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Comparative Evaluation of Routes of Administration and Efficacy of Monovalent and Bivalent Filarial Vaccine Candidates; BmALT-2 and WbGST

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MN, SK and KG conceived and designed the study. Author MN did experimental studies, collected data and maintained quality. Authors MN, RSY, SK and KG interpreted the data, did the analysis and drafted the article. Authors RSY, UP, SK and KG critically revised the article. Authors MN and UP re-drafted and gave final shape. All authors approved the final version to be published. Author SK supervised the project.

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ABSTRACT

Aims: To evaluate immunoprophylactic adequacy of monovalent and bivalent filarial vaccine candidates; BmALT-2 and WbGST through different routes of immunization *viz*., intramuscular (i.m.), intraperitoneal (i.p.) or subcutaneous (s.c.) way.
Study Design: *In vivo* and *in vitro* experimental studies using rodent model of filariasis.
Place and Duration of Study: Department of Biochemistry and J B Tropical Disease Research Centre, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Maharashtra, India, between June 2016 and July 2017.
Methodology: Male Mastomys (*n*=5-7/group) of 6-8 weeks age were immunized with 25 µg/dose of rBmALT-2, rWbGST, or rBmALT-2+rWbGST per animal along with alum as adjuvant through either

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of i.m., i.p. or s.c. routes. The control groups of mice received alum only. The protective immunity elicited in the animals was checked by antibody-dependent cellular cytotoxicity and micropore cytotoxicity assay. The splenocytes proliferation and cytokine profile were performed for the appraisal of cellular immune response.

Results: Our results demonstrated that immunization through i.m. route is more effective as compared to i.p. or s.c. routes and comparatively monovalent rBmALT-2 induces better protection as compared to monovalent rWbGST or bivalent dose consisting of rBmALT-2+rWbGST. Mastomys immunized through i.m. route with rBmALT-2 alone could induce 77.50% *in vitro* and 73.28% *in vivo* cytotoxicity ($P<0.05$) against *Brugia malayi* infective larvae (L3), whereas, rWbGST alone and rBmALT-2+rWbGST combined could induce 65% and 68% *in vitro* and 61% and 65% *in vivo* cytotoxicity ($P<0.05$), respectively. Our serological analysis showed that Mastomys immunized i.m. with rBmALT-2 or rWbGST or rBmALT-2+rWbGST induced IgG1, IgG2a and IgG3 antibody response, significantly higher than control groups. The protective effect was found to be associated with a predominantly increased level of IFN-γ and IL-4 in response to rBmALT-2.

Conclusion: Intramuscular route is the most effective way of administration of vaccine for better prophylactic response in filariasis. Additionally, co-administration of monovalent vaccine seems to interfere with the prophylactic effect of monovalent rBmALT-2 and rWbGST vaccines.

Keywords: Filariasis; antibody dependent cellular cytotoxicity (ADCC); vaccination; BmALT-2; WbGST.

1. INTRODUCTION

Lymphatic Filariasis (LF) is an incapacitating disease caused by *Wuchereria bancrofti, Brugia malayi,* and *B. timori.* LF is known as the second foremost reason for perpetual disability in 49 countries universally, affecting 893 million people [1]. In 2000, the WHO launched a Global Program for the Elimination of Lymphatic Filariasis to achieve the elimination of LF by 2020 through the use of annual mass drug administration (MDA) as preventive chemotherapy [2,3]. This approach extensively miniaturized the incidence of filariasis in several countries. Since continuous MDA renders parasites more susceptible to develop drug resistance, other types of intercessions are essential to be considered. Such research advocates for a greater supportable technique which includes a prophylactic vaccine alongside MDA to halt the transmission and control LF infection in prevalent regions [4-8].

