CHAPTER 2

CLONING, EXPRESSION AND PURIFICATION OF *P. vivax* ANTIGENS AMA1, MSP7 AND WARP

"Keep your face to the sunshine and you can never see the shadow"

-HELLEN KELLER

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INTRODUCTION

Apical membrane antigen 1 (AMA1) is a micronemal protein of apicomplexan parasites that appears to be essential during the invasion of host cells. AMA1 is a potential vaccine candidate as it has been observed in *in vitro* studies and in animal challenge models that the immune responses to *Plasmodium* AMA1 can have profound parasite-inhibitory effects. However, AMA1 is polymorphic, probably as a result of immune selection operating on an important target of naturally occurring immunity. AMA1 was first characterised as an integral membrane protein on the apical complex which is then transferred to the merozoite surface (Peterson et al., 1989).

AMA1 is a structurally conserved type I integral membrane protein (559-563 amino acids) in most *Plasmodium* species (Hehl et al., 2000 and Donahue et al., 2000). The cytosolic region comprises of 50 amino acids and the rest of the amino acid residues form the ectodomain of AMA1. The ectodomain, comprises of 16 conserved cysteines residues which contribute to disulfide bonding (Hodder et al., 1996). The pattern of the disulfide bonds is such that it leads to the formation of three domains (DI, DII and DIII). Structural amino acid conservation is observed among species and genera and, is particularly evident in DI, DII and DIII except in the DIII domain of *Babesia* spp. AMA1 and *Toxoplasma gondii* AMA1. Crystallographic studies reported that, the N-terminal region of domain DI extends to interact sequentially with the other two domains (DII and DIII). The core of DI comprises an extended folding motif of the plasminogenapple-nematode (PAN) domain family from which unstructured loops

protrude (Tordai et al., 1999). The pattern of this loop revealed a hydrophobic cleft that might serve as a receptor pocket (Bai et al., 2005).

The cell invasion machinery of parasites belonging to the Apicomplexa family is highly conserved and participates in the formation of moving junction (MJ) between the parasite and host cell membranes (Aikawa et al., 1978). The MJ formed passes through the apex to the posterior of the parasite. This causes the internalization into the parasitophorous vacuole (PV). The constituents of the moving junction are conserved and secreted from rhoptries (secretory organelles). RON2, RON4, RON5 and RON8 are the rhoptry neck proteins that is discharged during invasion to form a complex (Alexander et al., 2005, Lebrun et al., 2005 and Straub et al., 2009). The protein apical membrane antigen 1 (AMA1), is restrained at a different organelle, micronemes and discharged prior to the secretion of rhoptries, interacts with the RON complex during invasion (Carruthers et al., 1997).

Antibodies to AMA1 are found in most people exposed to malaria (Greenhouse et al., 2011). IgG1 with IgG3 (anti-AMA1) is predominantly found in naturally exposed populations. IgG2 or IgG4 levels are observed rarely (Rouhani et al., 2015). Antibody response to DI and DII are generally high as compared with DIII in individuals suffering from malaria. During infection the AMA1 antibody levels seem to be higher than other blood-stage antigen candidates (MSP119, GLURP and MSP3) (Nebie et al., 2008).

Merozoite surface appears as a thick fibrillar coat which is composed of integral and peripheral membrane proteins known as the merozoites surface proteins (MSPs) (Kadekoppala et al., 2010). MSPs interactions with the RBC is weak and reversible. But the interactions along with the proteins of the apical organelles, mediate a stronger

(irreversible) interactions which helps in the development of the moving junction. The basic structure of the MSPs have an eight membrane-bound proteins, with a glycosylphosphatidyl inositol (GPI) anchor for attachment, along with many peripheral membrane proteins which interacts with the integral membrane proteins. The peripheral membrane proteins can be divided in two groups: those that are tightly associated with an integral membrane protein and those that are largely soluble proteins of the parasitophorous vacuole and also interact with MSPs. The membrane-bound merozoite surface protein, which are GPI-anchored, include MSP1, -2, -4, -5, -10, Pf12, Pf38 and Pf92 (Sanders et al., 2005 and Gilson et al., 2006). Whereas the peripheral proteins include MSP3, -6 and -7. A non-covalent complex of three proteins- MSP1, MSP6 and MSP7 is the most abundant surface proteins on the surface of merozoite. In this complex, MSP1 is present as four polypeptide fragments – an 83 kDa N-terminal fragment ($MSP1_{83}$), two internal fragments of 30 and 38 kDa ($MSP1_{30}$ and $MSP1_{38}$) and a C-terminal 42 kDa fragment ($MSP1_{42}$) that carries the GPI anchor. Two polypeptides in the complex, $MSP6_{36}$ and $MSP7_{22}$, are derived from MSP6 and MSP7, respectively. MSP7 were first identified as 22 kDa polypeptide from the supernatants of cultured *P. falciparum* (Stafford et al., 1996, Pachebat et al., 2001 and Trucco et al., 2001).

The msp7 transcript in *P. falciparum* is detected from the onset of schizogony and the timing of msp7 transcription coincides with that of msp1 (Le Roch et al., 2001 and Pachebat et al., 2007). The 351-amino acid sequence of *P. falciparum* MSP7 can be divided into an N-terminal signal sequence, followed by an acidic region and a C-terminus sequence, which is a region of high sequence similarity. MSP7 is synthesised as a 48-kDa precursor that binds to MSP1 after its recruitment into the secretory pathway (Pachebat et al., 2007). MSP7 and MSP1 interacts in a pre-Golgi compartment

and this association is necessary for its trafficking to the surface of the merozoite. PfMSP7-1 undergoes at least two proteolytic processing events (Harris et al., 2005) starting with the removal of the signal peptide. Following this, the first proteolytic cleavage occurs and a N- terminal 20 kDa fragment is released ($MSP7_{20}$). While a 33 kDa fragment ($MSP7_{33}$) remains associated with the MSP1 precursor. $MSP7_{33}$ is cleaved again to give a 22-kDa C-terminal fragment ($MSP7_{22}$). The second cleavage occurs at the end of schizogony as the merozoites are released from the liver and coincides with the primary processing of MSP1 (Kadekoppala, 2010).

Apicomplexan parasites contains a secretory membrane bound organelle known as, Micronemes. These micronemes have molecules required for adhesion and locomotion on the host cells. During the host cell invasion, these molecules are presented or secreted on the apical surface (Dubremetz et al., 1998). Malarial parasites are fertilized in the midgut lumen of the mosquito vector and develop into a motile form, the ookinete. Ookinetes invade into and pass through the midgut epithelial cells to arrive at the basal lamina, where they develop into oocysts (Yuda et al., 2001). Micronemal proteins of apicomplexan parasites include soluble and transmembrane proteins. The transmembrane proteins can be divided into three parts, a single transmembrane domain, a large extracellular region and a short cytoplasmic tail and belong to the thrombospondin-related protein family members (Naitza et al., 1998). These proteins help in the locomotion of the parasites and are necessary to invade the host cell (Sultan et al., 1997 and Yuda et al., 1999). Soluble microneme proteins, also involved in the invasion of the host cell are present in the spore forming, coccidian parasites (Eschenbacher et al., 1993, Fourmaux et al., 1996 and Tomley et al., 1996). One of the soluble micronemal protein is von Willebrand Factor A Domain-related Protein or WARP. WARP has a von Willebrand factor type A module-like domain that is involved

in cell–cell interactions, cell–matrix interactions, and matrix formation in several proteins including von Willebrand factor, the alpha chains of integrins, and some types of collagen (Colombatti et al., 1993 and Thera et al., 2010).

