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ISOLATION, PREPARATION AND CHARACTERIZATION OF VACCINE FOR FILARIASIS

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Abstract

Objective: The present study was aimed on isolation and characterizing of antigen of filaria parasite for better immunomodulation effect of isolated antigen for make it available as ideal vaccine candidate.

Methods: Keeping in view their similarity of immune responses to human, the method as previously described in animal is used as model for experimental purpose.

Results: Antigen integrity was evaluated by performing the SDS -PAGE of the eluated protein. Antigens were found to be intact in the formulation stored at 4±1ºC after 30 days. Potential immune-effective fraction F6 is identified and isolated from Protein extract. SDS-Page of BmAII and F6 showed bands between 10.0 and > 180kDA and 54 and 68 kDA, respectively. The investigations proved that F6 as a potential source of vaccine candidate(s) and the present study is found satisfactory to select the F6 of *B. malayi* to recommend it as strong vaccine candidate for future study.

Conclusion: These results suggest that the F6 fraction of eluated protein is having promising responses towards vaccine development.

Keywords: Antigen, Filaraia Parasite, BmAFII, Isolation of Antigen, B. malayi.

1. INTRODUCTION

Lymphatic filariasis (LF) commonly known by the name elephantiasis is a mosquito-borne tropical disease caused by the filarial nematodes Wuchereria bancrofti, Brugia malayi and Brugia timori, elicits a wide spectrum of pathological disorders of the lymphatic system with varied clinical manifestations. The filarial parasites can survive in the human for many years causing

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permanent disability due to chronic syndromes such as lymphoedema, elephantiasis, and hydrocoele. The World Health Organization (WHO) ranks it as the second most common cause of long-term disability and estimated that over 1.25 billion people are at risk of the infection in 83 countries and territories (WHO, 2006a)[2]. Approximately 125 million already have been infected with LF, and over 40 million (WHO, 2004a)[3] are seriously incapacitated and disfigured by the disease (Ottesen et al., 1997)[4]. In 2002, it was estimated that LF is responsible for the loss of 4.52 million disability adjusted life years (DALYs) in men and over 1.42 million DALYs in women (WHO, 2004b)[5].

The methods to control and prevent the filarial infection include administration of antifilarials alone or combination of diethylcarbamazine (DEC)/ivermectin and albendazole and exposure control programs. In recent years, identification of several filarial antigens/proteins or molecules raised hopes for developing vaccines (Gregory et al.[6], 2000; Krithika et al.[7], 2005; Vedi et al.[8], 2008; Sahoo et al.[9], 2009; Joseph et al.[10], 2012) against lymphatic filariasis.

Novel adjuvants have been developed for enhancing antigen delivery and reducing the vaccine delivery to a single injection. For future human use, it is however necessary to use an adjuvant that is safe, biodegradable and which does not require repeated administration to produce the desired result.

Filarial parasites present a diverse array of antigens which elicit a complex and broad spectrum of immune and inflammatory responses in their hosts. Some of the responses are suppressive, some are protective and some are irrelevant. Several attempts have been made to identify and correlate the host immune responses with parasite antigens, but in the small number of experimental systems, such correlations remained inconclusive. Many host responses, both protective and non-protective, have the potential to cause pathological changes both locally and systemically and this, poses problems for the development of immuno-prophylactic strategies. Where pathology is associated with irrelevant responses, it may be possible to devise approaches that elicit only desirable protective responses. However, when protection and pathology reflect facets of the same response, the problems are much more complex to handle. Thus, to overcome such problems, precise identity of the molecules and delineation of the immune mediated pathways activated by the parasite molecules are prerequisites.

Studies conducted in laboratory revealed that of the two major Sephadex G-200 eluted fractions of *B. malayi* adult worm extract, BmAFII is protective *in-vivo* and stimulates predominantly pro-inflammatory cytokines to both adult worms and L3 while BmAFI facilitates parasite survival and stimulates predominantly IL-10 release (Dixit et al.[11], 2004; Dixit et al., 2006 [12]). Further, to narrow down to molecular entities that have cytokine release stimulating potential, *B. malayi* adult worm extract was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved fractions were blotted onto NCP and screened for the cytokine release potential. Some NCP-bound molecules corresponding to the molecular weight range of BmAFI and BmAFII, were found to possess proinflammatory and mixed pro-

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and anti-inflammatory cytokine stimulating potential *in-vitro* and provided a starting point for precisely identifying the functional molecules/proteins of interest (Dixit et al.[11], 2004).

