Studies on Sequence Diversity and Characterization of the Apical Membrane Antigen of *Plasmodium* in Indian Isolates

THESIS

Submitted in Partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

By
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Under the Supervision of
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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

2007

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

CERTIFICATE

This is to certify that the thesis entitled **"Studies on Sequence Diversity and Characterization of the Apical Membrane Antigen of *Plasmodium* in Indian Isolates"** submitted by **Vidya Rajesh, ID No. 2000PHXF018** for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.

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CONTENTS

ACKNOWLEDGEMENTS

First of all, I bow with esteem regards to the Almighty for listening to me in the time of need and showering his blessings to help me achieve all my goals.

It has been my great privilege to work with the inspiring and encouraging guidance of Dr. Ashis K. Das, Associate Professor and Group Leader, Biological Sciences Group. I owe to him my sincere thanks for his invaluable suggestions and constructive criticism in planning, execution and completion of this study. I shall forever be indebted to him for guiding me patiently, always understanding my limitations, and yet motivating me to accomplish the goals I set for this study.

With deepest regards, I wish to thank the Vice Chancellor Prof. L. k. Maheshwari, Deputy Director Prof. K.E.Raman, Deans Prof. Ravi Prakash, Prof. R.N.Saha, and Prof. A.K. Sarkar and Prof. G. Sundar for their ever willing guidance extended throughout the tenure of the present investigation.

With profound reverence, I express my gratitude to Prof. V.S. Rao, Director, BITS – Hyderabad campus for inspiring me, motivating me and for having indaunted confidence in my abilities, which made me reach heights in all spheres of my professional life whether it was teaching, division activities or giving my 100% to complete my doctoral study. His cheerful demeanour, ability to manage difficult situations and his ever positive attitude towards work and life has made him, my ideal role model.

My gratitude is due to Prof. S.K. Verma, Associate Professor - Biological Sciences Group, Chief Warden - BITS, Pilani and Member of my Doctoral Advisory Committee, for being my teacher, and a good advisor for all the professional problems I faced as faculty and as a doctoral student. I would also like to acknowledge the other learned member of the Doctoral Advisory Committee Prof. Suman Kapur, Professor - Biological Sciences Group, Chief of the Community Welfare Unit and International Affairs, BITS, Pilani, for her cooperation and timely suggestions.

My sincere thanks are due to Prof. Dhanpat Kochar, MD, Department of Medicine, S.P. Medical College, Bikaner, for helping in collection of blood samples required in this study.

I gratefully acknowledge the laboratory facilities used for sequencing of samples, rendered to me by CDFD, Hyderabad, especially Dr. Syed Hasnain, and Dr. Akash Ranjan.

I am grateful to Dr.Udhayakumar Venkatachalam, CDCP, Atlanta, for providing me the valuable peptides needed for this study. Major part of this study would not have been possible without his kind gesture.

I feel privileged in expressing high regards to the faculty, Biological Sciences Group, BITS, Pilani for their ever-willing help and support. I would like to specially acknowledge Dr. Shibashish Chaudhary and Arun Prasanna for all the help. I would like to make a special mention about my friend and colleague Neeru Sood, for being a wonderful listener in times of need.

I feel privileged in expressing high regards to Mrs. Das for her ever-willing help.

I owe a sincere thanks to my students Elamaran, Radhika, Ashish, Vidya, Gaurishankar, AartiRavindran for learning with me through most part of my thesis and for their fruitful company. Other labmates like Vijay, Ramachandran, Vishal, Shilpi, Deepak, Narayan, Ashwin, and Joy are duly acknowledged for their presence and support.

The assistance provided by the lab staff Mukesh, Kamlesh, Parameshwar Ji, and the office staff Ramakant Ji, Kanwar Singh Ji, Sharma Ji and Mathuram Ji is heartly acknowledged.

I extend my sincere thanks to my friends at BITS, Pilani - Dr. Sriram, P. Yogeeswari Padma, Smita, KVG Chandrashekar, Rajeshwari, and Petrichia, for their support and helping me out in times of need.

I have no words to express my gratitude to my foster family at Pilani, Pant uncle, Aunty and Deepu for their incessant moral support, prompt help, and timely suggestions. They have always been there for me as pillar of my strength and aspirations.

There is paucity of words to express my deepfelt gratitude and feelings for my family members – My husband Rajesh, for his timely comments and suggestions which always motivated me to perform better, my son Adarsh for bearing with me for my long hours at work and my absence, my two Sister in laws Usha akka, lalitha akka and their families for being my emotional anchors in times of personal difficulties, Siva, Rishab and their family for bearing with me for taking Soumya's long hours.

My acknowledgements would not be complete if I do not confess that this work would not have been fruitfully achieved without the support, ceaseless inspirations and encouragements of my parents and my sister. They have stood by me like a rock in times of need – both physically by taking care of Adarsh in Pilani for the major part of his infancy , as well as emotionally with all their positive words, innumerable phone calls and tireless motivations. Not a moment, I have felt alone or helpless in their presence. Thanks are due for all their love and affection, understanding and time-to-time gearing up.

Vidya Rajesh.

Abstract

Malaria parasites exhibit sequence diversity for a number of stage specific antigens. The Apical Membrane Antigen (AMA-1) is one of the important candidate vaccine antigens, expressed in the sporozoite and merozoite stage of infection. This protein is presumed to play a important role in invasion of host cells. Several Studies have proved that Apical Membrane Antigen (AMA-1) is an effective target eliciting a protective immune response. The sequence variation was investigated for both *Plasmodium falciparum* and *Plasmodium vivax* Apical Membrane antigen from different Indian isolates in this study. Our study revealed a much higher degree of sequence diversity than reported so far for both Pf AMA-1 as well as Pv AMA-1. Moreover, the presence of 5 novel haplotypes was also identified for the *P.vivax* AMA-1 among the Indian isolates studied. Hence, this study is significant in indicating the antigenic repertoire of isolates in a malaria endemic country like India.

Variant alleles were cloned and expression of the full length AMA-1 of *P. falciparum* was tried for immunological characterization. However, the expression of full length AMA-1 protein was not successful and hence synthetic peptides were designed against the variants of one already reported B cell epitope in the ectodomain region of AMA-1. The antibody response to the peptides delineating linear epitopes from five different variants of an epitope of AMA - 1 of *P.falciparum* was studied in naturally infected population from Rajasthan, India. Analysis of the antibody profiles of these 25 *P. falciparum* infected patients was also carried out.

