Liver stage Plasmodium Protein Pb122500 and Implication for Vaccine Design

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Abstract- In the mammalian host, invasion of the hepatocytes is the first step towards developing malaria disease. There are some signals which are exchanged between host and parasite. During early liver stage development, a sporozoite surface protein (CSP) is introduced in the hepatocytes cytoplasm. CSP then shuttles into and out of nucleus of hepatocytes and changes the host transcription profile. CSP export into the cytoplasm of infected hepatocyte requires the presence of a conserved pentameric motif-PEXEL (Plasmodium export elements) motifs. The CS protein out compete with the NFkb for nuclear import and down regulate the host genes regulated by NFkb which are involved in inflammation. These proteins could serve as good candidate for the drug development against malaria parasite. Marti et al. identified 22 sporozoite stage proteins in *P. falciparum* that had putative PEXEL motif. It has been identified that protein Pb122500 also contains that PEXEL motif and has a putative serine–thrionine kinase domain (so it is a drug candidate). The focus of current research is to study implications of Pb122500 gene as potential vaccine candidate by cloning and expressing the protein, characterizing the proteins, raise sera against them and try to localize these proteins in hepatocyte cells and find out further the host proteins interacting with these proteins so that we could find new antigen for vaccine against hepatic stages.

Index Terms- Cloning, Expression, Vaccine, SDS, Western, Vector, Pexel, Parasite, Liver.

I. INTRODUCTION

According to the center for disease control and prevention, it has been reported that each year 300-500 million people are infected by the malaria parasite and results in 3-5 million people death (1,11). Malaria in humans is caused by four species of *Plasmodium* (2) *Plasmodium vivax, Plasmodium falciparam, Plasmodium malariae, Plasmodium ovale*. *Plasmodium berghei* and *Plasmodium yoelii* are mouse malaria parasites. The complete life cycle of the malaria parasite is completd in three stages:Sporogonic cycle, Exo-erythrocytic cycle, Erythrocytic cycle. When the sporozoites invade the hepatocytes, they hide themselves in a specialized vacuolar compartment called parasitophorus vacuole (4). The membrane of this vacuole is derived from host cell plasma membrane and physically separates the parasite from host cell. Further development of parasite take place inside this parasito-phorus vacuole. Malaria parasites export hundreds of proteins to remodel the host cell in order to get access to nutrients, solute, ions and other things required for the parasite survival and its propagation inside the host cell (5). Parasite exported protein have to cross the two barriers in order to reach the host cytosol. These two barriers are parasite plasma membrane and PV-membrane (6). The parasite protein crosses the plasma membrane by vesicular transport (7) and reach to the space between PVM and parasite. In the infected RBCs, export of these parasite proteins into host cell depend on plasmodium export element (pexel) or vacuolar transport signal (VTS) (8). CS (Circumsporozoite) protein has the PEXEL motif by which it traverses the PV membrane. CS protein also has a NLS (Nuclear

Localization Signal) and a putative NES (Nuclear export signal) at C-terminus (9). NLS in CS uses alpha3 importin to enter into the nucleus. Binding of CS to importin alpha3 was abolished after deletion of the NLS. It has been proved that NLS has a role in the enhancement of EEF development. Hence plasmodium has PEXEL motif, to introduce the CS protein into the hepatocyte cytoplasm and a NLS to enter its nucleus. Once inside hepatocyte cytoplasm, CS out competesNFkB (involved in inflammation) nuclear import, thus down regulating the expression of many genes; controlled by NFkB. Thus CS inside hepatocyte creates a favorable niche for parasite growth. CS currently is a target antigen for vaccine against liver stage of malaria and it is in a phase-II clinical trials and have shown partial protection (~50%).

Marti et al. identified 22 sporozoite stage proteins in P. falciparum that had putative PEXEL motif. It has been identified that protein Pb122500 contains that PEXEL motif and has a putative serine–thrionine kinase domain (so is a drug candidate). We searched for orthologues in rodent malaria parasite, as our model system of study is rodent malaria. Marti et al. found that virulence proteins of infected RBCs have a trafficking signal. The first part of the signal is SS; responsible of export into the PV and second is a motif that targets the protein for onward transport into the erythrocyte proper. Marti et al. termed that motif as PEXEL (Plasmodium Export element) motif. It is also known as VTS (Vacuolar Transport Signal) or HT (Host Targeting) signal. Marti et al. also found the following features of the conserved pentameric motif - Positively charged hydrophilic amino acid in position 1, (Arg and Lys). A hydrophobic amino acid in position 3 (mainly Leu). Less conserved amino acid in position 5 (predominantly Asp, Glu and Gln). Non charged any amino acid in position 2 and 4.