In previous studies, abundant larval transcript-2 (ALT-2) and glutathione-S-transferase (GST) of *B. malayi* and *W. bancrofti* respectively, were analyzed to be conceivable vaccine candidates [9-12]. The 24 kDa ALT-2 protein among other recombinant filarial vaccine candidates' studies provided a considerably high defensive immune response to *B. malayi* infection [13,14]. In parasites, ALT-2 proteins are instigated to be expressed at the late L2 stage and peaks at the L3 stage [5]. Several studies have reported the presence of significant level of anti-ALT-2 antibodies in sera from endemic normal individuals [8,10,14]. BmALT-2 protein was thought to play an imperative role in the transmission and infectivity of the filarial parasite, as it was abundantly synthesized during the infectious phases of the parasite [13]. On the other side, at larval stage 3 (L3), *W. bancrofti* expresses GST as major enzymes, which are involved in neutralizing the host free radicals [12]. This study has also found increased level of anti-GST antibodies in sera from endemic normal individuals and as potential vaccine candidate. Studies on helminthic infections revealed that GST was a capable vaccine candidate [15-19]. However, these studies have not examined the effect of various routes of administration. Besides, studies have not been conducted to examine the synergistic effect of these two promising vaccine candidates together as a bivalent vaccine in the prophylaxis of lymphatic filariasis. In this study, we evaluated the immunogenicity and protective effectiveness of BmALT-2 and WbGST and compared the efficacy of the vaccines when given as a monovalent or as a bivalent by administering through different immunization routes in animal model Mastomys (*Mastomys coucha)*.

2. MATERIALS AND METHODS

2.1 Experimental Animals for Immunization and *In vivo* Study

Male *Mastomys coucha* (6-8 weeks) were bred and kept in the Central animal house facility of

Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). This study was allowed by the Institutional Animal Ethics Committee, MGIMS, Sevagram (MGIMS/IAEC/July/8/2014), which follows the USA NIH animal study guidelines [20,21].

2.2 *B. malayi* Parasites

L3 larvae (infective stage) of *B. malayi* were gathered using a Baermann technique as described previously [22,23].

2.3 Expression and Purification of Filarial Proteins (rBmALT-2 and rWbGST)

B. malayi rALT-2 and *W. bancrofti* rGST was expressed and purified as described previously [12]. Briefly, pRSET-A-Bm*alt*-2 and pRSET-A-Wb*gst* were constructed and transformed into *Escherichia coli* BL21(DE3) pLysS expression host and expression were induced by adding of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Merck Millipore, Bangalore) [12]. The immobilized affinity chromatography column bound to nickel (Thermo Fisher Scientific, Mumbai) was used for the purification of respective filarial proteins (BmALT-2 and WbGST). The concentration of purified filarial proteins was estimated by using the Bicinchoninic Acid protein assay kit (BCA) (Thermo Fisher Scientific, Mumbai) and the endotoxin (<0.2 EU/mL) content was calculated with Limulus Amebocyte Lysate test (LAL) kit (Fisher Scientific, Mumbai).

2.4 Immunization of *Mastomys coucha*

Male Mastomys of 6-8 weeks (*n*=5-7 each group) were divided into ten groups and immunized with (25 µg/dose) single protein or in combination as follows: Group I: immunized intramuscularly (i.m.) with rBmALT-2; Group II: immunized i.m. with rWbGST; Group III: immunized i.m. with rBmALT-2+rWbGST; Group IV: immunized intraperitoneally (i.p.) with rBmALT-2; Group V: immunized i.p. with rWbGST; Group VI: immunized i.p. with rBmALT-2+rWbGST; Group VII: immunized subcutaneously (s.c.) with rBmALT-2; Group VIII: immunized s.c. with rWbGST; Group IX: immunized s.c. with rBmALT-2+rWbGST; in 100 µL of 0.5 M phosphate buffered saline (PBS, pH 7.2) along with the equal volume of alum (Sigma-Aldrich, Mumbai) adjuvant. Respective control groups were administered with alum only. Each group was administered with three doses of respective proteins at every four weeks of hiatus followed by one booster dose. Sera samples were collected from each Mastomys by retro-orbital bleeding ten days after the booster dose and tested for the presence of anti-BmALT-2 and anti-WbGST Immunoglobulin (Ig)-G antibody level.