List of Tables **Page No.**

List of Figures — Page No.

Abbreviations

AMA	Apical Membrane Antigen
Pf	*Plasmodium falciparum*
Pv	*Plasmodium vivax*
P.	*Plasmodium*
Mab	Monoclonal Antibody
RBC	Red Blood Cell
Ab	Antibody
^{o}C	Degree Celsius
TRAP	Thrombospondin Related Adhesive Protein
CSP	Circumsporozoite Protein
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
SDS	Sodium dodecyl sulphate
dNTPs	deoxyribonucleoside triphosphates
Min	Minutes
M	Molar
ng	nano gram
µl	micro litre
µg	micro gram
rpm	revolution per minute
ml	milliliter
g	gram
µm	micro metre
kDa	Kilo Dalton
hrs	hours

Amino Acids

• Alanine	ala	A
• Arginine	arg	R
• Asparagine	asn	N
• Aspartic acid	asp	D
• Cysteine	cys	C
• Glutamine	gln	Q
• Glutamic acid	glu	E
• Glycine	gly	G
• Histidine	his	H
• Isoleucine	ile	I
• Leucine	leu	L
• Lysine	lys	K
• Methionine	met	M
• Phenylalanine	phe	F
• Proline	pro	P
• Serine	ser	S
• Threonine	thr	T
• Tryptophan	trp	W
• Tyrosine	tyr	Y
• Valine	val	V

Nucleotide bases

Adenine	A
Guanine	G
Cytosine	C
Thymine	T
Uracil	U

Chapter 1

Malaria – An Introduction

Malaria is probably one of the oldest diseases known to mankind. Deadly fevers, that were probably malaria, have been recorded since the beginning of the written word (6000-5500 B.C.). The symptoms of malaria were described in ancient Chinese medical writings. In 2700 B.C., several characteristic symptoms of what would later be named as malaria were described in the "Nei Ching", The Canon of Medicine. Details of this disease can be found even in the ancient Indian medical literature like Charaka Samhita and Sushrutha, which even associated these fevers with the bites of the Mosquitoes. However, it was only in the 5th century B.C., that malaria was clinically recognized and described by Hippocrates (Hockmeyer *et al*., 1988). He classified the fever types as quotidian (daily), tertian (alternate days) and quartan (fever three days apart). Malaria probably originated in Africa and accompanied human migration to the Mediterranean shores, India and South East Asia. In the past, malaria used to be common in the marshy areas around Rome and hence the name is derived from the Italian mal-aria or "bad air"; it was also known as Roman fever.

It was only at the turn of this century that many discoveries were made which helped us identify the causative agent and the vectors for this dreaded disease. In 1880, Laveran, a French physician working in Algeria, first identified the causative agent for human malaria. He was awarded the Nobel Prize in 1907. In 1885, Golgi identified *P. vivax* and *P. malariae*. Sakharov in 1889 and Marchiafava and Celli in 1890 identified *P. falciparum.* In 1891, Romanowsky described staining methods for identifying malarial parasites. In 1894, Manson hypothesised that mosquitoes transmit malaria On August 20th, 1897, Sir Ronald Ross, while working as a military physician in India, demonstrated the malarial oocysts in the gut tissue of female Anopheles mosquito, thus proving the fact that Anopheline mosquitoes were the vectors for malaria. (www.malariasite.com)

Significant control of the disease was accomplished in the 1930's and 1940's due to the advent of synthetic anti-malarial drugs and modern insecticides. The rise in incidence of drug resistant parasites and insecticide resistant vectors, respectively, has however been responsible for resurgence in malaria cases.

Prevalence of Malaria

According to the WHO Expert Committee on Malaria Twentieth Report (Geneva, 2000), about 100 countries or territories in the world are considered malarious. Large areas of Central and South America (Haiti and the Dominican Republic), Africa, the Indian subcontinent, Southeast Asia, the Middle East, and the Oceania are considered malaria-risk areas. The incidence of malaria world-wide is estimated to be 300-500 million cases each year resulting in 1.5 – 2.7 million deaths, of whom about 1 million are children under the age of five. About 40 % of the world's population is still at risk.

Malaria has been a problem in India for centuries. In the 1930's there was no aspect of life in the country that was not affected by malaria. The economic loss due to the loss of man-days due to malaria was estimated to be at Rs. 10,000 million per year in 1935. The annual incidence of malaria was estimated at around 75 million cases in 1953 with about 8 lakhs deaths annually. To combat this menace, the Govt. of India launched the National Malaria Control Programme in April 1953. The programme proved highly successful and within five years the incidence dropped to 2 million. Encouraged by this, the programme was changed to a more ambitious National Malaria Eradication Programme in 1958. By 1961, the incidence dropped to a mere 50,00 cases a year. But since then, the programme has suffered repeated setbacks due to technical, operational and administrative reasons and the cases of malaria started rising again. Malaria has now staged a dramatic comeback in India after its near eradication in the early and mid sixties. Presently it poses a major challenge with 2 to 2.5 million cases annually. (http://www.malariasite.com/malaria/MalariaInIndia.htm)

The malaria parasites responsible for causing the disease in humans are *Plasmodium falciparum* (responsible for most of the mortality); *Plasmodium vivax* (causing much morbidity), *Plasmodium malariae* and *Plasmodium ovale*. *P .falciparum* is found throughout tropical Africa, Asia and Latin America. *P.vivax* is observed worldwide in tropical and some temperate zones. *P.ovale* is mainly localized in tropical West Africa whereas *P.malariae* has a very patchy distribution worldwide.

P.vivax has been traditionally considered to be the dominant species in India. However, data from intensive studies undertaken in 1930 (Knowles *et al*., 1930) show an almost equal prevalence of *P.vivax* and *P.falciparum* on the basis of microscopic examination of blood smears. After the control and eradication programmes of the 1950's and 1960's the number of cases of both species went down considerably. However, according to some statistical data published by National Malaria Eradication programme (NMEP) in the year 1997, in India, the incidence of *P. vivax* malaria is 60 – 70 % and that of *P. falciparum* is 30 - 45%. Surprisingly, the "National Average" of *P. falciparum* malaria has risen to 35.5% from a meager 9.34% in 1972 (NMEP Survey report, 1995). It thus appears that in spite of various control measures, the malaria problem in the country persists and has, in fact, become more complex due to the appearance of drug resistant parasite forms and insecticide resistant vectors. This situation is further alarming in the view of reports of incidence of cerebral malaria by *P.falciparum* from different endemic foci in the country, and reports of severe malaria by *P.vivax* from Bikaner, Rajasthan (Kochar *et. al*. 2005).

