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Development of pilot scale production process and characterization of a recombinant multiepitope malarial vaccine candidate FALVAC-1A expressed in *Escherichia coli*

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ABSTRACT

Among the four human malarial species, *Plasmodium falciparum* causes most of the mortality associated with malaria. Several approaches are being pursued to develop a suitable malaria vaccine since it may be the most effective weapon to fight against malaria. A highly immunogenic, synthetic protein consisting of 21 epitopes from pre-erythrocytic and blood stages of *P. falciparum* (FALVAC-1A) was constructed and expressed in *Escherichia coli*. This vaccine candidate was highly immunogenic and induced protective antibodies in rabbits when produced through lab-scale processes in milligram quantities. In order to take this vaccine candidate for further clinical trial, we optimized the process for industrial scale production and purification. Here we describe various methods used in pilot scale production and characterization of FALVAC-1A. A fed-batch cultivation process in a bioreactor at 10-L scale was optimized to express the protein in high yields as inclusion bodies in *E. coli* cells with the recombinant plasmids. Methods to solubilize, capture and purify the target protein from the inclusion bodies were optimized and the resultant protein was >95% pure based on SDS–PAGE and RP-HPLC. This protein was then refolded and nativity was confirmed by Far-UV CD spectroscopy. Final purified protein was characterized to estimate yield, purity, mass and confirmed to be free of host cell proteins, nucleic acids and bacterial endotoxins. This study confirms that industrial scale clinical grade FALVAC-1A can be produced in a cost-effective manner for clinical trials.

More than a third of the world's population is at risk of contracting malaria, with over half a billion of these infections caused by *Plasmodium falciparum* [1–3]. Malaria is one of the main obstacles to socio-economic development in Africa; it is estimated that 200 people, primarily children, die of malaria every hour [4]. The recently noticed increase in the proportion of total mortality attributable to malaria in African pediatric population may be because of a general breakdown of public health services in many areas, antimalarial drug resistance, interactions with the human immunodeficiency virus and (perhaps) global climate change [5]. The ineffectiveness of malaria control and treatment strategies has resulted in the attention being targeted to development of a vaccine against malaria as a first order priority to combat the increasing incidence of the disease [6]. The underlying support for this effort comes from the evidence that long exposure to the parasite causes a partially protective immune response in people and such people have fewer and dense parasitaemias and are less likely to suffer when parasitaemic, providing significant protection from death [7].

Effective vaccines against malaria would have an enormous effect on reducing the negative impact of malaria. Although the complexity of the malarial parasite has made the malaria vaccine development process tenuous, advances in science and in the vaccine development process as well as increases in funding are encouraging [8]. The life cycle of the malarial parasite is complex but provides multiple points for interruption because several stages are morphologically and antigenically distinct, and immunity is stage specific [7]. The concept that vaccination may be a useful tool to control the disease is based on a number of observations. Individuals continually exposed to infection by the parasitic protozoan responsible eventually develop immunity to the disease, and passive immunization with antibody from immune donors can have a dramatic effect on blood stage parasitaemia [9]. Furthermore, inoculation of live attenuated parasites was shown to protect naïve volunteers against infection [10], and immunization with whole killed organisms gave protection in animal models [11]. Symptomatic, experimentally challenged volunteers with relatively high parasitaemia served in the development of the experimental malaria vaccine, SPf66, which was shown to delay or suppress the development of parasitaemia [25]. This molecule was developed based on the results of a mixture of three synthetic peptides (83.1, 55.1, 35.1), corresponding to fragments of the relative

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molecular mass 83,000 (83 K), 55 K and 35 K *P. falciparum* merozoite-specific proteins, which induced protection in *Aotus trivirpatus* monkeys experimentally infected with *P. falciparum* [12].

Twenty years of antigen identification, gene cloning and expression have produced many candidates for subunit vaccines [13]. Two of the principal approaches to the development of malaria vaccines target pre-erythrocytic and erythrocytic stages of the parasite life cycle [14]. A pre-erythrocytic (pre-blood stage, liver stage) vaccine candidate, known as RTS,S, has recently been shown to be effective against clinical and severe disease in children in Mozambique [15,16]. An effective vaccine against malaria that would protect non-immune individuals from the disease has long been a goal. Since *P. falciparum* is more virulent than the other common parasite *P. vivax* and is responsible for more than 95% of deaths due to malaria worldwide, it is the target of many vaccines against malaria [17]. Targeting proteins expressed at key life stages of the parasite has been the basis of many efforts by several workers in developing multistage, multiantigen vaccines that mimic natural protection against malaria. With the availability of the full sequence of *P. falciparum* genome [28,29], several efforts towards a multivalent vaccine approach are being pursued.

One such candidate vaccine is a synthetic protein named CDC/NII MALVAC-1 consisting of 21 epitopes ('12' B-cell and '9' T-cell epitopes derived from nine stage specific—sporozoite, liver stage and blood stage—antigens) of *P. falciparum* along with a Tetanus Toxoid helper epitope. Vaccine formulations of this candidate made with three different adjuvants produced good immune response in rabbits; the antibodies recognized antigens expressed at all the three different stages of the parasite and also produced significant anti-parasitic activity *in vitro* against the sporozoite and blood stages of the parasite [18]. In another study, the same antigen (renamed as FALVAC-1) was mixed with four adjuvants (Squalene/Span 80/CRL-1005 co-polymer water-in-oil emulsion, Montanide ISA-720, QS-21 and aluminum phosphate), injected to out-bred mice and shown to elicit immune responses to most of the epitopes [19].

