**% Larval death Mean ± SD**	
	***in vitro* ADCC** [a]	***in situ* micropore chamber** [b]
pVAX and alum controls	10 ± 4.98	5.9 ± 4.23
HSP12.6	74 ±5.84***	65 ±5.92***
ALT-2	76 ±7.13***	68 ±5.52***
TSP	68 ±4.10***	59 ±2.18***
HA	90 ± 4.4***	90 ± 11.23 ***
HT	82.30 ± 12.9 ***	80 ± 12.03 ***
TA	87.06 ± 9.8 ***	82 ± 11.21 ***
HAT	88.69±7.5 ***	94± 11.92 ***
anti-HAT depleted sera[c]	6.21±1.47***	**NA**

[a]***In vitro* ADCC assay** : 50 µl of pooled mice sera (n=10) samples were incubated with 2 x 10^5 normal peritoneal exudates cells and 10 *B. malayi* L3 at 37°C for 48 hrs. Following incubation, larval viability was determined and percent larval death calculated. Values represent Mean± SD of three wells. [b]**Micropore chamber method:** 20 *B. malayi* L3 in a micropore chamber were surgically implanted into the peritoneal cavity of each mouse. After 48 hrs, the chambers were removed aseptically and the larval viability was determined. Values represent Mean ± SD of

three experiments. N=10. Data is from one of three similar experiments showing comparable results. [c]Sera collected from the mice immunized with HAT depleted of antigen-specific antibodies against rBmHSP12.6, rBmALT-2 and rBmTSP. One way anova used for the multiple comparison of all the groups ($P<0.001$). Significant larval death *** ($P<0.001$) compared to control mice groups analyzed by one way anova followed by Dunnett's post anova test.


A.
***P<0.001
Absorbance at 450 nm
4
3
2
1
0
EN
NEN
B.
***P<0.001
Absorbance at 450 nm
4
3
2
1
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EN
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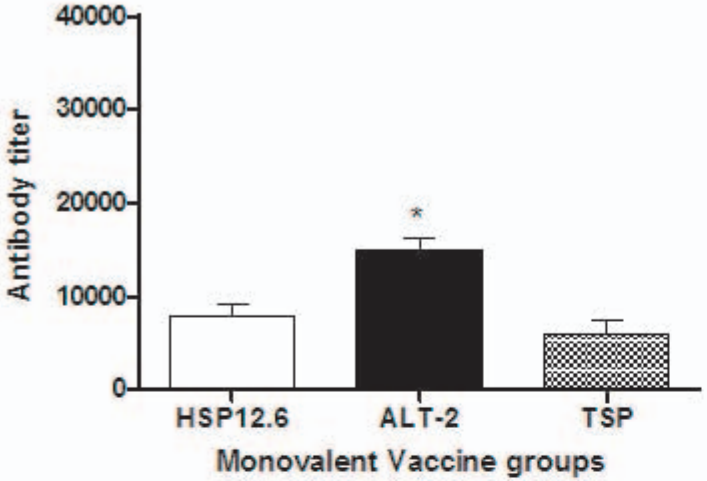
EN
NEN

A.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Antibody titer
*
HSP12.6
ALT-2
TSP
Monovalent Vaccine groups