Based on the above findings 6 molecular fractions (F1, F2, F5, F6, F10 and F14) were studied and F6 was selected for the present study to find out which of these fractions have the molecules potentially relevant to host protection or filarial pathology. The present work was aimed at characterizing parasite molecules with respect to their immunogenicity, involvement in the pathology of filariasis and to check it's potency via novel carrier delivery system.

There is an immediate need for the development of new and improved adjuvant and delivery system, which are potent, safe and can be used as a new generation vaccine. In the present study it is thought worthwhile to prepare liposomal-system having the potential benefits of reducing the number of dosages for primary immunization, reducing the total antigen dose required for effective immunization, enhancing both humoral and cell-mediated immune responses over a longer period of time, enabling combined vaccine administration and permitting effective primary or booster immunization. In addition, the integrity of the antigen is maintained by avoiding the use of organic solvents and a pH changes, preparation process is simple and easy to scale up for chemical studies and eventual manufacture.

The surface antigen(s) have important role in generation of protective immunity. Consequently, characterization of protective responses generated by surface antigen(s) that can be used as vaccine is worth considerable. A few body wall antigens have earlier protective and are potential vaccine candidates against filarial infections. The purified native protein or recombinant filarial protein might be more useful for achieving the desired immunity.

Therefore, the present study was aimed to isolate the purified native protein of parasite and to prepare

2. MATERIALS AND METHODS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were Sigma, USA. All other chemicals and reagents are available at in-house facility of CDRI, Lucknow.

2.1 Antigen isolation

2.1.1 Parasite

For laboratory experimental purpose, Brugia malayi, having many of the biological aspects similar to Wuchereria bancrofti, was used in the study. It is a sub periodic strain of human filarial infection and has successfully been transmitted to various vertebrate hosts including monkeys, cats and rodents. The infection is transmitted through black eyed susceptible strain of Aedes aegypti mosquitoes developed by McDonald (Liverpool School of Tropical Medicine and Hygiene, U. K.)[13].

2.1.2 Animal models

Rodents are preferred models for laboratory studies throughout the world. 'GRA' strain (Germany) of Mastomys coucha (belonging to family Muridae) as shown in Figure 1 is

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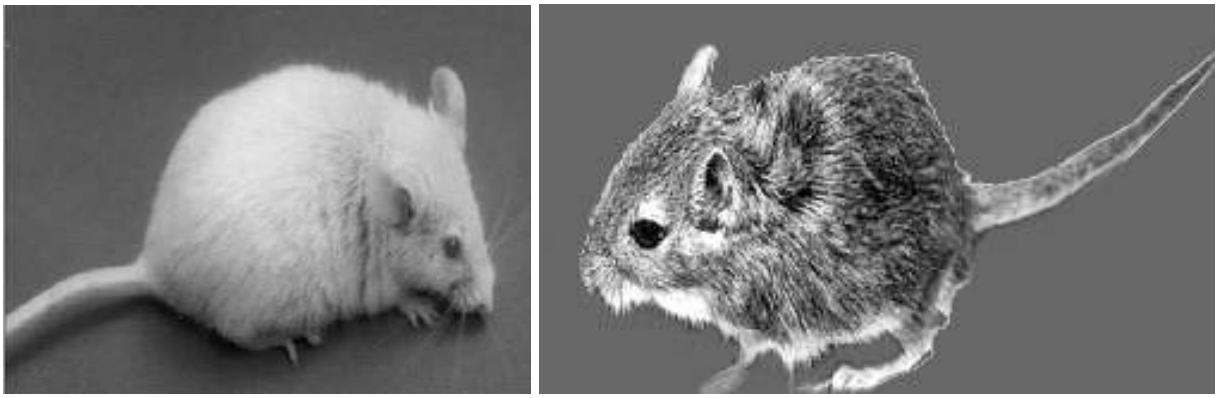
susceptible to B. malayi and is being maintained in the animal house of Central Drug Research Institute, Lucknow, India since last 35 years. It is a multimammate, prolific breeder with average litter size of 8 - 10 babies. The female may have her young at any time of the year and if conditions are right, may do so regularly at intervals of 33 days (Wilson and Reeder, 1993)[14]. M. coucha model is found amenable to perform chemotherapeutic and immunobiological investigations in experimental filariasis (Tyagi et al.[15], 1998). Keeping in view their similarity of immune responses to human, this animal is used as model for experimental purpose (Dixit et al.[11], 2004; Khan et al.[16], 2004).