Life Cycle of *Plasmodium*

The life cycle of malaria parasite is complex. The process has three phases in mosquito and two in the human host. (Fig. 1. 1)**.** The parasite undergoes a development stage in the mosquito and the female of the species requires a blood meal to mature her eggs. She bites a human and injects material from her salivary glands, which contains primitive malarial parasites called sporozoites, before feeding. These sporozoites circulate in the blood for a short time and then settle in the liver where they enter the parenchymal cells and multiply; this stage is known as pre-erythrocytic schizogony. After about 12 days there may be many thousands of young parasites known as merozoites in one liver cell, the cell ruptures and the free merozoites enter red blood cells. In the case of *P. vivax*, and *P.ovale* the liver cycle continues. *P.falciparum* on the other hand does not have a continuing liver cycle.

In the red blood cells the parasites develop into two forms, a sexual form and an asexual stage. The sexual stage consists of male and female gametocytes, which circulate in the blood and are taken up by a female mosquito when taking a blood meal. The male and female gametocytes fuse in the mosquito's stomach and form oöcysts in the wall of the stomach. These oöcysts develop over a period of days and contain large numbers of sporozoites, which move to the salivary glands and are ready to be injected into man when the mosquito next takes a meal. In the asexual cycle, the developing parasites form schizonts in the red blood cells, which contain many merozoites. The infected red cells rupture and release a batch of young parasites, merozoites, which invade new red cells. In *P.vivax*, *P.ovale* and probably *P.malariae,* all stages of development subsequent to the liver cycle can be observed in the peripheral blood. However, in the case of *P.falciparum* only ring forms and gametocytes are usually present in the peripheral blood. Developing forms appear to stick in the blood vessels of the large organs such as the brain and restrict the blood flow with serious consequences (Stanley. C. Oaks, 1991).

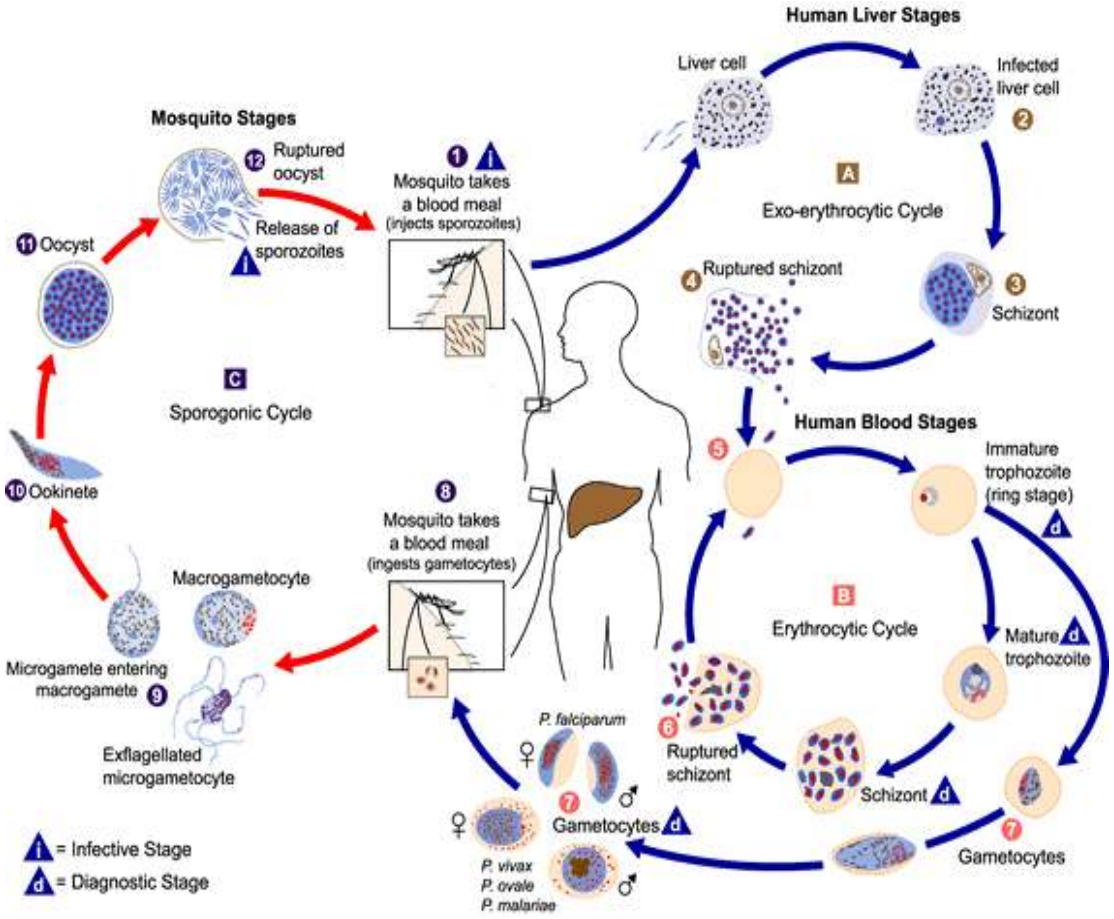


Figure 1.1 Schematic representation of the life cycle of malaria parasite

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony (A)), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony (B)). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites (1) into a new human host perpetuates the malaria life cycle.