2.5 Titer of Antigen-specific IgG Antibodies and Levels of IgG Isotypes in the Sera of Immunized Mastomys

The levels of total anti-BmALT-2 and anti-WbGST IgG antibodies in the sera samples of Mastomys were analyzed by indirect ELISA. In Brief, 96 wells ELISA plates (Thermo Fisher Scientific, Mumbai) were coated with 25ng/100µL/well of respective filarial proteins (rBmALT-2 or rWbGST) in the carbonate-bicarbonate buffer (100 mM, pH 9.5) and incubated at 4ºC for overnight. Plates were washed with PBST (0.05 M PBS having 0.05% of Tween 20, pH 7.2) once and blocked with 300 µL/well of blocking buffer (2% BSA in 0.05 M PBS) and incubated at 37ºC for 1 h. After blocking nonspecific binding sites, plates were incubated at 37ºC for 1 h with optimally diluted serum samples of immunized Mastomys (1:100; 1:500, 1:1 000 and 1:10 000 diluted in PBS; 100 µL/well). Followed by another washing, plates were incubated with 100 µL/well of secondary antibody (goat anti-mouse IgG antibody) conjugated with Horseradish Peroxidase [HRP] (1:10,000 dilution; diluted in PBS) for 45 min at 37ºC. By incorporating TMB, color has been developed and the reaction was stopped using 1.8 N H_2SO_4 and the absorbance was taken at 450 nm. Levels of antigen-specific IgG1, IgG2a, IgG2b, IgG3, antibodies against rBmALT-2, and rWbGST were determined in the sera of immunized Mastomys using indirect ELISA as described above using isotype-specific HRP labelled antibodies IgG1 (1:1 000), IgG3 (1:5 000), IgG2a, and IgG4 (1:15 000).

2.6 *In vitro* Cytotoxicity Assay with L3 Larvae of *B. malayi*

As illustrated previously [12,24,25], the *in vitro* ADCC assay of anti-BmALT-2 and anti-WbGST antibodies was adopted for evaluating the cytotoxicity effect against L3 larvae of *B. malayi*. The collective sera samples of Mastomys immunized by different routes *viz.*, i.m., i.p. and s.c. were used in this assay. Briefly, in 96 well

tissue cultures plates (Thermo Fisher Scientific, Mumbai), roughly 10-12 infective L3 were added to a suspension of 2×10^5 cells/well peritoneal exudates cells and 100 µL of RPMI medium were acquired from standard healthy Mastomys. About 50 µL pooled sera samples from immunized Mastomys were added in respective wells and the volume of each well was made up to 200 µL by the addition of the RPMI medium. The larval viability was examined under a light microscope after 48 h incubation (37ºC in 5% CO_2). The larvae (L3) which were straight and limpid with stagnation were considered as dead. The cytotoxicity percentage was interpreted as a proportion of inactive or dead larvae to the total number of larvae collected during the *in vitro* assay.

2.7 Micropore Chamber Assay

The prophylaxis potential of monovalent and bivalent vaccine formulations of rBmALT-2, rWbGST, or rBmALT-2+rWbGST, administered through different routes *viz*., i.m., i.p. and s.c. in Mastomys was determined by micropore chamber assay as described previously [4,25]. Briefly, adopting the micropore chamber (Millipore India, Bangalore), nearly 20 live infective *B. malayi* L3 in RPMI 1640 medium were surgically instilled into the peritoneal cavity of each experimental Mastomys. Mastomys was euthanized after 48 h of implantation and the chamber was recovered from the peritoneum and washed with normal saline. The contents of the glass slide were taken and the cell adhesion and cytotoxicity were microscopically tested. The percentage of cytotoxicity was calculated as a proportion of the total number of dead larvae out of the total number of larvae collected during the *in vivo* experiment.