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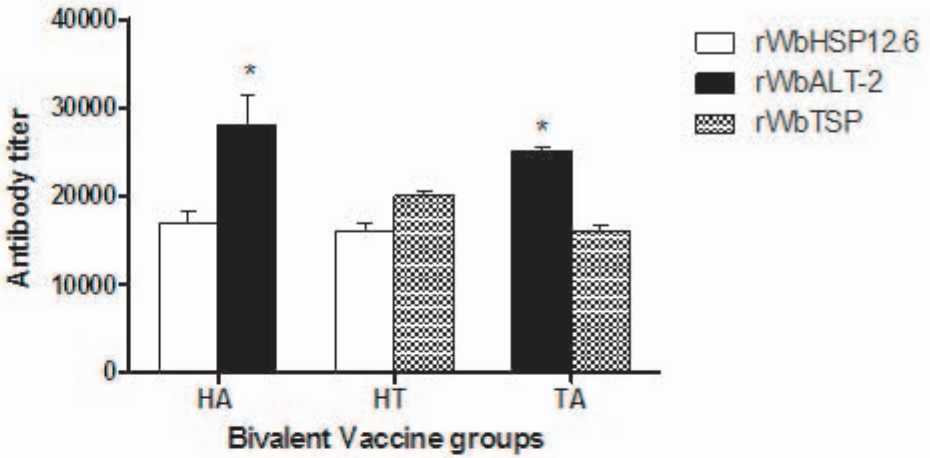
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30000
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*
HA
HT
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Bivalent Vaccine groups
rWbHSP12.6
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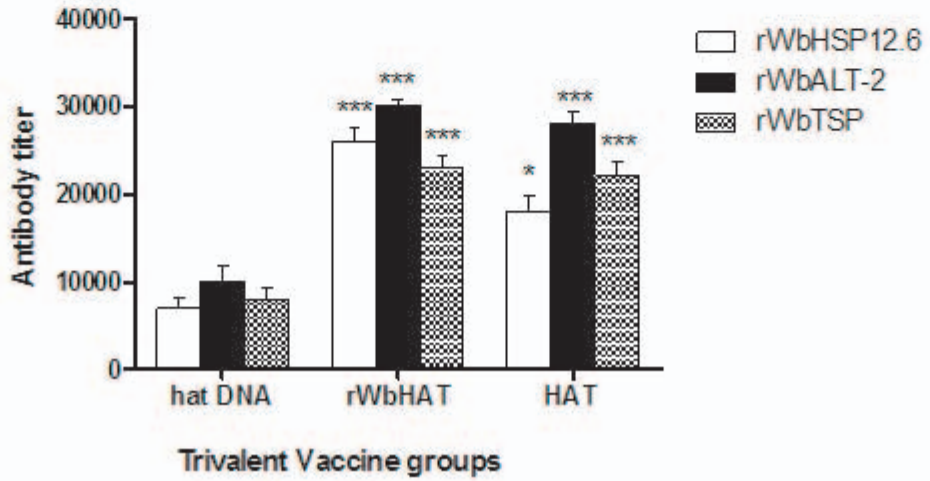
rWbTSP

C.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Antibody titer
***
***
***
*
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hat DNA
rWbHAT
HAT
Trivalent Vaccine groups
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rWbTSP