Content source: National Center for Infectious Diseases, Division of Parasitic Diseases, CDC, Atlanta.
http://www.cdc.gov/malaria/biology/life_cycle.htm

Sporozoite invasion of hepatocytes:

Plasmodium sporozoites are transmitted through the bite of infected mosquitoes and invade hepatocytes as a first and obligatory step of the parasite life cycle in man. Within minutes after transmission by an infected mosquito, malaria sporozoites invade hepatocytes, their initial site of replication in the liver. Hepatocyte invasion involves proteins secreted from parasite vesicles called micronemes, the most characterized being the thrombospondin-related adhesive protein (TRAP). Speed and selectivity of the sporozoite targeting to the liver have suggested a receptor-mediated mechanism. Sporozoites with their major surface proteins, the circumsporozoite protein (CSP) and the thrombospondin-related adhesive protein (TRAP), interact with heparan sulfate proteoglycans expressed on the basolateral cell surface of hepatocytes. Another microneme protein recently identified in the *Plasmodium falciparum* sporozoites, is the apical membrane antigen 1 (AMA-1). *P. falciparum* AMA-1 is also expressed in sporozoites and is lost after invasion of hepatocytes, and anti-AMA-1 antibodies inhibit sporozoite invasion, suggesting that the protein is involved during invasion of hepatocytes (O. Silvie *et.al*. 2004) . Although the molecular events involved in salivary gland and hepatocyte invasion, are studied at depth and though some information about the various antigens and cell proteins are known, the differential infectivity of oocyst and salivary gland sporozoites in vertebrate hosts still remains a mystery.

Erythrocyte Invasion

The interface between the host erythrocytes and the parasite merozoite is an important stage, giving an opportunity for blocking the entry of the parasite into the erythrocytes and, therefore, preventing any onset of clinical disease. However, the invasion is a highly complex process, involving several different receptor-ligand interactions, which pose many challenges, yet to be explored. The use of molecular, biochemical, and genetic approaches has yielded valuable insights into this complex process. Parasite proteins involved in these molecular interactions are promising candidates for malaria vaccines. A

clear understanding of these interactions is important for a rational design of vaccines, which could attempt to inhibit invasion and prevent malaria.

Invasion of human erythrocytes by *P.vivax* is dependent upon the presence of a single receptor, but *P.falciparum*, as well as some other species, exhibits the ability to utilize multiple alternative invasion pathways i.e.- there are a number of different molecules by which these parasites can enter erythrocytes. These alternative invasion pathways need to be understood at the molecular level in order to develop effective chemotherapeutics or vaccines aimed at blocking this critical event.

Erythrocytic Schizogony Merozoites

The erythrocytic merozoite is an ovoid cell and measures approximately 1.5 μm in length and 1 μm in width (Fig. 1.2 and Fig. 1.3). The apical end of the merozoite is a truncated cone-shaped projection demarcated by the polar rings. Three types of membrane bound organelles, namely rhoptries, micronemes, and dense granules are located at the anterior end of the merozoite. The contents of these organelles play a role in the binding and entry of the merozoite into the host cells. Extra cellular merozoites are intrinsically short-lived and must rapidly invade a new host erythrocyte.

Host Cell Entry

The malaria merozoite invasion process is complex (Fig.1.2) and involves a multi-step sequence, which can be divided into four phases: (a) initial recognition and reversible attachment of the merozoite to the erythrocyte membrane; (b) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of rhoptry-microneme substances with parasitophorous vacuole formation; (c) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat, and finally (d) re-

sealing of the parasitophorous vacuole membrane (PVM) and erythrocyte membrane after completion of merozoite invasion. The initial factor underlying recognition between merozoites and erythrocytes may occur between the merozoite surface coat filament and erythrocyte surface. Multiple different receptor-ligand interactions occur during the merozoite invasion process into an erythrocyte. Apical Membrane Antigen is one such ligand believed to have an important role in erythrocyte invasion. Triglia *et. al*. (2000) suggested a role of AMA-1 in erythrocyte invasion by using anti-AMA-1 antibody that inhibited erythrocyte invasion. They suggested several possible ways by which the anti - AMA-1 antibodies inhibit merozoite invasion.

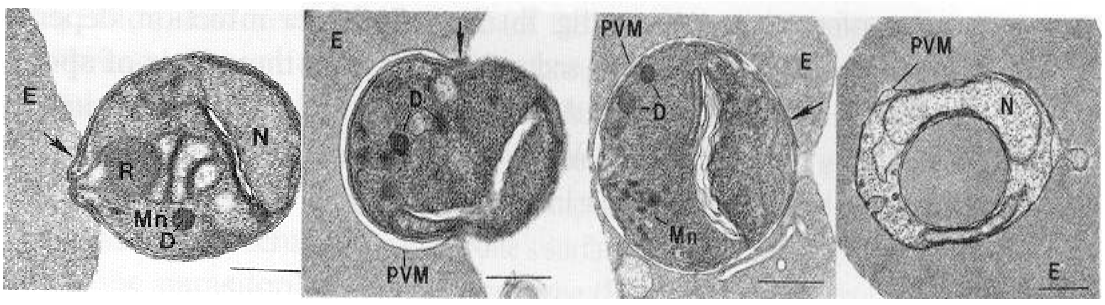
- Preventing the spreading of AMA-1 on merozoite surface
- Cross linking AMA-1 on the surface, thus preventing the processes requiring the mobility of macromolecules across the surface.
- Antibodies stearically hindering other merozoite - RBC interactions
- Direct blockage of AMA-1 function
- Antibody mediated agglutination of merozoites.

They concluded that immune response occurs by directly blocking the AMA-1 function and that AMA-1 is clearly an important molecule in invasion of erythrocytes. This is more true due to the fact that previously Urguiza *et. al*. (2001) had implicated AMA-1 as a erythrocyte binding protein, by identifying 8 peptides in AMA-1 having high binding activity to human erythrocytes. One major significantly important observation was revealed recently by Silvie *et. al* (2004), where they implicated AMA-1 in invasion of hepatocytes by *Plasmodium* sporozoites. They found that AMA-1 is expressed in sporozoites and is lost after invasion of hepatocytes and anti AMA-1 antibodies inhibit sporozoite invasion suggesting that the protein is also involved in invasion of hepatocytes. G.H. Mitchell *et. al.* (2004) have investigated the function of Apical Membrane Antigen in merozoite invasion by incubating *Plasmodium knowlesi* merozoites with red cells in the presence of a previously described rat monoclonal antibody (MAb R31C2) raised against an invasion-inhibitory epitope of *P. knowlesi* AMA-1 and then fixing the material for ultrastructural analysis. They have found that the random, initial, long-range (12 nm) contact between merozoites and red cells occurs normally in the presence of the antibody, showing that AMA-1 plays no part in this stage

of attachment. Instead, inhibited merozoites fail to reorientate, so they do not bring their apices to bear on the red cell surface and do not make close junctional apical contact. They concluded that AMA-1 may be directly responsible for reorientation or that the molecule may initiate the junctional contact, which is then presumably dependent on Duffy binding proteins for its completion.