AMA1 and MSP7 are vaccine candidates and AMA1 has been studied extensively for its ability to provide protection against malaria (Thera et al., 2010 and Garzón-Ospina et al., 2012). Thera et al., 2010 used FMP2.1 which is a recombinant protein (FMP2.1) based on apical membrane antigen 1 (AMA1) from the 3D7 clone as a vaccine. FMP2.1 is a lyophilized preparation of the ectodomain of the 3D7 clone of *P. falciparum* AMA1. One hundred children aged 1 to 6 years were used as test subject and observed the magnitude and duration of antibody responses to FMP2.1. Post immunization, the children in this trial had 100-fold increase, compared to a 5- to 6-fold rise in the adult trial and the levels were sustained for 1 year after the first dose was given. Another group (Bueno et al., 2009), observed that in naturally infected individuals the dendritic cell differentiation is impaired and antigen-presenting molecules (CD1a, HLAABC and HLA-DR), accessory molecules (CD80, CD86 and CD40) are down modulated, which resembles the effect of parasitized erythrocytes and hemozoin. They also observed that upon treatment of dendritic cells with recombinant AMA1 the antigen presenting molecules were upregulated and reached to a level close to those displayed by healthy control individuals. Also, Pv-AMA1 elicited a pro-inflammatory cytokine profile that was observed after stimulation of PBMCs from malaria-infected subjects. However, observation of Ouattara et al., 2010 were different. They used the malaria vaccine AMA1-C1, a bivalent vaccine comprised of recombinant AMA1 based on the 3D7 and expressed in *Pichia pastoris*. The phase 1 studies of AMA1-C1 in Malian adults and children showed that it was acceptably safe and tolerable and modestly immunogenic but Phase 2 clinical trial of AMA1-C1 vaccine in Malian children showed no impact of

vaccination on parasite density or clinical malaria. Later, Tomaz et al., 2015 also reported that those individuals who never had malaria had a low IgG antibody response against AMA1.

WARP has also been studied as a vaccine candidate. To investigate possible roles that WARP may play in ookinete invasion and differentiation into oocysts, (Abraham et al., 2004) fed mosquitos on an infected mouse which was passively immunized with anti-WARP antibodies. They observed that WARP antibody markedly reduced (69.5–92%) the oocyst formation, whereas pre-immune sera had little or no effect (0–29.6%).

This chapter mentions the cloning, expression and purification of *P. vivax* AMA1, MSP7 and WARP.

MATERIALS AND METHODS:

BACTERIAL STRAINS USED IN STUDY

E. coli DH5α: Genotype F^- ϕ80dlacZ ΔM15 (lacZYA-argF) U169 recA1 endA1hsdR17 (rk,mk+) phoAsupE44λ -thi-1 gyrA96 relA1. It is a RecA- and endA1 strain, which makes it highly transformable and doesn't produce endonuclease1, and also provides blue-white screening by alpha complementation and also allows maintenance of recombinant plasmids.

E. coli BL21(DE3): Genotype F^- ompT gal dcm lon $hsdS_B$ (r_B^- m_B^-) λ (DE3 [lacI lacUV5T7 gene1 ind1 sam7 nin5]). These strains are deficient in both lon and ompT proteases, resulting in a higher level of intact recombinant proteins. These strains are lysogens of bacteriophage DE3, a lambda derivative containing the gene for T7 RNA polymerase under control of the lacUV5 promotor. Induction with IPTG allows production of T7 RNA Polymerase, which then directs the expression of the target gene located downstream of the T7 promotor in the expression vector

CULTURE MAINTENANCE

E.coli DH5α and *E. coli* BL21(DE3) strains were maintained on Luria agar plates. *E.coli* DH5α bearing pJET1.2 was maintained on Luria agar plates containing 100µg/mL of ampicillin, as the vector has an ampicillin resistance gene. *E.coli* DH5α and E. coli BL21DE3 bearing pET30a (+) was maintained on Luria agar plates containing 40µg/mL of kanamycin, as the vector has a kanamycin resistance gene. Also the cultures were maintained at -20°C as glycerol stocks using 20% glycerol in distilled water

MEDIA AND ANTIBIOTICS

Table 2. 1 Media composition

S. No.	Name
1.	Luria agar (2%)
2.	Luria Bertani (2%)

ANTIBIOTICS

Table 2. 2 Antibiotics used for the study

S. No.	**Name**	**Stocks (mg/ml)**	**Final conc. in media (µg/ml)**
1.	Ampicillin	100	100
2.	Kanamycin	50	50

ETHICS STATEMENT AND BLOOD SAMPLE COLLECTION

All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. *P. vivax* infected blood sample was provided by the university health centre. This sample was

collected in EDTA coated vials (5 ml). Genomic DNA was isolated by the following method.

GENOMIC DNA ISOLATION

5 ml of blood was layered on equal volume of Histopaque 1077 (Sigma, USA) centrifuged at 800xg for 20 minutes, at room temperature. The upper layer except the RBCs was removed and discarded using a Pasteur pipette. 5ml of RBC lysis buffer (Sigma, USA) was added to the pelleted RBCs. It was maintained for 90 seconds at 37°C followed by centrifugation at 6000xg for 5minutes. The supernatant was removed and 125µl lysis buffer and 375µl distilled water was added to the pellet and incubated at 37°C for 3 hours. Post incubation, 100µl distilled water and 200µl phenol was added, mixed and centrifuged at 2000xg for 8 minutes. The supernatant was transferred carefully to a fresh centrifuge tube and 200µl of chloroform and 10µl of RNase was added and kept at 65°C for 30 minutes. 1/10 volume of sodium acetate and 2.5 volume of ethanol was added and the tube was left overnight at -20°C for precipitation. The precipitate containing the DNA was centrifuged at 200 g for 30 minutes at 4°C and washed once with 70% ethanol. The pellet was dried and gently resuspended in 50µl distilled water.

REAGENTS AND COMPOSITION FOR GENOMIC DNA ISOLATION

Lysis buffer:

- 40 mMTris–HCl (pH 8.0)
- 80 mM EDTA (pH 8.0)
- 2% SDS
- Proteinase K (Sigma- 20ng/ml)

Equilibrated Phenol (pH 7.0), Chloroform, RNase (Banglore Genei, 1.5mg/ml), 3 M sodium acetate (pH 5.0), Absolute ethanol and 70% ethanol.

AGAROSE GEL ELECTROPHORESIS

The genomic DNA isolated was analysed by electrophoresis in 1% agarose gel in 0.5X TBE followed by staining with (Ethidium Bromide) EtBr.

COMPOSITION OF TBE (5X): FOR 1 L SOLUTION

Tris 54 g

Boric acid 27.5 g

EDTA (0.5 M) 20 ml

Isolated gDNA quality was analysed in UV and quantified using a nano spectrophotomer. PCR amplification of the genes AMA1, MSP7 and WARP from the gDNA was performed using specific primers listed in table 2.3. The PCR was performed using a proof reading polymerase, Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Massachusetts, United States). These primers contained restriction enzyme sites for cloning and expression in the prokaryotic host. The PCR product was purified from an agarose gel using XcelGen DNA Gel/ PCR Purification Kit (Xcelris, INDIA) and cloned in pJET1.2 cloning vector using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Massachusetts, United States). The resulted recombinant plasmid was transformed into *E. coli* DH5α by heat shock. The transformants were plated on LB agar medium, containing ampicillin (100 µg/ml). The vector pJET1.2 contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Re-circularized pJET1.2/blunt vector molecules lacking an insert express a lethal restriction enzyme, which kills the host *E. coli* cell after transformation. Then, the recombinant pJET1.2 plasmid was isolated from the

putative clones using XcelGen Plasmid Isolation Kit (Xcelris, INDIA).Confirmation of the insert in the plasmid was done using restriction digestion as each primer used, has a distinct restriction enzyme site. Insert released were then ligated to pET30a (+) and transformed into *E. coli* DH5α. Plasmids were isolated from the putative clones obtained and confirmed by restriction digestion and PCR. Once confirmed the recombinant pET30a (+) was transformed into *E. coli* BL21 (DE3), followed by isolation of plasmid and confirmation of the insert as explained above. The positive clones were then sent for sequencing.

Table 2. 3 Primer sequences of AMA1, MSP7 and WARP used for cloning.

Primer name	**Primer sequence**	**GeneDB**
PvAMA1 *Nde I*	5′-CCATGGGGCCTACCGTTGAGAGAA-3′	PVX_092275
PvAMA1 *XhoI*	5′-CTCGAGTCATAGTAGCATCTGCTTGTT-3’	PVX_092275
PvMSP-7 *Bam*H I	5’-GGATCCATGAAAACAAAAGTACTAT-3’	PVX_082700
PvMSP-7 *XhoI*	5’- CTCGAGGTAGTAAAACATCGGGTT-3’	PVX_082700
Pv- WARP *Bam*H I	5’- GTGGGATCCAAAATAAACCTTGTGTCGC-3’	PVX_ 093675
Pv- WARP *HindIII*	5’-ACCCAAGCTTTCAGTCCGTAGAGTCGCTGTC-3’	PVX_ 093675

Table 2. 4 PCR system used for amplification of AMA1, MSP7 and WARP.