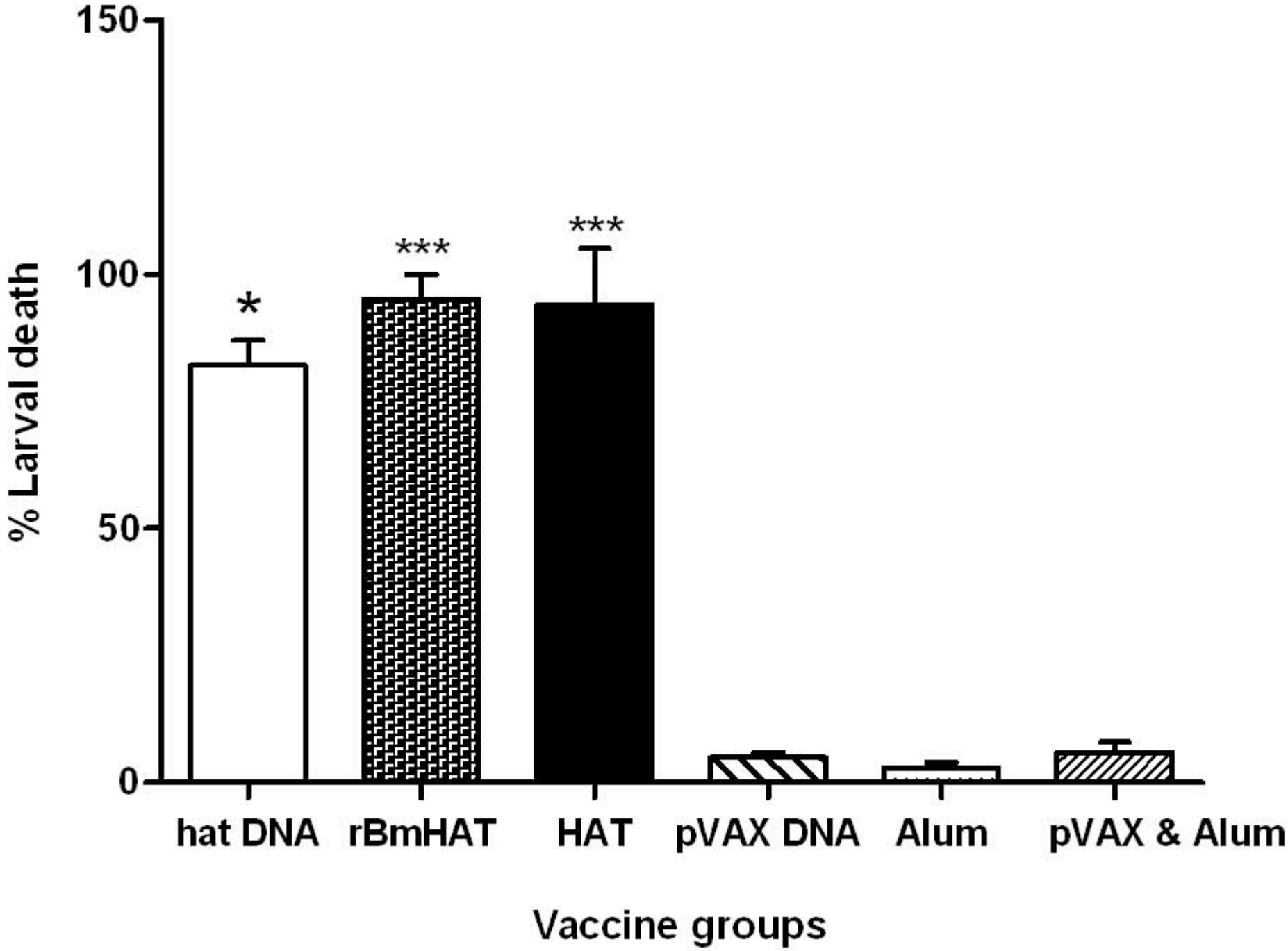
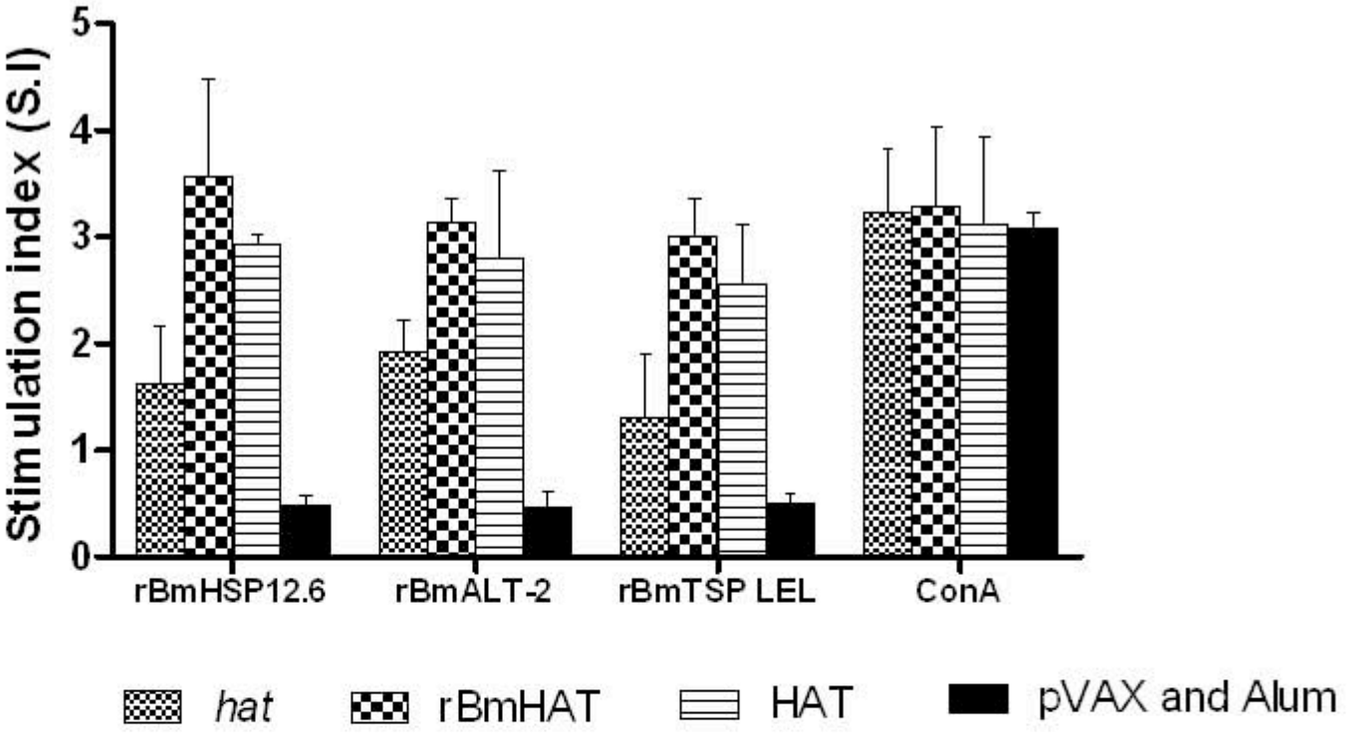