2.8 Splenocytes Proliferation and Cytokine Profile

The spleens were aseptically isolated from the Mastomys and crushed in RPMI 1640 medium (containing 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated fetal calf serum), pelleted, and resuspended in 0.1% ammonium hydrochloride (erythrocyte lysis buffer). Splenocytes were plated in triplicates (0.2×10^6 cells/well; 100 µL of RPMI media) in 96 well tissue culture plate (Thermo Fisher Scientific, Mumbai) and stimulated either with respective recombinant proteins (rBmALT-2 or rWbGST) (1 µg/well/100 µL), or Concanavalin A (1 µg/well/100 µL), (Con A; Sigma-Aldrich, Mumbai). After 48 h of incubation (37ºC in 5% CO_2), cell proliferation was calculated using an MTS assay kit (Promega, New Delhi). The stimulation index (SI) was determined by dividing the average absorbance of the cells stimulated by either antigens or mitogen by the average absorbance of the un-stimulated cells. For cytokine estimation, similar sets of cell (2×10^6 cells/mL/well) cultures were plated in 24 well tissue culture plates (Thermo Fisher Scientific, Mumbai) and cells were stimulated separately with 10 µg/well respective antigens (rBmALT-2 or rWbGST) or 2 µg/well Con A as a positive control or RPMI medium only serve as negative control and incubated for 72 h at 37ºC in 5% CO_2. After 72 h incubation period, spleen cells were pelleted and culture supernatants were collected for the cytokines estimation. The level of interleukin (IL)-10, IL-4, and interferon (IFN)-γ cytokines measured using ELISA kits (Invitrogen, Mumbai) as instructed by the manufacturer.

2.9 Statistical Analysis

By virtue of SPSS 21.0 (IBM, India) software, the statistical analysis was performed. One way ANOVA followed by Tukey *post-hoc* test for comparisons between multiple groups was used. $P<0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Antibody Response in the Sera of Vaccinated Mastomys

Sera of the test and control Mastomys were collected ten days after the last booster dose for the determination of IgG antibody response. Mastomys immunized *via* i.m., i.p. and s.c. routes with rBmALT-2 or rWbGST or in combination of both (rBmALT-2+rWbGST) showed significantly ($P<0.05$) higher IgG antibody response compared to control group (Fig. 1). The sera of Mastomys immunized by i.p. route with rBmALT-2 elicited the highest ($P<0.05$) IgG response compared to Mastomys immunized i.m. or s.c. group (Fig. 1).

Antigen-specific antibody isotypes analysis showed increased IgG1 and IgG3 responses irrespective of the immunization routes and were significantly higher ($P<0.05$) as compared to control-alum group (Table 1). Intraperitoneally immunized rWbGST group showed a significantly decreased level of IgG2a ($P<0.05$) compared to other vaccinated groups.

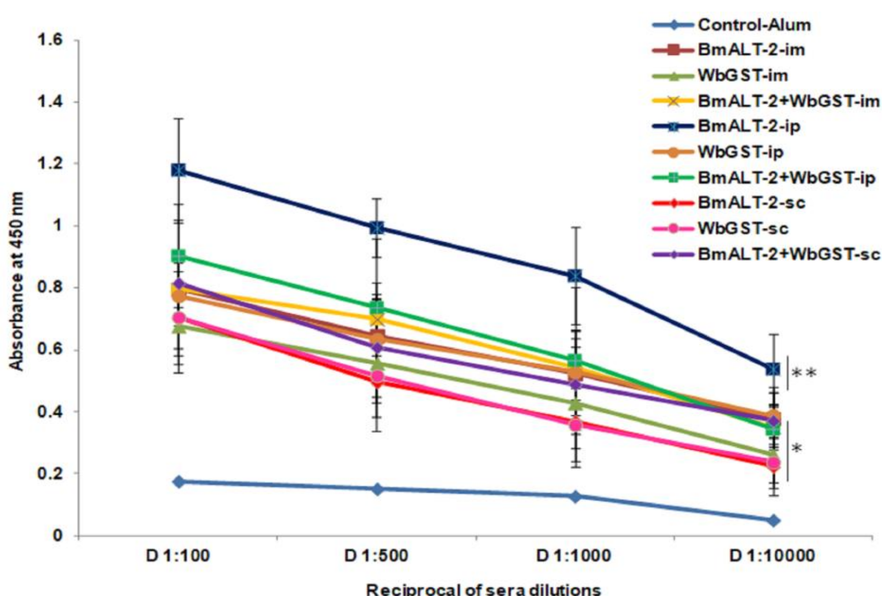

Fig. 1. Titres of anti-BmALT-2 IgG and anti-WbGST IgG antibodies in the sera of immunized Mastomys, ten days after the last booster dose; antibody titer was determined by ELISA