Component	**Volume (µl)**
Autoclaved MilliQ water (AMQ)	18
DNA	1
10X Buffer	2.5
10 mM dNTPs	0.5
Forward Primer (10 mM)	1.25
Reverse Primer (10 mM)	1.25
Taq Polymerase (2.0U/ µl)	0.5
Total	25

Table 2. 5 PCR conditions used for amplification of (i) AMA1, (ii) MSP7 and (iii) WARP gene.

Steps		Temparature (ºC)	Time	No. of cycles
1.	Pre-cycle denaturation	98	30 seconds	
2.	Denaturation	98	10 seconds	
3.	Primer annealing	47	30 seconds	34
4.	Primer extension	72	30 seconds	
5.	Post-cycle elongation	72	10 minutes	

(i)

Steps		Temparature (ºC)	Time	No. of cycles
1.	Pre-cycle denaturation	98	30 seconds	
2.	Denaturation	98	10 seconds	
3.	Primer annealing	44	30 seconds	34
4.	Primer extension	72	30 seconds	
5.	Post-cycle elongation	72	10 minutes	

(ii)

Steps		Temparature (ºC)	Time	No. of cycles
1.	Pre-cycle denaturation	98	30 seconds	
2.	Denaturation	98	10 seconds	
3.	Primer annealing	56	30 seconds	34
4.	Primer extension	72	30 seconds	
5.	Post-cycle elongation	72	10 minutes	

(iii)

COMPETENT CELLS PREPARATION

The protocol from Sambrook et al., 1989 was modified and was used for preparation of competent *E. coli* with an efficiency of ~10^6 transformed colonies/µg of plasmid DNA. The reagents used in the preparation of competent cells are mentioned in table 2.6.

Table 2. 6 Reagents and composition for competent cell preparation.

S. No	Name
1.	0.1 M $CaCl_2$
2.	0.1 M $CaCl_2$ in 20 % glycerol.

A single bacterial colony [DH5α/BL21 (DE3)] was inoculated in 5ml LB and the culture was incubated overnight at 37°C with vigorous shaking. 1ml of overnight grown culture was transferred into 100 ml LB and incubated at 37°C with vigorous shaking till OD600nm reached 0.3-0.4. The bacterial cells were transferred to sterile, 30 ml oak ridge tubes (Tarsons, INDIA). The cells were pelleted down by centrifuging at 3500rpm for 5 minutes at 4°C. The medium was decanted from cell pellet, in sterile conditions. Pellet was resuspended by adding 10 ml of ice-cold 0.1 M $CaCl_2$ solution. The cells were centrifuged at 3000rpm for 5minutes at 4°C and the supernatant was discarded and this step was repeated thrice. Pellet was finally resuspended in 1ml of 20% glycerol in 0.1 M $CaCl_2$. 100 µl of the competent cells were used for each transformation. Rest of the cells were stored at -80°C. For all ligation systems the vector to insert molar ratio was 1:3.

Table 2. 7 Ligation system of AMA1 (PCR product) in pJET 1.2.

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	4
2.	pJET1.2/blunt Cloning Vector (50 ng/µl)	1.0
3.	AMA1 (30 ng/ µl)	1.5
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	2X ligation buffer	7.5
6.	Total	15

Table 2. 8 Ligation system of AMA1 in pET30a (+)

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	7.5
2.	pET30 a(+) (50 ng/µl)	2.0
3.	AMA1 (30ng/µl)	3.0
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	10x ligation buffer	1.5
6.	Total	15

Table 2. 9 Ligation system of MSP7 PCR product with pJET1.2 vector.

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	1.5
2.	pJET1.2/blunt Cloning Vector (50 ng/µl)	1.0
3.	MSP7 (15ng/µl)	4.0
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	2X ligation buffer	7.5
6.	Total	15

Table 2. 10 Ligation system of MSP7 in pET30a (+).

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	5.9
2.	pET30 a(+) (50 ng/µl)	2.0
3.	MSP7 (15ng/µl)	4.6
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	10x ligation buffer	1.5
6.	Total	15

Table 2. 11 Ligation system of WARP PCR product with pJET1.2.

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	4.0
2.	pJET1.2/blunt Cloning Vector (50 ng/µl)	1.0
3.	MSP7 (15ng/µl)	2.5
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	2X ligation buffer	7.5
6.	Total	15

Table 2. 12 Ligation system of WARP in pET30a (+)

S. No	Components	Volume (µl)
1.	Autoclaved MilliQ water	8.9
2.	pET30 a(+) (50 ng/µl)	2.0
3.	MSP7 (15ng/µl)	1.6
4.	T4 DNA ligase (5weiss/µl)	1.0
5.	10x ligation buffer	1.5
6.	Total	15

TRANSFORMATION

The protocol from Sambrook et al., 1989 was modified and was used for transformation of an *E. coli* culture with a particular plasmid. 100µl competent cells were used and the ligation system was added to the tube and the contents were mixed gently. The tube was maintained in ice for 30 min. Following the incubation the tube was then transferred to a 42°C dry bath and incubated for 90 seconds without shaking. The tube was rapidly transferred to an ice-bath to chill for 2 minutes. 900µl LB was added to the tube and incubated for 45 minutes at 37°C, on a shaker. The tube was centrifuged at 10,000rpm for 1 minute and the supernatant was discarded by inverting the tube under sterile conditions. The pellet was resuspended in the remaining supernatant of the tube

and plated on the pre-warmed LB plates containing antibiotics. The plates were incubated at 37°C for 16-18 hours.

PLASMID ISOLATION BY ALKALINE LYSIS METHOD

Table 2. 13 Composition of reagents for plasmid isolation by alkaline lysis.

S. No.	Reagent	Composition
1.	PI	Tris-Cl 25mM (pH 8.0), EDTA 10 mM (pH 8.0), 200µg/ml lysozyme (freshly prepared from 500 mg/ml stock)
2.	PII	0.2 N NaOH (freshly diluted from 10 N stock), 1% SDS (from 20% stock)
3.	PIII	3M Potassium acetate, pH 5.5 (adjusted by glacial acetic acid)

A single colony of transformed bacteria was inoculated in 5ml of LB with 5µl of antibiotic. The culture was incubated overnight at 37°C with vigorous shaking. Following day the culture was centrifuged at 13,000 rpm for 1 minute and supernatant was discarded and then the pellet was resuspended in 250µl of ice-cold P-I solution followed by incubation at 37°C for 20 minutes. 250 µl freshly prepared P-II solution was added & and the contents of the tube were mixed by inverting and incubated for 5 minutes (till it becomes transparent), 350µl of PIII solution was added, followed by incubation in ice for 10 minutes. The tubes were centrifuged at 4°C for 10 minutes at 10,000 rpm and the supernatant was transferred to a fresh microfuge tube and 500µl of chloroform-isopropanol (24:1) was added and incubated on a vortex mixer for 15 seconds. The remaining supernatant was transferred carefully to a fresh microfuge tube and 0.6 volumes of isopropanol was added. The tube was incubated at -20°C for minimum 45 minutes and centrifuged at 13,000 rpm for 10 minutes and the supernatant was carefully discarded. 1 ml of 70% alcohol was added & inverted gently 2 times and the mixture was centrifuged for 10 minutes at 13,000rpm. Supernatant was discarded

and the pellet was dried at 37°C and the dried pellet was re-suspended in 50µl of autoclaved distilled water. 1µl RNase (stock 1.5mg/ml) was added and kept at 65°C in water bath for 30 minutes for DNA to dissolve and remove all RNA contaminations, if any. The efficiency of plasmid preparation was observed on 0.8% agarose gel by gel electrophoresis.

RESTRICTION DIGESTION

For all the restriction digestions 1µg of DNA (final concentration) was used and the systems were kept at 37ºC for 2 hours and then at 70ºC for 5 minutes to inactivate the enzymes. Post incubation the entire system of 50 µl was run on a 0.8% agarose gel.

Table 2. 14 Restriction digestion system used for AMA1/pJET1.2.