150
100
50
0
% Larval death
*
***
***
hat DNA
rBmHAT
HAT
pVAX DNA
Alum
pVAX & Alum
Vaccine groups

Figure 4.

A.


Stimulation index (S.I)
0
1
2
3
4
5
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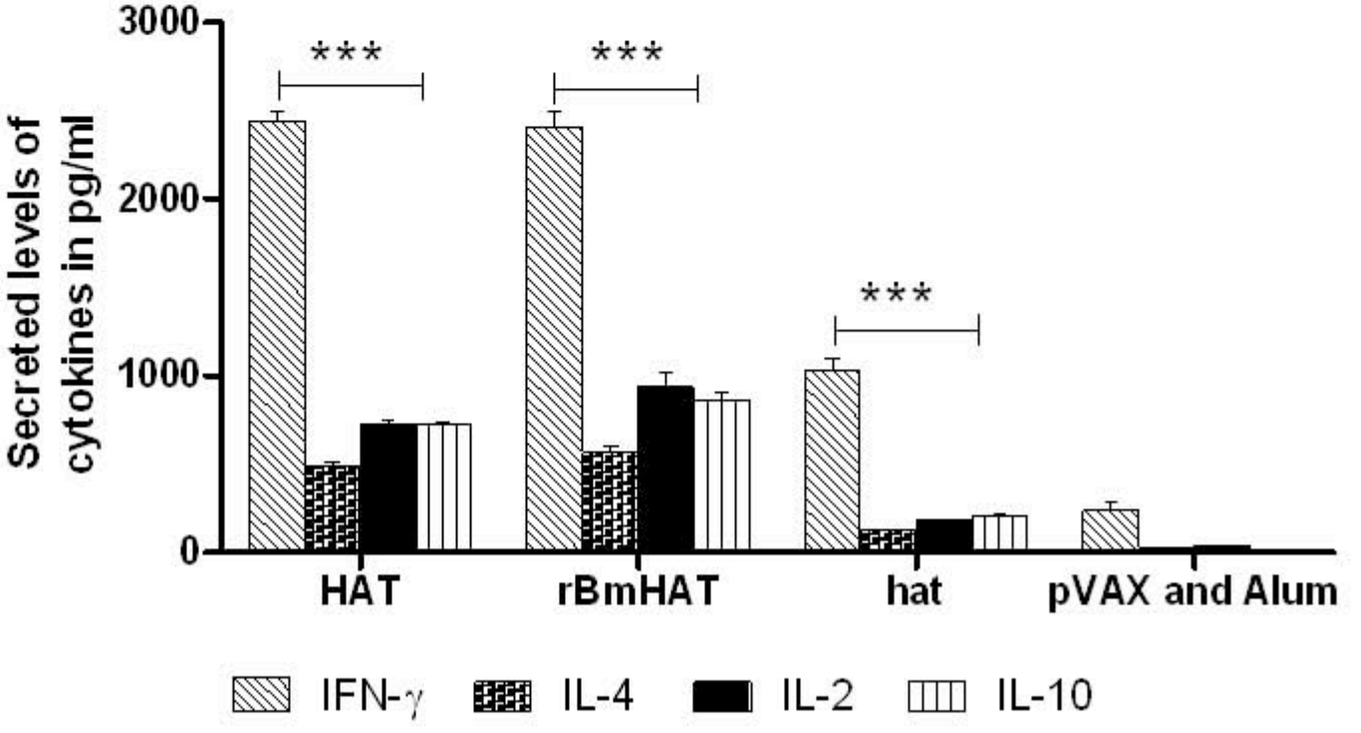
rBmALT-2
rBmTSP LEL
ConA
hat
rBmHAT
HAT
pVAX and Alum

B.