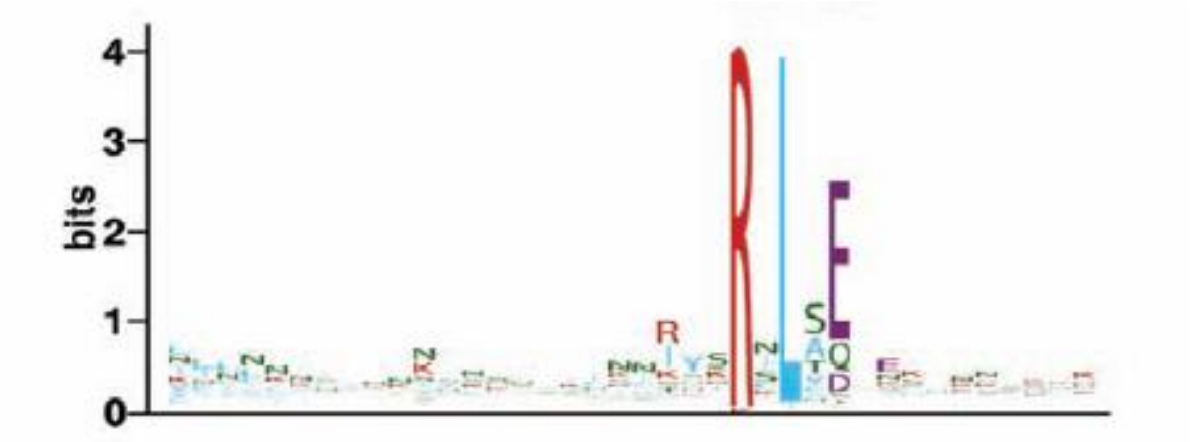

Figure 1: Pexel motif [Consensus: (R/K) X (L) X (E/D/Q)]

II. MATERIALS & METHODSETHODS

Materials

***E. coli* strains**: *E. coli* expression strain BL21, cloning strain DH5alfa and XL1-Blue has been used.

Plasmids: The *E. coli* expression vector pGEX6P1, pET28a, cloning vectors-TOPO vector, pUC19 has been used.

Media and other materials: Luria Bertani broth, LB Agar, Glycerol, from Hi media, nucleic acid extraction and purification kits from QAIGEN, Ampicillin, and Chloramphenicol were obtained from Sigma. Restriction Enzymes, buffers, were purchased from New England Bio-labs, USA. DNTPs, Taq polymerase HiFi DNA polymerase, Deep vent and their buffer were purchased from NEB.

Methods:

***E.coli* culture and its preservation:** All the bacteria were grown in LB media at 37ºC with shaking in the presence of appropriate antibiotic. The medium was sterilised by autoclaving at pressure of 15-lbs/square inch for 15 min. For preserving the bacterial cultures, cells were allowed to grow in LB media with appropriate antibiotic concentration, and when cells reach log phase, 300µl of the culture was added into the micro centrifuge tube containing300µl of 50% glycerol solution. The cells were mixed thoroughly till the solution become homogenous and stored at -80°C.

Expression check of Pb268 in PGEX-6p1: *E. coli* strain BL21 was used for checking the expression of the recombinant protein Pb268 optimized (previous name of Pb122500). Pb268optimized in PGEX in BL21 strain glycerol stock streaked on LB plates (AMP+CMP). 8 colonies streaked on another LB plate (AMP+CMP) and also inoculated in 10ml LB media each containing (AMP+CMP). The falcons were incubated at 37°C in a shaker incubator till OD_{600} 0.6 was attained. IPTG was added to the culture at a final concentration of 1 mM. The cultures were incubated, for 12 hr at 37°C, prior to harvesting by centrifugation at 13000 rpm for 1.30 min at 4°C. The post induction cell pellet was resuspended in native lysis buffer (added Triton X-100, PMSF) at 2-5 ml per gm weight of the pellet. Lysozyme was added to 1 mg/ml and of cell pellet and incubated on ice for 30 min. The suspension was sonicated on ice (seven 3sec bursts with a 1sec cooling period between each burst) for 2-3 minutes for each of 8 samples at amplitude of 60. The lysate was centrifuged at 13000rpm for 2-3minutes at 4ºC to pellet the cellular debris

SDS PAGE for expression check: Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method described by Laemmle (1970). The stacking gel containing 5 % acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8) were mixed and polymerized. The separating gel had 12% or 15% acrylamide depending on the case and 0.1% SDS. Running buffer consisted of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 containing 0.1% SDS. The protein samples were prepared in sample buffer [0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and 5% β-mercaptoethanol (Laemmli, 1970) and were denatured by boiling at 100ºC for 10 min in lyses buffer. The electrophoresis was carried out at 30 mA. Wide dual range protein marker was run simultaneously to calculate the subunit molecular size of the proteins.