*Mastomys immunized i.m., i.p., and s.c. with recombinant proteins BmALT-2 or WbGST or BmALT-2+WbGST and Control-alum. Each data point represents Mean±SD (n=5-7). *P<0.05, **P<0.001compared to Control-alum group as analyzed by Kruskal–Wallis test followed by Tukey's post hoc multiple comparisons test*

Table 1. Levels of anti-BmALT-2 and anti-WbGST IgG isotype

Groups	IgG1	IgG2a	IgG2b	IgG3
rBmALT-2-im	0.285±0.04*	0.157±0.02*	0.052±0.00	0.190±0.04*
rWbGST-im	0.363±0.05**	0.197±0.01*	0.047±0.00	0.242±0.06**
rBmALT-2+rWbGST-im	0.254±0.06*	0.174±0.02*	0.049±0.00	0.187±0.03*
rBmALT-2-ip	0.372±0.05**	0.316±0.06**	0.064±0.01	0.296±0.04**
rWbGST-ip	0.266±0.08*	0.086±0.01[ns]	0.089±0.01**	0.127±0.02
rBmALT-2+rWbGST-ip	0.267±0.02*	0.163±0.02*	0.124±0.02**	0.148±0.01*
rBmALT-2-sc	0.268±0.07*	0.155±0.04*	0.050±0.00	0.177±0.05*
rWbGST-sc	0.349±0.09*	0.217±0.01*	0.049±0.00	0.137±0.02
rBmALT-2+rWbGST-sc	0.300±0.06*	0.189±0.01*	0.050±0.00	0.285±0.04**
Alum-control	0.121±0.01	0.095±0.01	0.034±0.01	0.079±0.01

**P<0.05, **P<0.001 compared to Control-alum group as analyzed by Kruskal–Wallis test followed by Tukey's post hoc multiple comparisons test*

3.2 Splenocyte Proliferation and Cytokine Profile Analysis

The cellular immune response was evaluated by analyzing splenocytes proliferation, and cytokine profile in response to protein stimulation. Spleen cells from immunized Mastomys stimulated with either rBmALT-2 or rWbGST were proliferated significantly (Stimulation index, i.e. SI 3.73±0.44 and SI 1.36±0.17, respectively) compared to control alum group (SI 0.89±0.16). The rBmALT-2+rWbGST immunized Mastomys spleen cells respond to both rBmALT-2 (SI 2.19±0.26) and rWbGST (SI 1.94±0.19) signifying that a resilient cellular response to both rBmALT-2 and rWbGST is achieved.

The cytokine response of the antigen-responding cells was determined by measuring level of IL-10, IL-4, and IFN-γ in the supernatant, collected from culture of spleen cells of immunized Mastomys, triggered with respective antigens (rBmALT-2 or rWbGST or combination of both). The splenocytes of Mastomys immunized *via* i.m., i.p. or s.c. routes with rBmALT-2 or rWbGST alone or combination of both the antigens

showed elevated levels of IL-10, IL-4, and IFN-γ cytokines except rWbGST-s.c. group compared to control alum group (Table 2). The Mastomys immunized with rBmALT-2 alone or the combination of both the antigens through i.m. route showed increased levels of IL-4, IFN-γ, and IL-10. The Mastomys immunized i.m. with rWbGST alone or in combination with rBmALT-2 showed significantly increased levels of IL-10 ($P<0.001$) cytokines compared to Mastomys immunized i.p. or s.c. (Table 2). Moreover, Mastomys immunized i.m. with rBmALT-2 or rWbGST alone or combination of two antigens showed significantly elevated levels of IL-4 ($P<0.001$) cytokines compared to i.p. or s.c. immunized groups of Mastomys (Table 2).