S.No.	Reagent	Volume (clone 1) (µl)	Volume (clone 2) (µl)
1.	Plasmid (DNA) (1µg)	1.0	3.0
2.	10X buffer	5.0	5.0
3.	10X BSA	5.0	5.0
4.	*Nde I* (10U/µl)	1.0	1.0
5.	*Xho I* (10U/µl)	1.0	1.0
6.	Autoclaved MilliQ water	37.0	35.0
7.	Total	50.0	50.0

Table 2. 15 Restriction digestion system of AMA1/pET 30a (+) in Dh5α.

S.No.	Reagent	Volume (µl)
1.	Plasmid (DNA) (1µg)	1.0
2.	10X buffer	5.0
3.	10X BSA	5.0
4.	*Nde* I (10U/µl)	1.0
5.	*Xho* I (10U/µl)	1.0
6.	Autoclaved MilliQ water	37.0
7.	Total	50.0

Table 2. 16 Restriction digestion system for AMA1/pET30a (+) in BL21 (DE3).

S.No.	Reagent	Volume (clone 1) (µl)	Volume (clone 2) (µl)
1.	Plasmid (DNA) (1µg)	2.0	10.0
2.	10X buffer	5.0	5.0
3.	10X BSA	5.0	5.0
4.	*Nde* I (10U/µl)	1.0	1.0
5.	*Xho* I (10U/µl)	1.0	1.0
6.	Autoclaved MilliQ water	36.0	28.0
7.	Total	50.0	50.0

Table 2. 17 Restriction digestion system for MSP7 /pJET 1.2

S.No.	Reagent	Volume (µl)
1.	Plasmid (DNA) (1µg)	2.0
2.	10X buffer	5.0
3.	10X BSA	5.0
4.	*Bam*H I (10U/µl)	1.0
5.	*XhoI* (10U/µl)	1.0
6.	Autoclaved MilliQ water	36.0
7.	Total	50.0

Table 2. 18 Restriction digestion system for MSP7/pET30a (+)Dh5α.

S.No.	Reagent	Volume (µl)
1.	Plasmid (DNA) (1µg)	1.0
2.	10X buffer	5.0
3.	10X BSA	5.0
4.	*Bam*H I (10U/µl)	1.0
5.	*XhoI* (10U/µl)	1.0
6.	Autoclaved MilliQ water	37.0
7.	Total	50.0

Table 2. 19 Restriction digestion system for MSP7/pET 30a BL21 (DE3).

S.No.	Reagent	Volume (clone 1) (µl)	Volume (clone 2) (µl)
1.	Plasmid (DNA) (1µg)	6.0	2.0
2.	10X buffer	5.0	5.0
3.	10X BSA	5.0	5.0
4.	*Bam*H I (10U/µl)	1.0	1.0
5.	*XhoI* (10U/µl)	1.0	1.0
6.	Autoclaved MilliQ water	32.0	36.0
7.	Total	50.0	50.0

Table 2. 20 Restriction digestion system for WARP/pJET1.2**.**

S.No.	Reagent	Volume (µl)
1.	Plasmid (DNA) (1µg)	3.0
2.	10X buffer	5.0
3.	10X BSA	5.0
4.	*Bam*H I (10U/µl)	1.0
5.	*HindIII* (10U/µl)	1.0
6.	Autoclaved MilliQ water	35.0
7.	Total	50.0

Table 2. 21 Restriction digestion system for WARP/pET30a (+) Dh5α.

S.No.	Reagent	Volume (µl)
1.	Plasmid (DNA) (1µg)	10.0
2.	10X buffer	5.0
3.	10X BSA	5.0
4.	*Bam*H I (10U/µl)	1.0
5.	*HindIII* (10U/µl)	1.0
6.	Autoclaved MilliQ water	28.0
7.	Total	50.0

Table 2. 22 Restriction digestion system WARP/pET 30a (+) in BL21 (DE3).

S.No.	Reagent	Volume (clone 1) (µl)	Volume (clone 2) (µl)
1.	Plasmid (DNA) (1µg)	15.0	12.0
2.	10X buffer	5.0	5.0
3.	10X BSA	5.0	5.0
4.	*Bam*H I (10U/µl)	1.0	1.0
5.	*HindIII* (10U/µl)	1.0	1.0
6.	Autoclaved MilliQ water	23.0	26.0
7.	Total	50.0	50.0

EXPRESSION ANALYSIS

A single positive clone for each antigen were considered for expression. Clones were cultured in LB medium containing 40 µg/ml kanamycin at 37℃ with shaking for 3 hours to reach OD 0.4, then 1 mM of IPTG, as a gene expression inducer, was added to the culture and the culture was continued for another 3 hour. After incubation, the culture was centrifuged and the cell mass was resuspended in Laemmli sample buffer. The bacterial lysate were then run on a 10% SDS-Polyacrylamide gel electrophoresis. Gel was stained with coomassie blue. Clone induced without IPTG and an empty vector

was used as a negative and vector control respectively. Upon destaining of the gel, an overexpressed protein was observed in the clones induced with IPTG.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents for SDS-PAGE:

- Tris-Cl, pH 8.8, 1.5 M (for resolving gel)
- Tris-Cl, pH 6.8, 1.0 M (for stacking gel)
- 30% gel stock – {29g acrylamide + 1g bis acrylamide }
- 10% Ammonium persulphate (APS) – stored in dark at 4°C
- N,N,N,N' tetra ethylene diamine (TEMED)

5X Sample Buffer

- Tris-Cl (pH 6.8) 250 mM
- SDS 10%
- Glycerol 50%
- Bromophenol Blue 0.5%
- β-mercaptophenol 500mM

1X Tank Buffer

- Tris base 25mM
- Glycine 250mM
- SDS 0.1%

Table 2. 23 Components and their composition used for 10% resolving gel.

S.No.	Component	Volume
1.	Distilled water	1.9 ml
2.	30% Gel stock	1.7ml
3.	1.5 M Tris-Cl (8.8)	1.3ml
4.	10% SDS	50 µl
5.	10% APS	50 µl
6.	TEMED	7.5 µl

Table 2. 24 Components and their compositions used for 5% stacking gel.

S.No.	Component	Volume
1.	Distilled water	1.4 ml
2.	30% Gel stock	330 µl
3.	1 M Tris-Cl (8.8)	250 µl
4.	10% SDS	20 µl
5.	10% APS	20 µl
6.	TEMED	5 µl

The sample was loaded in the gel and run at 50V till the sample reaches the end of stacking gel and the moment the sample enters resolving gel the voltage is increased to 100V. SDS-PAGE gels were then subjected to either coomassie blue staining or silver staining as explained below.

COOMASSIE BRILLIANT BLUE STAINING

The gels were incubated with 0.25% coomassie brilliant blue R 250 (Fluka) prepared in 30% methanol and 10% acetic acid for 1 hour. Post incubation the coomassie was

recollected for reuse and the gels were repeatedly washed with destaining solution (30% methanol and 10% acetic acid) till the bands are distinctly visible.

SILVER STAINING

The gel was kept in the fixative solution (30% methanol and 10% acetic acid) for 30 minutes. A wash was given with 5% methanol for 10 minutes. Following this three washes were given with distilled water for 5minutes each. 50ml of 0.02% sodium thiosulphate was added for 2minutes. Again three washes were given with distilled water for 1minute each. Then 250µl of 20% $AgNO_3$ solution was added to 50ml of distilled water to the gel and left for 20-30 minutes. A quick wash was given with distilled water for 15-20 seconds. The chilled developing solution was added to the gel till the bands appeared. When the protein bands were stained the developing solution was removed and 1% acetic acid was added to stop the reaction.

Reagents used for silver staining method is as follows:

Table 2. 25 Components and their compositions used for silver staining**.**

S.No.	Reagent	Composition (%)
1.	Methanol (100%)	5
2.	Sodium carbonate	2.19
3.	Sodium thiosulphate	0.02
4.	Silver nitrate (20 %)	0.2
5.	Acetic acid (100%)	1
6.	Formaldehyde	0.46

DEVELOPING SOLUTION

1ml of 0.02% sodium thiosulphate was added to 90ml of 2.19% sodium carbonate and kept in a refrigerator to chill. 125 µl formaldehyde was added and the volume was made up to 100 ml before use.