Coomassie brilliant blue staining:SDS-Polyacrylamide gels containing more than 200ng protein concentration were visualised by standard "Coomassie Brilliant Blue R 250 (CBB R 250)" staining solution {0.1% (w/v) CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water}, followed by de-staining in 25% (v/v) methanol and 10% (v/v) acetic acid in water.

Western blot: The proteins separated by SDS PAGE were transferred from the gel to nitrocellulose membrane with the help of semidry transfer system (Bio-Rad, USA). In brief, after removal of stacking gel, the resolving gel was soaked in transfer buffer for 5 min at 4ºC. The nitrocellulose membrane and pieces of Whatman 3 mm filter papers were soaked in transfer buffer for 10 min at 4ºC. The membrane was placed in the transfer apparatus after three layers of Whatman filter sheet. The gel was placed over the membrane followed by three layers of Whatman filter sheet. Care was taken avoid trapping any air bubble between the gel and

nirocellulosemembrane. The semidry transfer was carried out at 20 V for 30 min at room temperature. Following transfer, the membrane was carefully removed from the blotting apparatus and blocked with PBS containing 2% milk protein for 1 h at room temperature. The membrane was washed thrice with PBST and incubated with the primary antibody (anti -Flag) diluted 1: 3000 in PBS containing 0.5% milk protein of 1 h with constant rocking. The membrane was washed thrice with PBST and incubated with HRP-labeled anti mouse Ig antibody (diluted 1:4000 in PBS containing 0.5% milk protein) for 1 h. Following incubation, the membrane was washed five times with PBST and developed using DAB along with H_2O_2 in PBS.

III. RESULTS & DISCUSSIONS

Expression of C-terminal 490-510 amino acids of Pb 122500 (Previously Pb000268.02.0) in pGEX6p1 in RIL strain of *E.coli* at 370C with 1mM IPTG:

Pb000268.02.0 optimized in pGEX in RIL strain glycerol stock streaked on LB plates (AMP+CMP). Colonies were streaked on another LB plate (AMP+CMP) and also inoculated in LB media containing (AMP+CMP) and kept on shaker incubator on 370C. IPTG was added after OD600 0.6 was attained. The post induction cell pellet was resuspended in native lyses buffer. Lysozyme was added to cell pellet and incubated on ice. The suspension was sonicated. The lysate was centrifuged to pellet the cellular debris. 20µl of protein sample (each pellet and supernatant) with 5µl of 5X Protein Loading Dye and after giving heat treatmentwas loaded on 5% stacking, 10% resolving SDS-PAGE.