Secreted levels of cytokines in pg/ml
0
1000
2000
3000
***
***
***
HAT
rBmHAT
hat
pVAX and Alum
IFN-γ
IL-4
IL-2
IL-10

Supplementary data

Figure 1A. Schematic representation of DNA vaccination or protein vaccination regimen in mice. Control animals in the DNA vaccination regimen received pVAX vector alone and controls in protein vaccination regimen received alum alone plus PBS.

[a] Immunized Intradermally; [b] Immunized subcutaneously

Figure 1B. Schematic representation of prime-boost vaccination regimen in mice. Control animals received either two doses of pVAX vector alone on day 0 and day 15, and/or alum plus PBS on day 30 and day 45.

[a] Immunized Intradermally; [b] Immunized subcutaneously

Figure 2. Total IgG levels against rBmHSP12.6, rBmALT-2 or rBmTSP LEL in **A)** endemic normal individuals (en) and /or anti-BmHAT antibodies depleted en sera. (n=10), **B)** Mice immunized with rBmHAT vaccine and /or anti-BmHAT antibodies depleted mice sera. Each bar represents the Mean ± S.D. Significant **($P<0.05$) antibody titer compare to depleted sera (Students t-test).

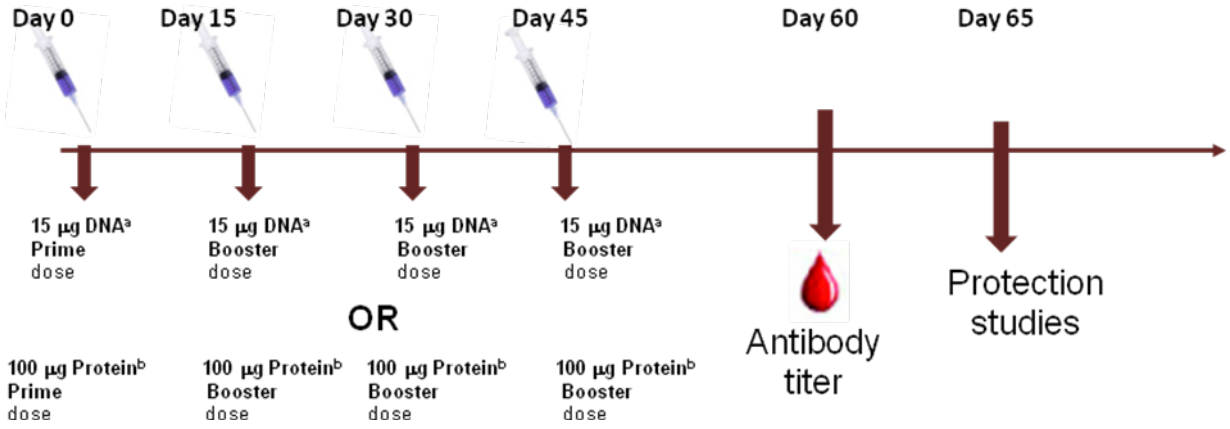
Figure 3. PCR amplified product of *Bmhat* gene is 850 bp in size. 1. 100 bp DNA ladder, 2. PCR product of *Bmhat* (850 bp) gene. Codon optimized genes of *hsp, alt* and *tsp* were cloned into pVAX 1 vector. The cloned product of *hat* was confirmed by PCR amplification using *hsp* specific forward primer and *tsp* specific reverse primer.

Figuer 4. Immunoblot analysis of rBmHAT: A. CBB stained pure fraction rBmHAT eluted from IMAC , **B**. Immunoblot of rBmHAT with 1) anti-HSP12.6, 2) anti-ALT-2, 3) anti-TSP LEL and/or 4) anti- HAT antibodies from mice. Antibodies against all the four antigens recognized the rBmHAT fusion protein.

Supplementary data

Figure 1.