This recombinant protein was expressed in the baculovirus expression system [20], however, the protein yield was too low (0.1–1.0 mg/L) for efficient production. By contrast, a construct with codons optimized for *E. coli* expression yielded 3-fold more protein and also promoted host cell growth after IPTG induction [21]. However, this construct was unsuitable for further clinical development because of concerns about stability, potential homology of one of the epitope sequences with human sequences and low yield. Therefore the construct was further modified by adding spacer determinants imparting breaks in the secondary structures to achieve greater physicochemical stability. The resultant amino acid sequence was then optimized for expression in *E. coli*. This was named as FALVAC-1A. This was produced at lab-scale reproducibly and was stable at physiological pH at workable concentrations at high purity. The expressed protein showed a high degree of sequence fidelity [22]. But the same methods that are used in the lab-scale processes did not produce the protein at required yield and antigenicity in larger scales, which is mandatory if the molecules have to be taken for field use. We report here a process to produce sufficient quantities of FALVAC-1A protein at pilot scale to enable its use in clinical studies.

Materials and methods

Transformation, expression check and cell banking

Competent *E. coli* BLR (DE3) cells (Novogen) were prepared and transformed with the plasmid pET24d—FALVAC-1A using calcium chloride method [23]. The cells were plated on Luria Bertani (LB)[1] agar with Kanamycin (30 μg/ml) and plates were incubated overnight at 37 °C. Single isolated colonies with better growth were selected and inoculated into 5-ml LB broth in tubes with Kanamycin (LB-Kan); this and all liquid cultures were grown in shaker at 37 °C at 200 rpm. A part of the overnight grown culture was subcultured into 25-ml LB-Kan broth in a 100-ml flask and grown until OD_{600} was between 0.6 and 1.0 and FALVAC-1A protein expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside); another part of the overnight grown culture was subcultured into 50-ml LB-Kan broth in a 250-ml flask and grown for 10–12 h. The induction phase of the 25-ml culture was stopped after ~4 h; 1 ml of culture was centrifuged at 10,000*g*, 4 °C for 10 min and was resuspended with sample buffer for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) containing SDS and β-mercaptoethanol, boiled for 5 min. and loaded onto two 12% SDS–PAGE gels as per standard Laemmli Protocol [24]. One gel was stained with Coomassie Brilliant Blue-R250 (Sisco Research Labs, Mumbai, India), destained and visualized; the second gel was used for Western blotting as follows: proteins were electrophoretically transferred onto Immobilon-P (hydrophilic PVDF, Millipore, USA) membrane. This membrane was blocked with 1% milk powder, 0.05% Tween-20 in 10 mM phosphate-buffered saline (PBS, pH 7.2). The blocked membrane was treated with ~25 ng/ml of rabbit antibodies to the NANPNANPNANP epitope of the circum-sporozoite protein (CSP) antigen for 1 h, washed with PBS-Tween 5 times and incubated with 1:4000 dilution of goat anti-rabbit HRP conjugate for 1 h at 37 °C. The Western blot was developed with diamino-benzidine (DAB) and hydrogen peroxide (H_2O_2) to confirm the identity of the expressed protein. The best expressing clone was selected for further studies. To prepare glycerol stock, the 50-ml culture of corresponding clone already grown at 37 °C was sub-cultured into 200-ml LB-Kan medium in shaker-flask and grown for 8–10 h. Cells were harvested by centrifugation at 4 °C, 10,000*g*, mixed with glycerol and the 50% glycerol stock of these cells was aliquoted in 2-ml cryovials and stored frozen at −70 °C for use in further work. The prepared glycerol stock was used to reconfirm the expression of the recombinant FALVAC-1A.

Cultivation in bioreactor

Glycerol stock (500 μl) was inoculated into 25-ml semi-defined medium containing the following ingredients: yeast extract (10 g/L), potassium dihydrogen phosphate (4 g/L), dipotassium hydrogen phosphate (4 g/L), disodium hydrogen phosphate, $2H_2O$ (3.5 g/L), ammonium sulphate (1.2 g/L), ammonium chloride (0.2 g/L), manganese sulphate, $7H_2O$ (2 g/L), dextrose (2 g/L), cobalt chloride, $6H_2O$ (2.5 mg/L), manganese chloride, $4H_2O$ (15 mg/L), copper sulphate (1.5 mg/L), borax (3 mg/L), sodium molybdate, $2H_2O$ (2.5 mg/L), zinc acetate, $2H_2O$ (13 mg/L), ferric citrate (100 mg/L) and kanamycin (30 mg/L), pH 6.9 ± 0.1. The culture was grown overnight. Ten milliliters of the overnight grown culture was inoculated into 190-ml of the broth in 500-ml flask and grown for 7–9 h. Culture from two such flasks (400 ml) was inoculated into 9.6 L of sterile broth in a 19 L bioreactor. The batch phase of the cultivation lasted for 6 ± 1 h and the OD_{600} reached 20 ± 5. The dissolved oxygen (DO) level was maintained at 20–40 during this phase. Then fed-batch was started with a mixture of yeast extract (80 g/L), ammonium sulphate (72 g/L), ammonium chloride (12 g/L), dextrose (600 g/L)

[1] *Abbreviations used:* LB, Luria Bertani; IPTG, isopropyl-β-D-thiogalacto-pyranoside; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CSP, circum-sporozoite protein; DAB, diamino-benzidine; DO, dissolved oxygen; IMAC, immobilized metal affinity chromatography; EBA, expanded bed adsorption; PES, polyethersulfone; CD, circular dichroism; SEC, size-exclusion chromatography; RP-HPLC, reverse phase-high performance liquid chromatography.