Harris *et. al.* (2005) isolated a 20 residue peptide (R1) from a random peptide library, which binds to a 'hot spot' on AMA-1 where several other peptides and monoclonal Abs bind and cause inhibition of merozoite invasion into host erythrocyte. The study of these interactions helped in indentifying the molecular mechanisms of invasion better and identifying several critical functions of AMA-1, which will aid in further development of of novel antimalarial strategies.

So, based on these data, AMA-1 definitely has a predominant role in the life cycle of the malarial parasite and inhibition of AMA-1 has proved to offer protective immunity implicating it as an important vaccine candidate.



a b c d

Figure 1.2 Malaria merozoite invasion process

a Apical end of a *P; knowlesi* merozoite attaches to an erythrocyte (E). The erythrocyte membrane becomes thick at the attachment site (arrow). ***b*** Further advanced stage of erythrocyte entry by a *P; knowlesi* merozoite. The junction (arrow), formed between the thickened erythrocyte membrane and the merozoite, is always located at the orifice of the merozoite entry. No surface coat is visible on the portion of the merozoite surface, which has invaginated the erythrocyte membrane, while the surface coat is present behind the junction (arrow) site. ***c*** Erythrocyte entry by a *P; knowlesi* merozoite is almost complete. The junction (arrow) has now moved to the posterior end of the merozoite. An electron-opaque projection connects the merozoite's apical end and erythrocyte membrane. ***d*** A trophozoite (ring form) stage of *P; falciparum* is surrounded by the parasitophorous vacuole membrane (PVM). R = Rhoptry; D = dense granules; Mn = micronemes; E = erythrocyte; N = nucleus. Bars = 0.5 ~m.

Content Source: Malaria Immunology. Karger Publishers, 2002. p.3

Apical Membrane Antigen AMA-1

Integral Membrane proteins of *Plasmodium falciparum* sporozoites and merozoites are potential components of a malarial vaccine. The identification of the antigenic integral membrane proteins of asexual blood stages of *P.falciparum* was done by Smythe. *et. al.* (1988) using temperature dependent phase separation with the non-ionic detergent Triton X - 114 coupled with affinity purification of human Abs. They characterized two intergral membrane proteins of Molecular weight 45,000 and 55,000 respectively. One was identified as MSA – 1 and the other, unidentified was found to be located in the rhoptries of the merozoite.

Parasite molecules contained within the apical complex of merozoites having a membrane bound organelle called rhoptries discharge their contents at the time of schizont rupture and have a role to play in erythrocyte invasion. Peterson *et. al (*1989) described the Apical Membrane Antigen (AMA-1) as a blood stage antigen of *Plasmodium falciparum.* AMA-1 was characterized as a type I integral membrane protein with a molecular weight of 83 kDa. It was observed that, this protein lacks repetitive sequences unlike other *Plasmodium* antigens. It contained two hydrophobic stretches, one near the N terminal, which may act as signal peptide and a second located 55 amino acids from the C terminus. This consists of predominantly hydrophobic residues and lacks charged residues. It has 17 cysteine residues preceding the membrane-spanning domain. Except the most N terminal cysteine, the other 16 cysteines form intra molecular disulphide bonds for maintaining the three dimensional structure of protein (Hodder *et. al.* 1996)**.**

Regarding the localization of this antigen in the merozoite, a report by Narum and Thomas (1994) established the differential localization of full length and processed form of *Plasmodium falciparum* AMA-1. They reported that the full length 83 kDa molecule remains apically restricted following merozoite release (Fig. 1.3). However the processed form of 66 kDa circumferentially associates with the merozoite surface at or around the time of schizont rupture and merozoite release.

Homologues of the AMA-1 gene have been identified in all the commonly studied species of *Plasmodium* (Cheng and Saul 1994, Dutta *et. al*. 1995, Kappi and Adams 1996, Marshall *et. al.* 1989, Peterson. *et. al*. 1989, 1990., Kocken *et. al*. 2000)**.** It has been found that a structural homologue is also present in the micronemes of the parasite *Toxoplasma gondii* (Donahue *et. al*. 2000). The AMA-1 protein from *Plasmodium falciparum* has recently identified to be expressed in sporozoites also in addition to merozoites and is lost after invasion of hepatocytes. (Silvie *et. al*. 2004).

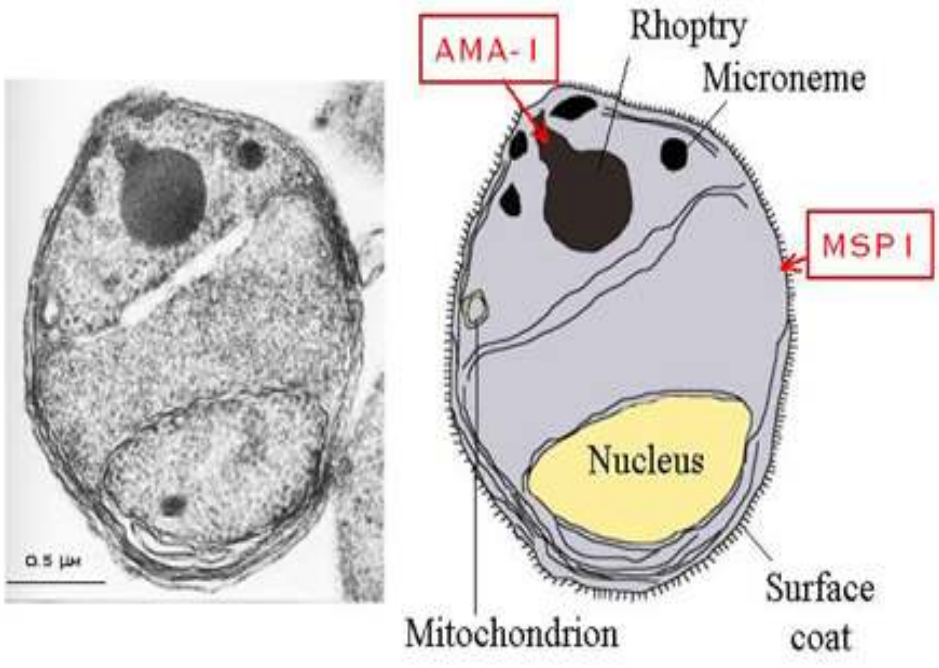


Fig 1.3 Electron micrograph and schematic representation of a *Plasmodium falciparum* merozoite. The vaccine candidate antigens AMA-1 and MSP1 are indicated in red.