A



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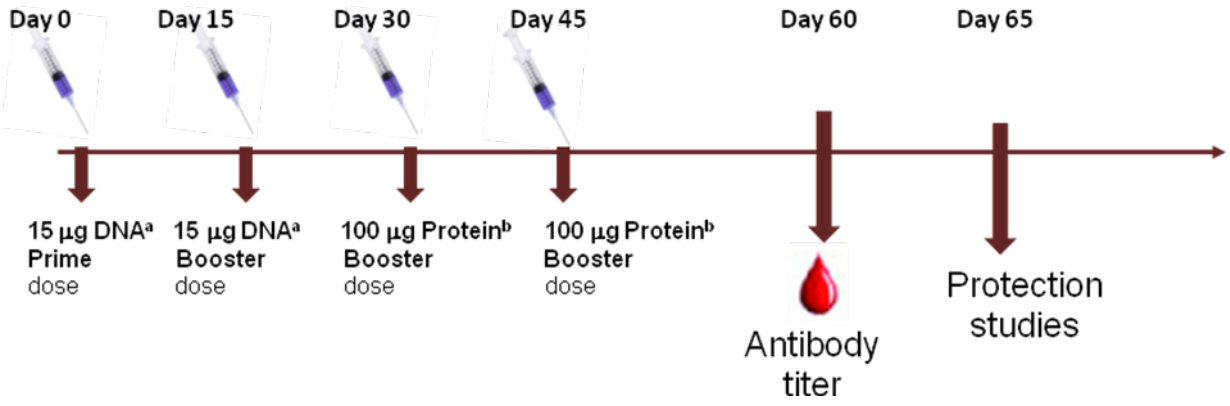

Figure 2.

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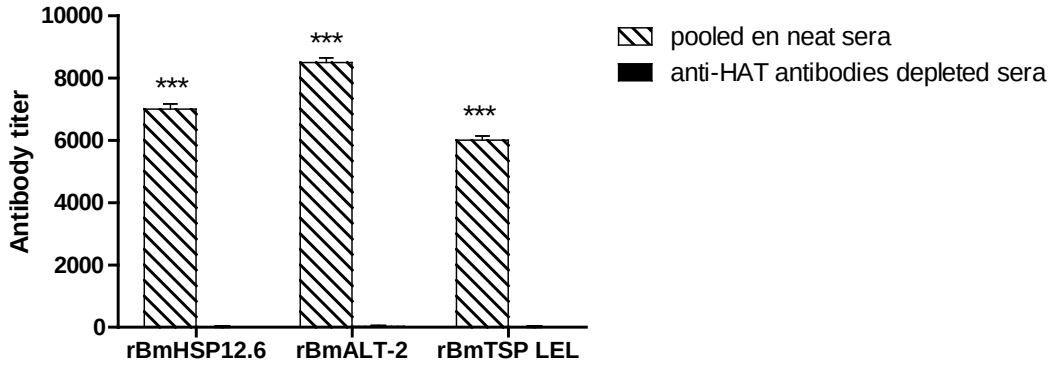
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8000
6000
4000
2000
0
Antibody titer
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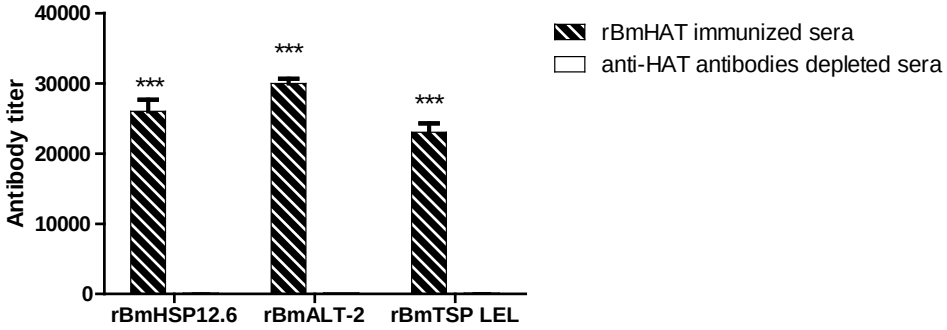
rBmHSP12.6
rBmALT-2
rBmTSP LEL
pooled en neat sera
anti-HAT antibodies depleted sera

B.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Antibody titer
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rBmALT-2
rBmTSP LEL
rBmHAT immunized sera
anti-HAT antibodies depleted sera

Figure 3.

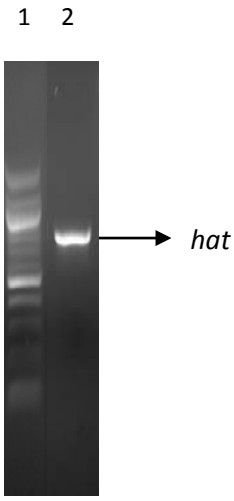

Figure 4.

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