Ni-AFFINITY CHROMATOGRAPHY

The cell pellet harvested after the IPTG induction was mixed and resuspended in 15 ml of the sonication buffer and was lysed during sonication cycles consisting of 9 sec burst at high intensity and 9 sec cooling period on ice for 10 minutes.. The supernatant was collected in a syringe and passed through a 0.4 µm filter assembly onto the activated column and kept on a rocker at 4ºC for 30 minutes. The column was then removed from the rocker and allowed to stand for 20 minutes. After this a fraction was collected which was labelled as unbound fraction. Then the column was washed with 50ml wash buffer. 1ml of elution buffer was added and allowed to stand for 15 minutes. 500 µl fractions were collected and labelled as elution fraction 1 and 2 respectively. This step was repeated again and thereby elution fraction 3 and 4 were collected.

COLUMN WASHING AND STORAGE

After elution was carried out, in order to remove any residual protein that was left bound to the column, lysis buffer (15 ml) was passed through the column followed by 50 ml of distilled water. The column was then incubated with 0.5N NaOH for 30 minutes to remove any cell debris. After 30 minutes of incubation the 0.5N NaOH was passed through the column followed by 50 ml of sterile distilled. Columns were stored in 20% ethanol at 4ºC. The purification samples were proceeded for observation by SDS PAGE. To remove salts and detergents from the purified samples they were subjected to chloroform methanol extraction.

REAGENTS FOR Ni-AFFINITY CHROMATOGRAPHY

Nickel affinity chromatography is based on the principle that the histidine residues present in the protein binds to the nickel beads present in the chromatography column. The reagents used for the chromatography are as follows.

Table 2. 26 Composition of lysis buffer 1 used for the solubilisation.

S.No.	Reagent	Volume
1.	NaCl (3M)	1.5 ml
2.	Tris-Cl (1M), pH 8.0	750 µl
3.	Imidazole (2M)	75 µl
4.	PMSF (100mM)	150 µl
5.	Sarcosine	0.15 g
6.	Triton X	75 µl
7.	Triethanolamine	52 µl
Make the volume upto 15 ml with AMQ		

Table 2. 27 Composition of lysis buffer 2 used to equilibrate the column.

S.No.	Reagent	Volume
1.	NaCl (3M)	5 ml
2.	Tris-Cl (1M), pH 8.0	2.5 ml
Make up the volume upto 50ml with AMQ		

Table 2. 28 Composition of wash buffer used to remove the unbound proteins present in the column.

S.No.	Reagent	Volume
1.	NaCl (3M)	5 ml
2.	Tris-Cl (1M), pH 8.0	2.5 ml
3.	Imidazole (2M)	500 µl
Make up the volume upto 50ml with AMQ		

Table 2. 29 Composition of elution buffer used to elute out the protein that was bound to nickel beads present in the column.

S.No.	Reagent	Volume
1.	NaCl (3M)	500 µl
2.	Tris-Cl (1M), pH 8.0	250 µl
3.	Imidazole (2M)	750 µl
Make up the volume upto 5ml with AMQ		

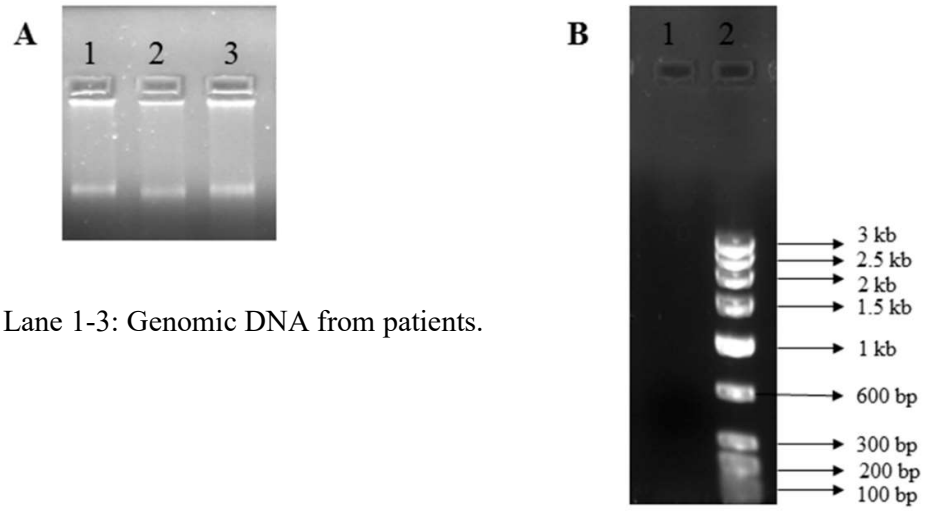
COLUMN PREPARATION

750 µl nickel slurry was added to the column and was allowed to stay till two separate layers were visible. The nob was opened from the bottom and the remaining solution was allowed to pass through. The column was activated by passing 50ml of distilled water followed by 30 ml lysis buffer.

RESULTS

CLONING

All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M.S University of Baroda. Genomic DNA was isolated from patients (Figure 2.1A). PCR amplification of human β2 microglobulin gene, using the isolated DNA as a template, was carried out. No amplification was observed confirming the absence of any human DNA contamination (Figure 2.1B). Specific primers were designed for the cloning of Apical Membrane Antigen-1 (PvAMA1), Merozoite Surface Protein 7 (PvMSP7) and von Willebrand factor A (PvWARP) and are listed in Table 2.31.



Lane 1-3: Genomic DNA from patients.

Lane 1: PCR product of β_2 m from *P. vivax* DNA

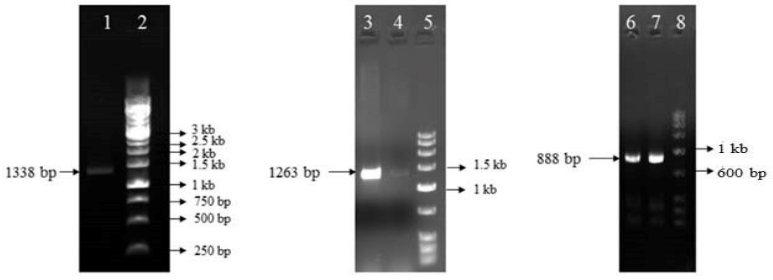
Lane 2: GeNeI™ Low Range DNA Rule

Figure 2. 1 Isolation and analysis of genomic DNA from *P. vivax* infected individuals. Ethidium bromide stained 0.8% agarose gel electrophoresis of (A) Genomic DNA isolated from infected individuals and (B) PCR amplification of human β_2 microglobulin gene from the *P. vivax* genomic DNA (negative control).

Table 2. 30 Primer sequences used for specific amplification of genes from *P. vivax* genomic DNA

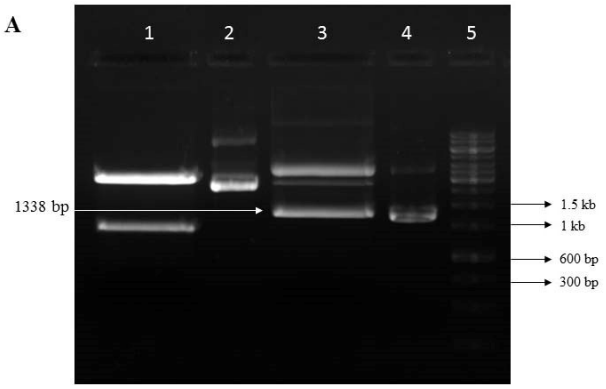
Primer name	Primer sequence	GeneDB
PvAMA1 *Nde I* (Cloning primer)	5′-CCATGGGGCCTACCGTTGAGAGAA-3′	PVX_092275
PvAMA1 *Xho* I (Cloning primer)	5′-CTCGAGTCATAGTAGCATCTGCTTGTT-3’	PVX_092275
PvMSP-7 *Bam*H I (Cloning primer)	5’-GGATCCATGAAAACAAAAGTACTAT-3’	PVX_082700
PvMSP-7 *Xho* I (Cloning primer)	5’- CTCGAGGTAGTAAAACATCGGGTT-3’	PVX_082700
Pv- WARP *Bam*H I (Cloning primer)	5’- GTGGGATCCAAAATAAACCTTGTGTCGC-3’	PVX_093675
Pv- WARP *HindIII* (Cloning primer)	5’-ACCCAAGCTTTCAGTCCGTAGAGTCGCTGTC-3’	PVX_093675

Above mentioned primers were used to amplify the gene segments and PCR products of 1338 bp (AMA1), 1263 bp (MSP-7) and 888 bp (WARP) was observed on a 0.8% agarose gel stained with EtBr (Figure 2.2). The amplified products were gel eluted and purified using DNA Gel/PCR purification kit (Xcelris) so as to avoid any nonspecific bands. ORF coding for PvAMA1, PvMSP7 and PvWARP was cloned into *E. coli* DH5α using the CloneJet cloning kit. The clones of these library were randomly selected to confirm the presence of desired insert. Plasmid was isolated from the colonies obtained using plasmid isolation kit (Xcelris). Plasmids from which release of 1338 bp (AMA1) with *Nde* I and *Xho* I, 888 bp (WARP) with *Bam*H I and *Hind*III and 1263 bp (MSP7) with *Bam*H I and *Xho* I were considered to harbour the desired insert (Figure 2.3). Inserts released were then ligated with pET-30a(+) vector (Invitrogen). Transformation of these ligated products in *E.coli* DH5α yielded more than 100 colonies. Colonies were randomly screened using double restriction digestion and PCR (Figure 2.4). The confirmed constructs were then transformed into expression host *E. coli* BL21 (DE3).