Another rodent, the Mongolian gerbil (Meriones unguiculatus, family Gerbillinae) as shown in Figure 1 has proven to be an excellent permissive rodent model for the study of lymphatic filariasis using B. pahangi or B. malayi. The animal (called 'jird') is used for the propagation of B. malayi parasites (McCall et al.[17], 1973).

All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. The animals are kept in plastic cages and were housed in animal quarters under controlled climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light-dark cycles). They were fed with standard rodent diet pellets and had free access to drinking water.



Experimental Filarial models – *Mastomys coucha* **&** *Meriones unguiculatus*.

2.1.3 Maintenance of *B. malayi* infection

2.1.3.1 Rearing and breeding of A. aegypti colony

In the laboratory the mosquitoes were reared and bred in an insectarium maintained at controlled temperature (26 ± 1°C) and humidity (80 ± 5%). The adult mosquitoes were kept in nylon mesh cages and provided 10% glucose solution with vitamin B supplement, soaked in cotton for feeding. From time to time female mosquitoes were fed on normal *M. coucha* blood to promote egg laying. A beaker containing water was kept in the cage for egg laying. The eggs layed after about 40 hrs blood feeding were filter separated and stored after drying at same temperature. Eggs can be preserved under such condition for 3 - 4 months. For maintenance of mosquito life cycle the eggs were transferred to enamel bowl containing tap water. The larvae hatch out in the

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water within 24 hrs and these were provided with feed containing dog bix and yeast powder. The larvae usually took about 8-10 days to become pupae, which ultimately developed into adult mosquitoes within 48 hours.

2.1.3.2 Feeding of mosquitoes on mf positive M. coucha

B. malayi infected *M. coucha* showing 100-200 mf/10 µl of blood were used as donors. The feeding of mosquitoes on donors was carried out between 12 noon and 1:00 PM (peak microfilaraemia time). Mosquitoes starved for 2-3 hrs were allowed to feed on the donors, which was kept inside the mosquito cage in a wire netting immobilized cage. After 1 hr of feeding the donor animal was removed and mosquitoes were provided with glucose solution as mentioned above. In 9 - 10 days time the mf in the mosquitoes developed into L_3.

2.1.4 Isolation of L_3 from mosquitoes

On day 9 or 10 post feeding, the mosquitoes were paralyzed and crushed gently in 4-5 ml of 0.6% insect saline (IS) and transferred to Baerman's apparatus which consisted of glass funnel, muslin cloth and transparent rubber tubing with a pinch cock. The funnel was filled with lukewarm IS. Crushed mosquitoes were then put onto muslin cloth and allowed to stand for half an hour with light provided from top by a table lamp. The L_3 released from the mosquitoes move away from light, traverse through the muslin cloth and settled down at the bottom of the tube. These were collected by opening the pinchcock and washed with IS several times to remove the mosquito debris. The larvae were counted and used immediately for exposure to animals.

2.1.5 Inoculation of L_3 to M. coucha or jird

For infection purpose 6-8 weeks old male *M. coucha* were inoculated with active and motile L_3 (100 per animal) subcutaneously. Establishment of successful infection was examined in blood smear after day 90 post larval inoculation and thereafter monitored at regular intervals. Animals showing desired levels of infection were used for transmission to healthy animals. Thus, the cycle was continued. Similarly, in jirds of the same age group about 200 L_3 were inoculated intraperitoneally. The larvae develop into adult worms in about three months and can be harvested thereafter when required.

2.1.6 Preparation and fractionation of antigen

The worms were washed several times and crushed in phosphate buffered saline (PBS) in cold followed by sonication (Soniprep 150) for 10 cycles of 30 seconds each at 10-micron amplitude with intermittent gap of 1 minute. The total homogenate was mixed with equal amount of 2X sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (0.125 M Tris-HCl, pH 6.8; 4% SDS, 5% β-mercapto ethanol, 20% glycerol and 0.01% bromophenol blue) and boiled in water bath for 5 min. The protein samples thus prepared were centrifuged to remove any particulate residue before loading to gel.