3.3 *In vitro* Cytotoxicity Assay

Further, we determined if an ADCC assay can kill *B. malayi* L3 in the vaccine-induced antibodies. Our results showed that Mastomys immunized i.m. with rBmALT-2 demonstrated the highest larval killing effect (77.50%; $P<0.05$) compared to sera of Mastomys immunized with rWbGST and control alum group through either of the route i.e. i.m., i.p. or s.c. (Table 3). Mastomys immunized rWbGST alone via any route showed 62 to 65% cytotoxicity ($P<0.05$) compared to control alum group. Whereas, combination groups showed 64 to 68% cytotoxicity ($P<0.05$) compared to control alum group. When compared among antigens, only rBmALT-2 immunized Mastomys sera showed the highest activity against L3 irrespective of the route of immunization (Table 3).

3.4 Micropore Chamber Assay

We also evaluated the immunoprophylactic efficacy of rBmALT-2 or rWbGST or rBmALT-2+rWbGST vaccine candidates *in vivo* using a micropore chamber approach. The microscopic examination of micropore chambers instilled in the peritoneum of vaccinated Mastomys revealed the migration of host macrophages and polymorphonuclear cells within the chambers projecting to their adherence and killing of larvae (L3). *In vivo* study of Mastomys vaccinated via i.m. route with rBmALT-2 induced significantly high cytotoxicity (73.28%; $P<0.05$) compared to control alum group (11.56%) (Table 3). Whereas, Mastomys immunized via i.p. or s.c. with rBmALT-2 gave around 67% cytotoxicity compared to control alum group. Mastomys immunized with rWbGST antigen alone gave 61% ($P<0.05$) larvicidal activity against *B. malayi* L3 irrespective of the administration route. Whereas, rBmALT-2+rWbGST vaccinated group showed a 62 to 65% ($P<0.05$) cytotoxicity effect compared to control alum group (Table 3).

4. DISCUSSION

As suggested by several reports, despite increasing progress towards lymphatic filariasis elimination all over the world through MDA, still several LF endemic pockets could not be covered by antifilarial chemotherapy, which can be responsible for LF transmission and disease re-emergence. Developing a potential vaccine against LF has not been easy due to the complex life cycle of the parasite and dynamic antioxidant protection against the innate immune activity of the host. As a result, even after the active research since the last two decades, no single vaccine successfully emerged with high antifilarial efficacy. In the present work, we examined the adequacy of monovalent and bivalent filarial vaccine candidates; BmALT-2 and WbGST through different routes of immunization viz., intramuscular (i.m.), intraperitoneal (i.p.) or subcutaneous (s.c.) way.

Table 2. Cytokine responses in culture supernatants of spleen cells of vaccinated mastomys

Groups	IL-10	IL-4	IFN-γ
rBmALT-2-im	128.19±13.52	328.37±5.83**	826.94±173.68
rWbGST-im	216.18±57.75**	320.85±13.24**	644.31±411.51
rBmALT-2+rWbGST-im	184.62±24.90**	356.74±17.36**	1193.07±203.37**
rBmALT-2-ip	120.16±17.57	197.17±3.91	596.95±253.78
rWbGST-ip	121.03±18.03	206.27±23.63	669.76±225.38
rBmALT-2+rWbGST-ip	123.59±24.84	198.45±12.74	533.24±137.06
rBmALT-2-sc	111.61±13.27	262.55±13.84**	954.70±217.60*
rWbGST-sc	72.01±8.64^{ns}	269.79±12.80**	883.66±114.16*
rBmALT-2+rWbGST-sc	109.77±22.46	269.79±12.14**	977.97±389.84*
Control-alum	88.15±12.19	185.64±11.87	134.59±18.60