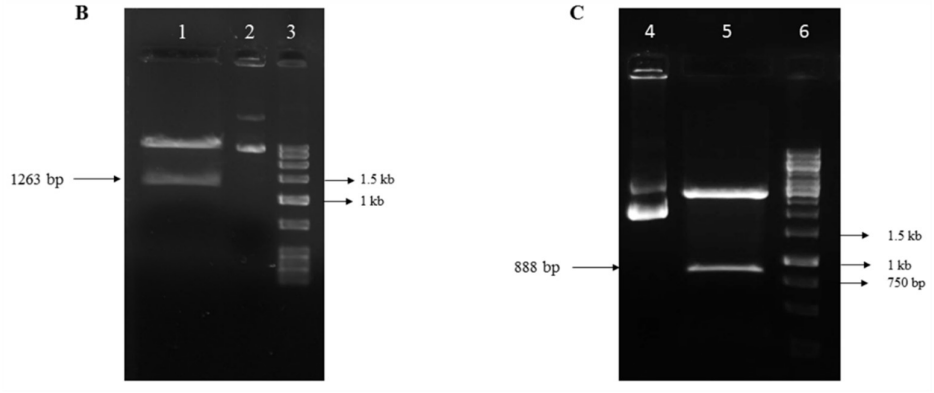


Lane 1: PCR product of AMA1
Lane 2: 1kb DNA ruler (Thermo-scientific)
Lane 3-4: PCR product of MSP7
Lane 5 & 8: GeNeI^{1M} Low Range DNA Rule
Lane 6-7: PCR product of WARP

Figure 2. 2 Analysis of PCR products from *P. vivax* genomic DNA.
Ethidium bromide stained 0.8% agarose gel electrophoresis of (i) AMA-1 (ii) MSP7 and (iii) WARP PCR products

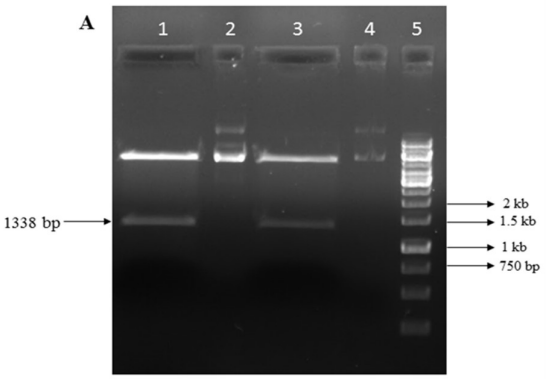


Lane 1: pJET-AMA1/*Nde* I, *Xho* I (Clone-1)
Lane 2: Undigested pJET-AMA1 (Clone-1)
Lane 3: pJET-AMA1/*Nde* I, *Xho* I (Clone-2)
Lane 4: Undigested pJET-AMA1 (Clone-2)
Lane 5: Low Range DNA Rule

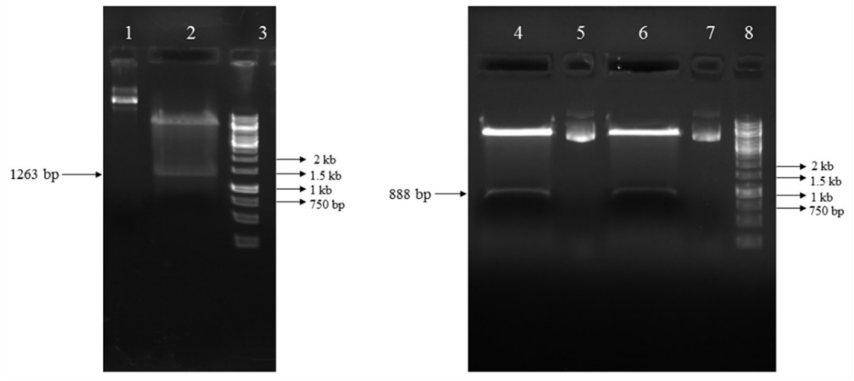


Lane 1: pJET-MSP7/*Bam*H 1, *Xho* I
Lane 2: Undigested pJET-MSP7
Lane 3: Low Range DNA Rule
Lane 4: Undigested pJET-WARP
Lane 5: pJET-WARP/*Bam*H 1, *Hind*III
1kb DNA ruler (Thermo-scientific)

Figure 2. 3 0.8% agarose gel stained with EtBr showing confirmation of AMA1 (A), MSP7 (B) and WARP (C) inserts in pJET1.2 vector by restriction digestion.



Lane 1: pET 30a-AMA1/*Nde* I, *Xho* I (Clone-1)
Lane 2: Undigested pET 30a-AMA1 (Clone-1)
Lane 3: pET 30a-AMA1/*Nde* I, *Xho* I (Clone-2)
Lane 4: Undigested pET 30a-AMA1 (Clone-2)
Lane 5: 1kb DNA ruler (Thermo-scientific)

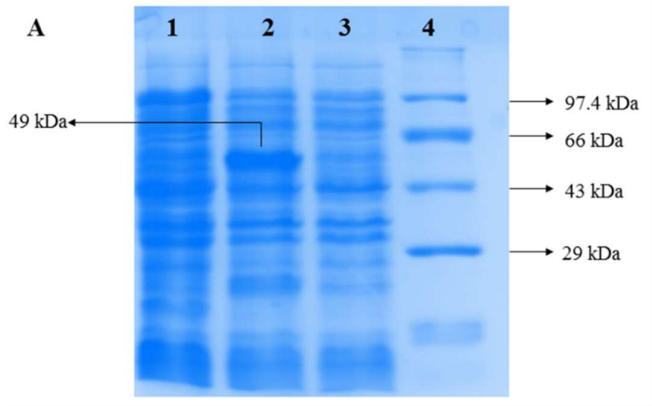


Lane 1: Undigested pET 30a -MSP7
Lane 2: pET 30a -MSP7/*Bam*H I, *Xho* I
Lane 3 & 8: 1kb DNA ruler (Thermo-scientific)
Lane 4: Undigested pET 30a –WARP (Clone 1)
Lane 6: pET 30a -WARP/*Bam*H I, *Hind*III (Clone 2)
Lane 5: Undigested pET 30a –WARP (Clone 2)

Figure 2. 4 0.8% agarose gel stained with EtBr showing confirmation of AMA1 (A), MSP7 (B) and WARP (C) inserts in pET30a (+) vector by restriction digestion.

EXPRESSION AND PURIFICATION

The expression of AMA1, MSP7 and WARP was induced with 0.5mM IPTG at log phase and the cells were harvested after 3 hours. The cells were lysed using laemmli sample buffer and the expression was analysed on SDS-PAGE. Bands corresponding to 49kDa (AMA1), 66kDa (MSP7) and 40 kDa (WARP) was observed in the lane with *E. coli* BL21 (DE3) transformed with the recombinant plasmids (Figure 2.5). *E. coli* BL21 (DE3) transformed with control plasmid pET30a (+) was used as a negative control that contained the similarly induced sample. Hexahistidine tag at the N terminus in the case of MSP7, WARP and C terminus in the case of AMA1 was exploited for their purification. Recombinant His-tagged proteins were selectively adsorbed to HIS-Select® Nickel Affinity Gel (Sigma) and then eluted using high concentration of imidazole. The eluted band corresponding to their molecular weight were distinctly visible on the coomassie and silver stained gels.