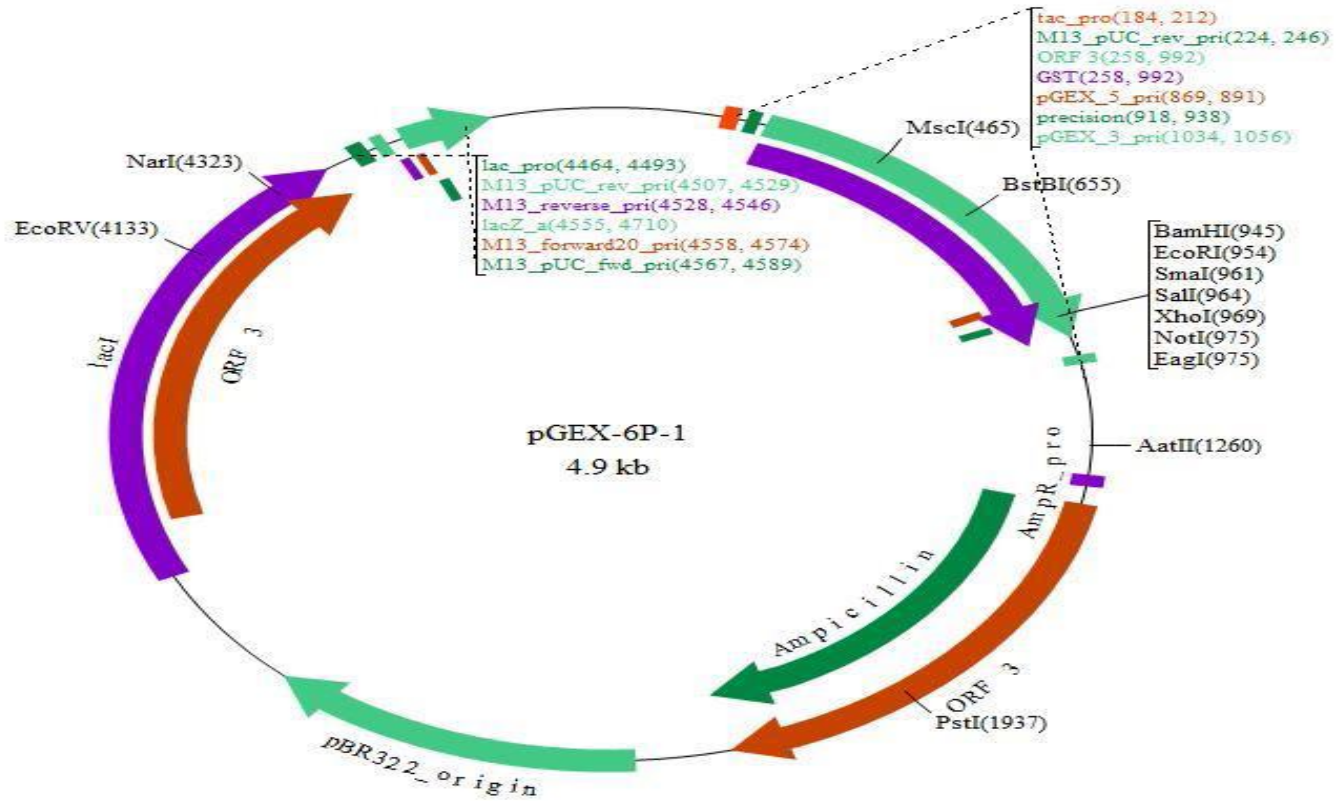

Figure 2: PGEX VECTOR: STRUCTURE

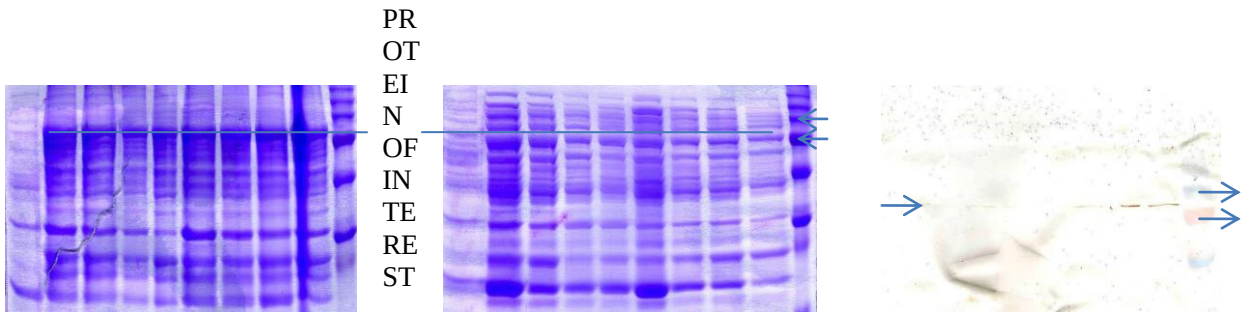

Figure 3: EXPRESSION CHECK (with 1mM IPTG & growth at 37 Degree temp) IN BL21 STRAIN OF Ecoli OF Pb268 IN PGEX VECTOR by SDS PAGE WESTERN BLOT.

(a) PELLET -1-UNINDUCED, 2 to10 INDUCED, 10-RECOM DUAL RANGE PROTEIN MARKER

(b) SUPERNATANT- 1-UNINDUCED, 2 to10 INDUCED, 10-RECOM DUAL RANGE PROTEINMARKER

(c) WETERN BLOT-WEAK EXPRESSION IN 4, 7, 8, 9, LADDER at 10.

IV. CONCLUSIONS

The fig. 3 shows that our protein of interest of 75 Kda is partially coming in pellet fractions (fig 3a) and partially in supernatant fractions (fig 3b). Because we wanted to obtain our protein in supernatant fractions, we performed western blot of supernatant fractions to further confirm expression of desired protein. In western blot (fig. 3c), it can be seen that very weak expression in lane 4,7,8,9 were coming. The possible reasons could be:

- Initially we were trying to express Pb000268.02.00 (only C-terminus) in pGEX vector in *E. coli* (BL21 strain).Later on we found that gene's database had been updated and an N-terminus was also included in its database and new gene was renamed as Pb122500. So it justifies weak or no expression of pb000268.02.0 in *E. Coli*(BL21 strain).

- Since this gene codes for a "putative kinase domain" containing protein, a basal/leaky expression is sufficient to interfere host metabolism and to kill the host cell.

- Large insert size may lead to unstable plasmid. Due to large vector-insert construct, host is not able to replicate, so plasmid is lost and only weak/leaky expression is obtained..

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