The structure and conformation of any protein is closely related to its function. Moreso, in case of immunogenic molecules, like the candidate vaccine antigens of the malarial parasite *Plasmodium*. The Apical membrane antigen of *P. falciparum* is also an important vaccine candidate under consideration for inclusion in vaccine and hence the structure of this molecule is of prime importance. Various studies have been done to identify the structure of AMA-1 and to identify the functional role this protein in erythrocyte invasion.

Structurally, the division of the *Plasmodium falciparum* AMA - 1 protein of 622 amino acids was done into 4 domains by Verra and Hughes, 2000 - the signal and prosequence domain, the ectodomain, the transmembrane domain, and the cytoplasmic domain. The signal and prosequence domain extends from amino acids 1 – 137. The ectodomain extends from amino acids 138 – 535. The ectodomain is further further divided into 3 domains based on disulphide bonds into :
Domain I (Amino acid 138 – 308),
Domain II (Amino acid 309 – 420),
Domain III (Amino acids 421 – 535).
The transmembrane and the cytoplasmic domain extends from amino acids 536 – 622.
The clear-cut demarcation between the domains was not established at this stage as the crystallographic structure of the entire molecule had not been elucidated.

The disulphide bond structure of this protein (AMA – 1) was given by Hodder *et. al* .in 1996, where he showed the presence of 8 disulphide bonds between the conserved 16 cysteine residues of the molecule of AMA - 1. He also showed that the three domains of the ectodomain have molecular weight of 19 kda, 13 kda and 14 kda respectively. Two disulphide bonds were stabilizing domain II, whereas domain I and domain III had three disulphide bonds each. Six of the eight disulphide bonds were unequivocally assigned by Hodder *et. al.* and other two bonds were established later by Nair *et. al.* (2002).

The characterization of the disulphide bonds in the ectodomain is as follows:

Domain I	Cys 217 – Cys 247
	Cys 263 – Cys 275
	Cys 149 – Cys 302
Domain II	Cys 320 – Cys 418
	Cys 337 – Cys 409
Domain III	Cys 443 – Cys 502
	Cys 490 – Cys 507
	Cys 492 – Cys 509

The disulphide bond conformation is part of both the secondary and tertiary structure of the protein. This disulphide bonds forms the basis for the conformation of AMA-1 and is conserved across species and also across strains of the same species. Fig 1.4 (a and b).

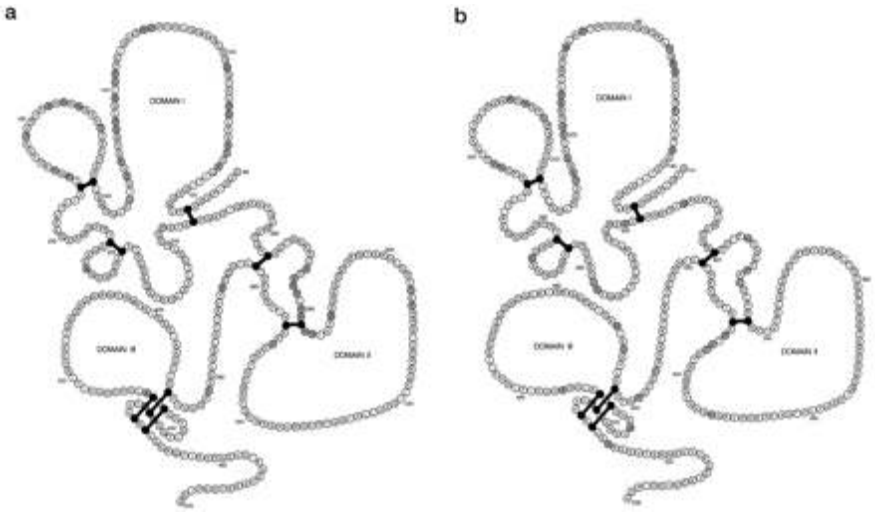


Figure 1. 4 **.** *a*. The sequence shown is that for *P. chabaudi* DS AMA-1 ectodomain (residues 86-479). *b*, The sequence shown is that of *P. falciparum* 3D7 AMA-1 ectodomain (residues 141-538). *Black circles* represent disulfide-linked Cys residues, and *shaded circles* represent the location of amino acid substitutions within the primary sequence as given by Hodder *et. al*. 1996.

The recently elucidated solution structure of the domain III of the ectodomain using NMR spectroscopy by Nair *et. al.* (2002) shows the 14 kda peptide to contain a well defined disulphide stabilized core separated by disordered loop. Within the disulphide stabilized core, residues 443 – 447 form turn of the helix and residues 495 – 498, 503 – 506 form an anti parallel Beta sheet with a type I B turn centered on residues 500 – 501 producing β hairpin type structure. The structured region of the molecule includes all three disulphide bonds.

The three dimensional structure of Domain I and Domain II of Pf AMA-1 determined by Bai *et.al.* (2005) revealed that domains I and II contained of two intimately associated PAN domains. They also reported that the polymorphisms at these domains in AMA-1 are located on one side of the molecule, presumably because this region of AMA-1 is most accessible to antibodies reacting with parasite surface. Moreover the most highly polymorphic residues were present surrounding a conserved hydrophobic trough that is ringed by domain I and domain II loops. This is anticipated to be the receptor binding pocket.

Just before this, Pizarro *et. al*. (2005) published the crystal structure of *Plasmodium vivax* AMA-1 Domain I and II. They also reported the presence of PAN domains in the structure of Domain I/II and also proposed that, Domain II was important for the biological function of AMA-1. Kato *et.al.* (2005) showed that Domain III binds to the Kx protein of human erythrocyte and concluded that AMA-1 plays an important but not exclusive role in invasion of human erythrocytes.