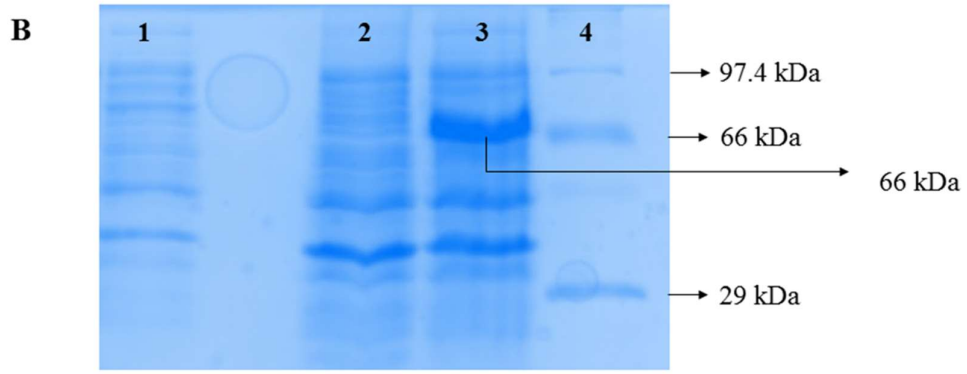


Lane 1: whole cell lysate of pET30a (+)-AMA1

Lane 2: whole cell lysate of induced pET30a (+)-AMA1

Lane 3: whole cell lysate of vector control [pET30a(+)]

Lane 4: High molecular protein marker

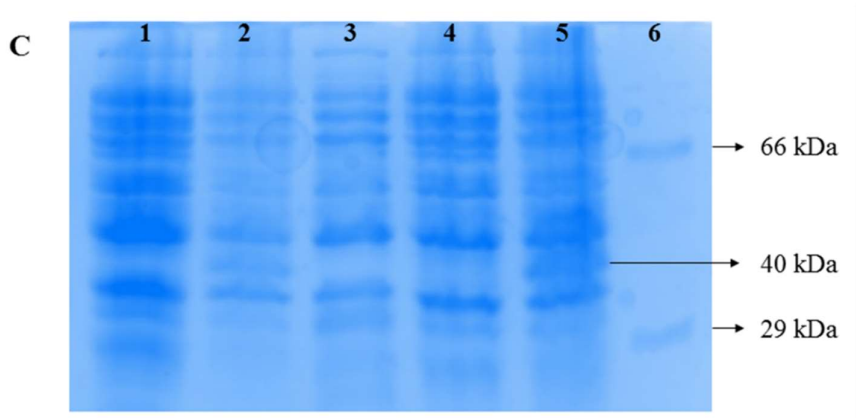


Lane 1: whole cell lysate of vector control [pET30a(+)]

Lane 2: whole cell lysate of pET30a (+)-MSP7

Lane 3: whole cell lysate of induced pET30a (+)-MSP7

Lane 4: High molecular protein marker



Lane 1: whole cell lysate of pET30a (+)-WARP (Clone 1)

Lane 2: whole cell lysate of induced pET30a (+)- WARP (Clone 1)

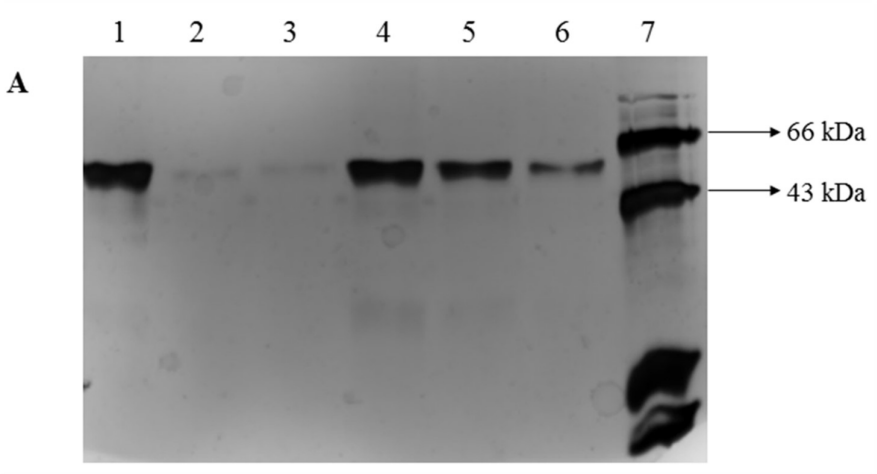
Lane 3 whole cell lysate of vector control [pET30a(+)]

Lane 4: whole cell lysate of pET30a (+)-WARP (Clone 2)

Lane 5: whole cell lysate of induced pET30a (+)- WARP (Clone 2)

Lane 6: High molecular protein marker

Figure 2. 5 Coomassie stained 10% SDS-PAGE with whole cell extracts of *E. coli* BL21 (DE3) transformed with either pET30a(+) or pET30a(+)-AMA-1 (A), pET30a(+)-MSP7 (B) and pET30a(+)-WARP (C)

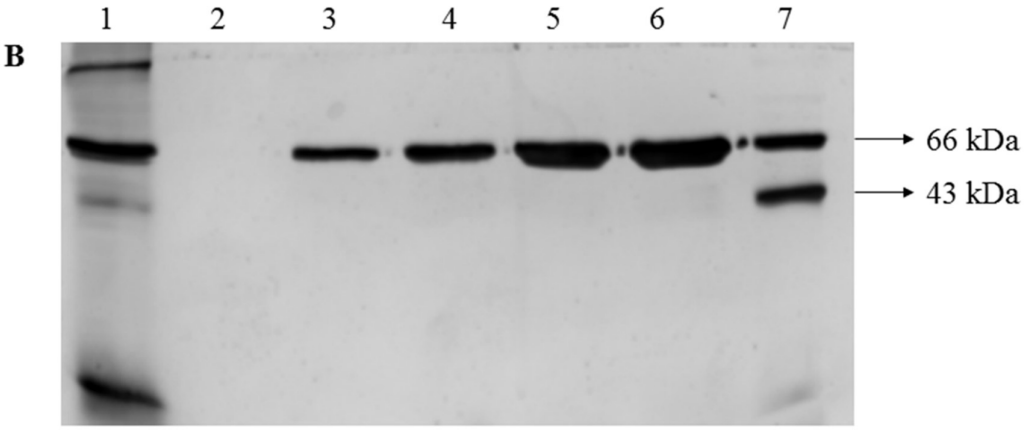


Lane 1: Unbound fraction

Lane 2: Wash fraction

Lane 3-6: Purified AMA1

Lane 6: High molecular protein marker

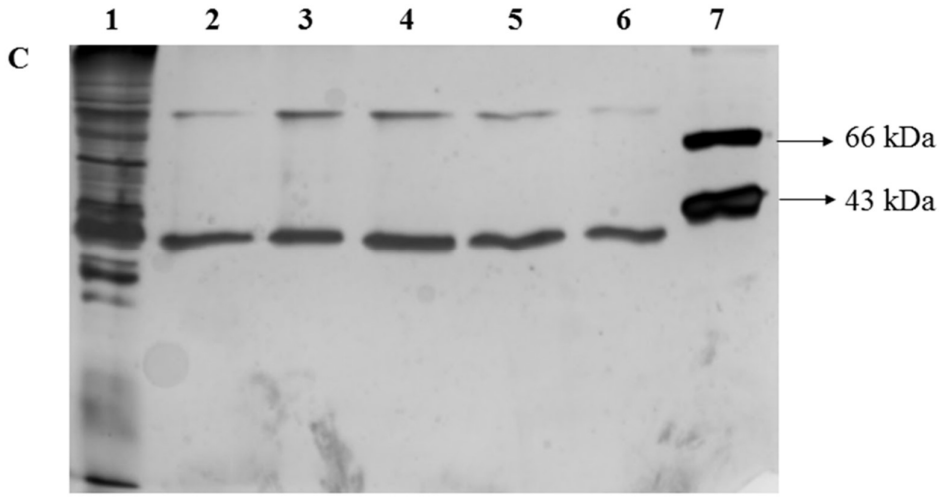


Lane 1: Unbound fraction

Lane 2: Wash fraction

Lane 3-6: Purified MSP7

Lane 6: High molecular protein marker



Lane 1: Unbound fraction Lane 2-6: Purified WARP

Lane 7: High molecular protein marker

Figure 2. 6 Silver stained 10% SDS-PAGE of purified AMA-1, MSP7 and WARP by nickel affinity chromatography

DISCUSSION

Plasmodium vivax AMA1 is encoded by a single copy gene on chromosome 9 while MSP7 and WARP are each encoded by a single copy gene on chromosome 12 and 1 respectively. AMA1 is present in sporozoites and helps to penetrate hepatocytes while MSP7 helps in the attachment of the parasite to RBC. WARP mediates the attachment of ookinete to the mosquito midgut followed by its differentiation to oocyst. All the three genes are present as a single gene without any introns. Thus RNA isolation and preparation of cDNA is not required.