**P<0.05, **P<0.001compared to Control-alum group as analyzed by Kruskal–Wallis test followed by Tukey's post hoc multiple comparisons test*

Table 3. The number of larvae recovered from different sets of experiments

Vaccination groups	Percent larvae recovered (Mean±SD)	
	In vitro (ADCC assay) (%)[a]	*In vivo* (micropore assay) (%)[b]
rBmALT-2-im	77.50±3.54**	73.28±3.47*
rWbGST-im	65.15±2.14*	61.72±2.94*
rBmALT-2+rWbGST-im	68.30±2.36*	65.96±2.70*
rBmALT-2-ip	69.70±4.29*	67.77±5.34*
rWbGST-ip	62.60±1.48*	61.26±4.16*
rBmALT-2+rWbGST-ip	64.10±3.63*	62.37±3.15*
rBmALT-2-sc	71.40±1.93*	67.15±8.16*
rWbGST-sc	65.20±2.14*	61.36±2.37*
rBmALT-2+rWbGST-sc	68.30±2.36*	64.25±6.01*
Control	11.80±0.98	11.56±5.53

**P<0.05, **P<0.001compared to Control-alum group as analyzed by Kruskal–Wallis test followed by Tukey's post hoc multiple comparisons test*

A large body of evidence unveiled the potential of BmALT-2 and WbGST as vaccine candidates against various parasitic infections [5,14,15, 18,26]. Several studies demonstrated the presence of antibodies against these proteins in endemic normal individuals [8,10,12,14]. BmALT-2 has several important features of a vaccine candidate and it is particularly expressed in the L3 stage of *B. malayi.* Similarly, GST is also a significant vaccine contender since it plays a crucial position in parasite survival by assisting xenobiotics metabolism for detoxification and in combating host-induced assault [27]. Previous vaccination studies using either of these proteins reported significant protection against filariasis in Jirds and Mastomys [10-12,28].

Based on available evidence, *in vitro* ADCC assay and *in vivo* micropore chamber method are contemplated as the key immunological rationales for the clearance of circulating filarial parasites [6,23,24,29-33]. Herein, the present study demonstrated that Mastomys immunized i.m. route with monovalent rBmALT-2, elicit enhanced protective efficiency against infective larvae of *B. malayi* compared to Mastomys immunized i.p. or s.c. It was observed that protective response in Mastomys immunized i.m. with rBmALT-2 alone elicited stronger protection (77.50%) against *B. malayi* infective larvae compared to either with monovalent rWbGST (65.15%) or bivalent (rBmALT-2+rWbGST) vaccine (68.30%), administered i.m. Mastomys immunized by i.m. route using rBmALT-2 alone also showed significantly stronger protection (77.50%) compared to i.p. or s.c. based immunization with rWbGST (63%-65%) alone or in combination (rBmALT-2+rWbGST). However, bivalent vaccines-induced higher protective efficacy compared to monovalent rWbGST administered through i.m., i.p. or s.c. routes. Combining two antigen rBmALT-2 and rWbGST as a bivalent vaccine improved the vaccine-induced protection in Mastomys, which is in accordance with other studies [4,6,7,28-31]. We observed similar results when performed *in vivo* micropore chamber experiments.

It is interesting to note that the protective efficacy of co-administered BmALT-2 and WbGST as a bivalent vaccine was higher (as demonstrated in both ADCC and micropore chamber experiments) compared to WbGST, which administered alone as monovalent vaccine. However, the protective efficacy of this bivalent vaccine was lower compared to the monovalent BmALT-2 vaccine. A clear interference was observed when BmALT-2 and WbGST administered together. At present, we cannot put aside the possibility that a direct interaction between BmALT-2 and WbGST negatively affected the immunogenicity of BmALT-2. The antigenic epitopes on BmALT-2 might have been masked by WbGST, leading to the downregulation of the efficacy of BmALT-2 in the combination vaccine [6,34]. A similar result observed in malaria where two recombinant Merozoite Surface Proteins (MSPs) of *Plasmodium yoelli*, PyMSP-1_{42}, and PyMSP-8, were tested for their protective efficacy, and it was found that the immunogenicity of PyMSP-1_{42} was reduced when mice were immunized with a combination of PyMSP-1_{42} and PyMSP-8 [34]. Similar results were also observed in a filarial vaccine study where *Bm*TGA and *Bm*ALT-2 tested for their protective efficacy in Jirds and it was found that immunizing with the combination of these two antigens conferred only 47% protection against *B. malayi* L3 larvae [6]. Thus the choice of antigens for a cocktail mix is critical,

since not all antigen mixtures are successful [6].