Proteolytic processing of the *Plasmodium falciparum* AMA-1:

The processing details of the Pf AMA-1 were given by Howell *et. al*. in the year 2001 based on direct microsequencing and mass spectrometric peptide mass fingerprinting . The 83 kDa precursor which is localized to the secretory organelles at the apical end of merozoite is proteolytically processed to give a 66 kDa form of the protein. The processing involves N – terminal truncation of the 83 kDa protein between residues Serine 96 and Isoleucine 97. This suggested that the beginning of Domain I is from amino acid 98 onwards as against 138 as suggested by Verra and Hughes (2000) and Hodder *et. al.* (1996). The only other species, which shows a similar truncation in AMA-1, is *Plasmodium reichnowii*. The other known species of *Plasmodium* do not exhibit any such processing and are synthesized directly as 66 kDa proteins. (Kocken, C. H. M. *et. al*. 1998, Deans, J. A. 1984). Following its translocation to the merozoite surface, the 66 kDa form is further cleaved in either of the two sites – one between domains II and III, the other lying at the membrane proximal position at the C terminal end of domain III. This yields two fragments of 44 kDa and 48 kDa, which are released in soluble form into cultural supernatant. From the relative yields of the two forms Howell *et. al.* proposed that cleavage at the membrane proximal position of domain III appears to be slightly more favoured over cleavage at a alternative site (Fig 1. 5). Comparison of the processing of *Plasmodium falciparum* AMA-1 with *Plasmodium knowlesi* shows that the 66 kDa homologue in *Plasmodium knowlesi* gives two fragments of 42 and 44 kDa forms (Deans, J. A. *et. al*. 1984)**.**

A similar process also occurs in *Toxoplasma gondii* system (Hehl. A. B. *et. al*., 2000)**.**

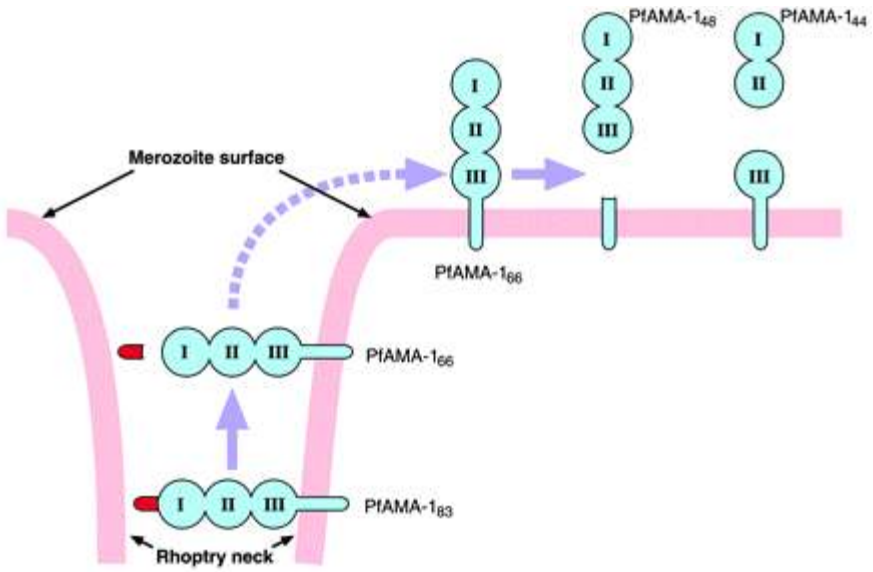


Figure. 1. 5. Proteolytic processing of PfAMA-1.

A model for the proteolytic processing of PfAMA-1 is shown. Conversion of PfAMA-1_{83} to PfAMA-1_{66} by loss of the prosequence (*red*) takes place in the rhoptry neck. Translocation of PfAMA-1_{66} to the merozoite surface ensues. The molecule is then cleaved at one of two alternative positions: either at a membrane-proximal site downstream of domain III to produce PfAMA-1_{48} or between domains II and III to produce PfAMA-1_{44}. The cleaved fragments are shed from the parasite surface, leaving the transmembrane and cytoplasmic domain, with or without domain III attached, still anchored within the parasite membrane to be carried into the invaded host cell.

Thus the pattern that emerges shows that translocation of AMA-1 to the surface of parasite and its subsequent shedding as a result of controlled specific proteolytic processing is a conserved process that transcends species barrier. Similar processing has been reported in sporozoites as well. As observed with TRAP, AMA-1 is initially mostly sequestered within the sporozoite. Upon microneme exocytosis, AMA-1 and TRAP relocate to the sporozoite surface, where they are proteolytically cleaved, resulting in the shedding of soluble fragments. A subset of serine protease inhibitors blocks the processing and shedding of both AMA-1 and TRAP and inhibits sporozoite infectivity, suggesting that interfering with sporozoite proteolytic processing may constitute a valuable strategy to prevent hepatocyte infection (Silvie, O. *et. al.* 2004)**.**

S. Dutta *et. al.* in one of the studies in 2004 proved that that antibodies to AMA-1 capable of inhibiting erythrocyte invasion act by disrupting proteolytic processing of AMA-1. They proved that invasion-inhibitory polyclonal AMA-1 antibodies inhibit secondary proteolytic processing and surface redistribution of AMA-1 on merozoites.

Aims and Objectives

There have been only a few studies on the important vaccine candidate Apical Membrane Antigen of *Plasmodium* in the Indian context. This is very essential in a malaria endemic country like India, where there are nearly 2 - 2.5 million cases reported annually. The study gains more significance as attempts to eradicate the disease by means of vector control and parasiticidal drugs have met with difficulties, owing to the growing resistance of anopheles mosquitoes to insecticides and of the parasite to available antimalarial drugs. For these reasons, development of malarial vaccine is of high priority and diversity of antigens among different parasite isolates is one of the obstacles in vaccine development. This study was undertaken with the intention of investigating the genetic diversity in the Apical Membrane Antigen of both the prevalent species of *Plasmodium* - *Plasmodium falciparum* and *Plasmodium vivax* in the Indian context. The information from such a study was evaluated experimentally to indicate the effectiveness of this molecule in vaccine-based strategies.

The major objectives of the present investigation were:

1. Polymorphism studies in the Apical Membrane Antigen of *P. vivax* from Indian Isolates.
2. Investigation of genetic diversity of the Apical Membrane Antigen of *P. falciparum* in isolates from Indian sub-continent
3. Cloning and Expression studies of the full length AMA-1 gene of *P.falciparum* in prokaryotic systems.
4. Evaluation of antibody profiles in naturally infected individuals, to peptides based on different AMA-1 epitope variants.