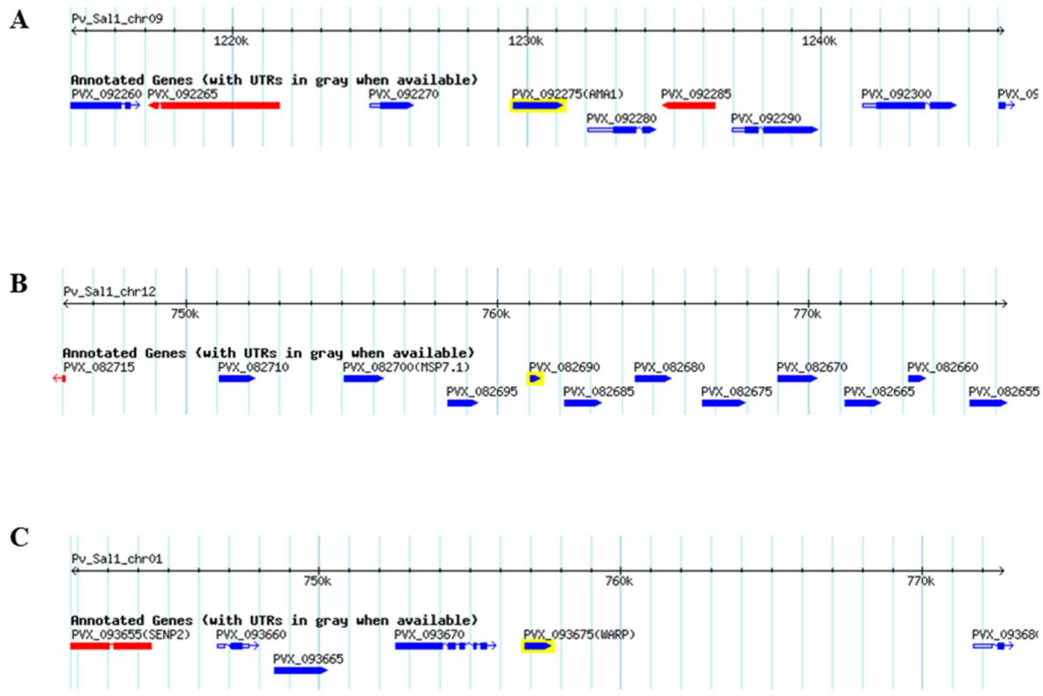


Figure 2. 7 AMA1, MSP7 and WARP gene position in P. vivax genome. All the genes are highlighted with yellow background. (Maps were generated in www.plasmodb.org)

Primers were designed using online tools like BLAST, oligonucleotide calculator and ORF finder. For directional cloning, restriction enzyme sites were incorporated in the flanking regions. Restriction enzyme sites were chosen accordingly, so that they do not occur between the ORFs using an online tool NEB cutter. *Nde* I and *Xho* I were chosen for AMA1, but *Nde* I was present in the reading frame of MSP7 and WARP. So *Bam*H I and *Xho* I was chosen for MSP7 and *Bam*H I and HindIII for WARP (as *Xho* I was present in the reading frame of WARP).

Strategy for cloning included amplification of the complete ORFs of AMA1, MSP7 and WARP by PCR. Phusion DNA polymerase (Thermo Scientific, USA) was used for the amplification as it is a proof reading polymerase. The PCR product obtained were then cloned in pJET1.2 vector followed by subcloning into pET30a (+) vector for protein

expression (Figure 2.8). As suitable restriction sites were not available in the flanking regions of the ORFs, restriction sites were incorporated within the flanking regions of primers. Upon incorporation of these sites in the flanking regions of the PCR products the optimum digestion using restriction enzymes was difficult to achieve. So the PCR products were cloned in a pJET1.2 vector (high copy number and a blunt ended vector). Insert was released from pJET1.2 vector and ligated to pET30a (+) vector.

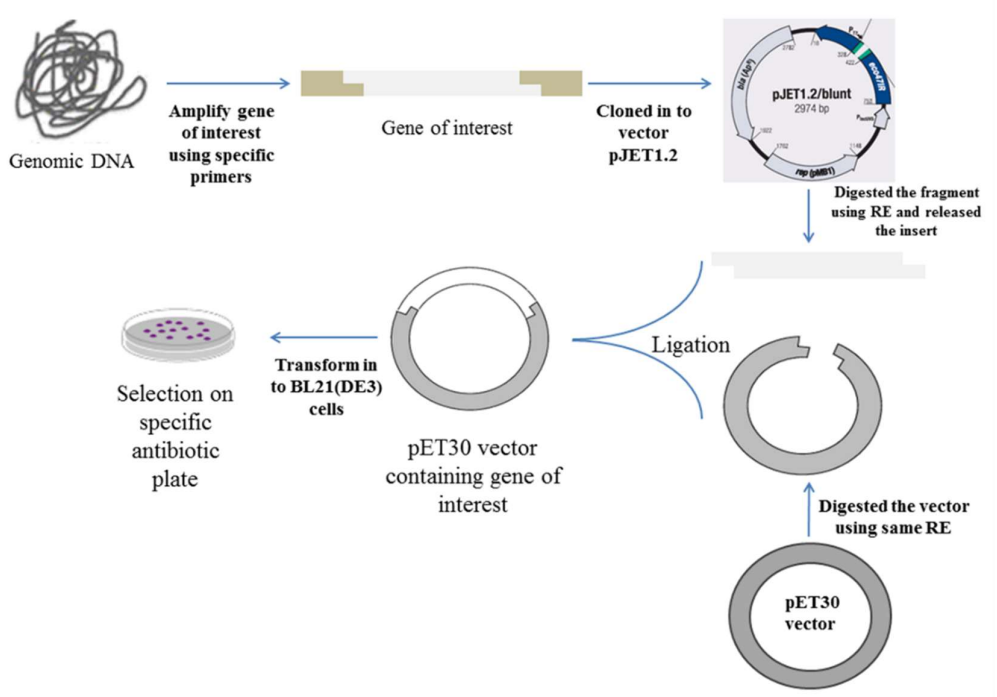


Figure 2. 8 Schematic representation of the cloning strategy.

pET 30a(+) along with BL21(DE3) was used for heterologous expression of the *P. vivax* genes. One of the key features of pET system is the ability to clone target genes under conditions of extremely low transcriptional activity such as the absence of a source of T7 RNA polymerase. The host RNA polymerase does not initiate expression from the T7 promoters so the background expression is minimal in the absence of T7 RNA polymerase. For protein production the recombinant plasmid is transferred to a

host that are lysogens of bacteriophage DE3 and carries a fragment containing the lacI gene, the lacUV5 promoter and the gene for T7 RNA polymerase. This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. The recombinant cultures were induced by the addition of IPTG at log phase. IPTG acts upon the lac promoter on the T7 RNA polymerase gene present in the genomic DNA of BL21 (DE3). This T7 RNA polymerase produced will act on T7 promoter of pET30a (+) and initiate the target gene synthesis. The hexahistidine tag was provided at the C-terminus of AMA1 as the *Nde* I site was used for cloning. *Nde* I site was not used for cloning of MSP7 and WARP gene so the hexahistidine tag was provided at the N-terminus. These tag were sufficient for the purification of the desired proteins using nitriloacetic acid (Ni-NTA) resin based affinity chromatography. The resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins. The molecular weight of the protein (AMA1 49 kDa, MSP7 66 kDa and WARP 40 kDa) being expressed is higher than expected for MSP7 and WARP, due to the additional nucleotide sequences in the reading frame from the vector itself. Yuda M et al., (2001) showed that WARP exist in the form of oligomer. When reduced with β mercaptoethanol, dimers and monomers were visible. We observed that our WARP gave comparable results.