The Mastomys immunized through three different routes with rBmALT-2 or rWbGST or rBmALT-2+rWbGST developed significantly higher titer of anti-BmALT-2 and anti-WbGST antibodies. IgG1 and IgG3 antibodies are associated with Th2-type immune response and Th1 response favors IgG2a production [35]. IgG isotype profile against BmALT-2 or rWbGST or combination in the sera of i.m. immunized Mastomys showed predominance for IgG1, IgG2a, and IgG3 antibodies. Increased level of Mastomys IgG1 and IgG2a isotypes has the proficiency in fixing complement and attach to protein antigens, although, IgG3 principally recognized carbohydrate epitopes [36,37]. Immune complexes of mouse IgG3 and IgG2a can fasten effectively to FcγRI and bring out receptor-mediated responses [38], signifying that IgG3 and IgG2a isotypes could play an important part in the vaccine-induced protection through possible implication in ADCC reactions against invading pathogens.

Cytokine signals are critical determinants of both protective immunity and immunopathology. BmALT-2 and WbGST antigen-specific cytokine profile of vaccinated Mastomys has shown the elevated levels of IL-10, IL-4, and IFN-γ which suggested a mixed Th1/Th2 response. BmALT-2 mostly induced IFN-γ and IL-4 response followed by the low response of IL-10 while, WbGST shows high IL-10 and IFN-γ response. IL-4 and IL-10 produced in Th2 response selectively stimulate the production of IgG1 and IgG3 antibodies. While increased IFN-γ reflects Th1 immune response [38]. The immunized Mastomys results have shown unbiased Th1/Th2 immune response for both the vaccine candidates. Interestingly, IL-10 secretion by cells from Mastomys immunized i.m. with rBmALT-2 alone was comparatively lower as compared to Mastomys immunized i.m. with rWbGST alone or combination vaccine (rBmALT-2 & rWbGST) and we observed higher protection efficiency in this rBmALT-2 alone immunized Mastomys. Cells from Mastomys immunized i.m. with a combination vaccine (rBmALT-2 & rWbGST) produced a greater level of IL-4 and IFN-γ compared to Mastomys immunized i.m. with rBmALT-2 alone, but could not induce better protection as reflected *in vitro* and *in vivo* cytotoxicity experiments. IL-10 has been reported to inhibit the production of TNFα and reactive oxygen species by infected macrophages [39-44], reducing both parasite killing and toxicity to the local environment. Interestingly, IL-10 impairs macrophage destruction of both intra and extracellular parasites, as demonstrated *in vitro* with *Toxoplasma gondii* [41], *Trypanosoma cruzi* [45], *Leishmania donovani* [43], and extracellular larval stages of *S. mansoni* [46,47]. The immune responses of Th1 type are shown to be necessary for stimulating the immune protection against lymphatic filarial infection [48]. Besides, studies have also identified Th2 responses as conducive to intestinal helminth resistance [49-51]; however, it is difficult to extrapolate such results to parasites such as filariae.

5. CONCLUSION

Intramuscular route is the most effective way of administration of vaccine for better prophylactic response in filariasis. Additionally, the co-administration of monovalent vaccines seems to interfere with the prophylactic effect of monovalent rBmALT-2 and rWbGST vaccines. Overall, this study advocate for a larger field-based trial with this candidate vaccine to ascertain its translational prospect.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 15-8013, revised 2015) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Institutional Animal Ethics Committee (IAEC).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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