2.2 Sodium dodecyl sulphate -polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. SDS-PAGE

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simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix.

In order to isolate dominating fractions which have been identified to be stimulators of pro- and mixed pro- and anti-inflammatory cytokine release (Dixit *et al.*[11] 2004; Table 3.1) extract of adult worms was used. The extract was resolved in 10% gels (Laemmli, 1970)[18]. Preparative (13.8×13 cm slab) gels were run in gel with a dual-gel electrophoresis chamber (AE-6220, Atto Japan). Resolved fractions (six) of interest were cut with the help of pre-stained molecular weight markers run along the side. These were designated as F1, F2, F5, F6, F10 and F14. The fractions in gel strips were stored as such in gel at -10^{0}C till elution.

Table 2.2: Cytokine-release stimulating potential of NCP-bound molecules of adult *Brugia malayi* soluble extract in THP-1 cell system

Fraction (MW kDa)	**Cytokine**
F1 (>180)	TNF-α (++), IL-10 (++)
F2 (169-180)	TNF-α (+++), IL-10 (+++)
F5 (67.8-84.3)	IL-1β (+++)
F6 (54.3-67.8)*	IL-1β (+++), IL-6 (+++), TNF- α (+)
F10 (38.44-41.84)	IL-1β (++)
F14 (17.0-22.5)	IL-10 (+)

+, ++ & +++ indicate ascending grade of predominance.

* selected for present study.

2.3 Electro-elution of proteins from gel

Proteins from gel strips were electro-eluted by micro electro eluter (Millipore, USA) as per method described by manufacturer. Briefly, about 75 % of the perforated tube was filled with SDS PAGE gel strips in small pieces. The tube carrying gel was fitted into centricon™ tubes having a membrane filter of required cut off limit. After filling both upper and lower chambers of the microeluter with Tris-glycine buffer, electricity (~200V) was applied for 2 to 4 hrs depending on the size-based mobility of the fraction ensuring near complete elution from the gels. After elution is over the centricon tube was disassembled from the slot and the gel carrying tube was removed. Protein solution retained in the centricon was centrifuged at 2000g in cold (4 °C) in an angular rotor till the volume reached to required level. The eluted fractions were run in SDS-PAGE to confirm their molecular weight. Finally the protein solution thus obtained was filter sterilized with 0.22µ membrane filter and stored at -20°C until used.

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2.4 Antigen for future Study

2.4.1 Bovine serum albumin (BSA) as a model antigen

BSA is a white to light tan colored powder that contains not more than 3.0% w/w of water, containing about 96 % protein. It has a molecular weight of 67KDa. It consists of a carbohydrate free polypeptide chain connecting four globular segments of unequal size. It must be protected from light and moisture and store at temperature between 2° C and 25° C.

2.4.2 *Brugia malayi* adult worm protein extract (F6) as a candidate antigen

F6 is a sephadex G-200 eluted fraction of B. malayi adult worm extract. It has a molecular weight of 54.3-67.8 KDa. It has five proteins namely heat shock protein (HSP60), NAD dependent epimerase/dehydratase, intermediate filament (IF), elongation factor 2 (EF2), hypothetical protein CBG00623. It must be protected from light and moisture and store at temperature -20ºC.

3. RESULTS & DISCUSSION

3.1 Isolation and Characterization of Antigens

Potential immune-effective fraction F6 is identified and isolated from Protein extract. SDS-Page of BmAII and F6 showed bands between 10.0 and > 180kDA and 54 and 68 kDA, respectively. The investigations proved that F6 as a potential source of vaccine candidate(s) and thae present study is found satisfactory to select the F6 of *B. malayi* as a strong vaccine candidate for future study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This protocol was approved by the Institutional Animal Ethical committee of CSIR-CDRI which implements the national guidelines of CPCSEA for use and handling of animals.

HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human subjects performed by any of the authors; all institutional and national guidelines for the care and use of laboratory animals were followed.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

Not applicable

INFORMED CONSENT STATEMENT

Not applicable

AUTHOR CONTRIBUTIONS

The corresponding author designed and performed the experiments. The co-authors supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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