Chapter 2

Materials and Methods

Study site and malaria infected blood sample collection

P. falciparum :

The two study regions were Rajasthan and West Bengal; in the western and eastern parts of India, about 2000 Km apart. Both are known as endemic for *P.falciparum* malaria. Samples were collected from 10 malaria patients with their informed consent. Five patients (providing isolates R1-R5) live in Rajasthan (Kota, Jaipur, Sikar) and the other five (Isolates S1 - S5) live in West Bengal (Siliguri). Blood samples (1-5 ml) were collected from each patient and stored at -70^{o}C until DNA extraction.

P.vivax :

The samples were collected from the Bikaner region of Rajasthan in the western part of India. This region shows unstable episodes of *P. vivax* and *P. falciparum* malaria especially after rainy season. About 2 ml to 5 ml of infected blood was sampled in 16% ACD (Acid Citrate Dextrose) solution from malaria patients with their informed consent. For this proper assistance was taken from trained clinicians. Among the various samples collected, eleven *P. vivax* infected samples, as reported by the microscopic diagnosis were used for the study. Three of the eleven samples belong to patients suffering from severe malaria. Blood samples were collected and stored at -70^{o}C until DNA extraction.

Parasite Infected Blood DNA Extraction

1) Lysis Buffer A: 10 mM NaCl
   50 mM Tris
   10 mM EDTA

2) Lysis Buffer B: 10 mM NaCl
   50 mM Tris
   10 mM EDTA
   1% SDS
3) 1 X PBS Buffer
4) Proteinase K
5) Phenol – Tris saturated
6) 1M Sodium Chloride solution
7) 70 % and 100 % Ethanol
8) Chloroform
9) 1X TE Buffer

The *Plasmodium* infected frozen malaria positive blood samples were thawed at 37^{o}C and washed with 1X PBS to get a clear pellet. The extraction procedure was based on lysis of the cells in a mixture of 10 mM NaCl, 50 mM Tris, 10 mM EDTA in the presence of 1% SDS. The pellet was incubated at 37^{o}C for 1 hr, thoroughly re-suspended by tapping or gentle vortexing at regular intervals. The lysate was thoroughly mixed by inversion and incubated with 100 µg/ml Proteinase K for 1 hr at 50^{o}C. A conventional phenol : chloroform extraction was then performed and the DNA in the aqueous phase was precipitated by addition of chilled ethanol in the presence of 0.2 M NaCl. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer (Sambrook *et. al*., 1989).

Polymerase Chain Reaction (PCR)

1) Taq Polymerase (Bangalore Genei)
2) 10 X Reaction Buffer
3) 1M $MgCl_2$
4) dNTPs 10mM stock Solution in water (Bangalore Genei)
5) Primers (Commercially synthesized and resuspended in Nuclease free sterile water)

Genomic DNA was extracted from *Plasmodium* infected blood samples collected from different regions in India using standard protocol (Sambrook *et. al*., 1989). A diagnostic PCR was done to confirm *P.falciparum* and *P.vivax* infections. The diagnostic PCR test, based on the 18s rRNA of *Plasmodium* was performed to differentiate and confirm the clinically positive *P. falciparum* and *P. vivax* samples using the conditions specified earlier. (Das *et . al* 1995). The PCR reaction utilized the following primers - one genus specific 5' primer and 2 species-specific 3'primers in the same reaction cocktail

5' ATCAGCTTTTGATGTTAGGGTATTT 3' - Genus specific
5' TAACAAGGACTTCCAAGC 3' - *P. vivax* specific
5' GCTCAAAGATACAAATATAAGC 3' - *P. falciparum* specific

After confirming the identity of samples as *falciparum and vivax* (Fig 2.1), the AMA - 1 gene was amplified from both *P. falciparum* and *P.vivax* as per the details given below:

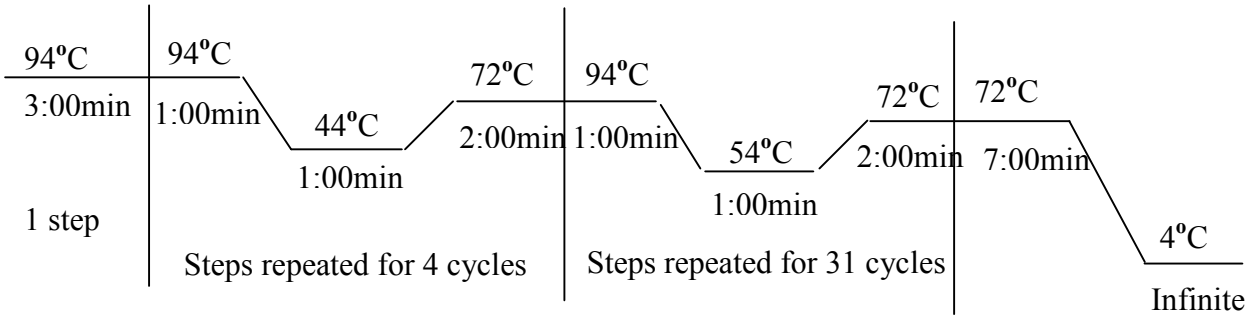
P. falciparum:

A 1.8 kb fragment of *P.falciparum* AMA - 1 of *P.falciparum* was amplified (Fig 2.2) using primers designed based on the flanking regions of the gene. Forward and reverse primers used are (5'GCG GGA TCC ATG AGA AAA TTA TAC TGC 3', and 5' GCG AAG CTT ATA GTA TGG TTT TTC CAT CA 3') having an additional GCG overhang and Bam HI and Hind III sites at their respective ends. 100 ng of parasite DNA was taken

as target for each amplification reaction, which utilized standard components for a 50-µl-reaction volume.

Samples were subjected to 35 cycles of amplification. The initial four cycles comprised of denaturation at 94°C for 1.0 min, annealing at 44°C for 1.0 min and extension at 72°C for 2 min. The rest of the 31 cycles consisted of denaturation at 94°C for 1.0 min, annealing at 54 °C for 1.0 min and extension at 72 °C for 2 min.

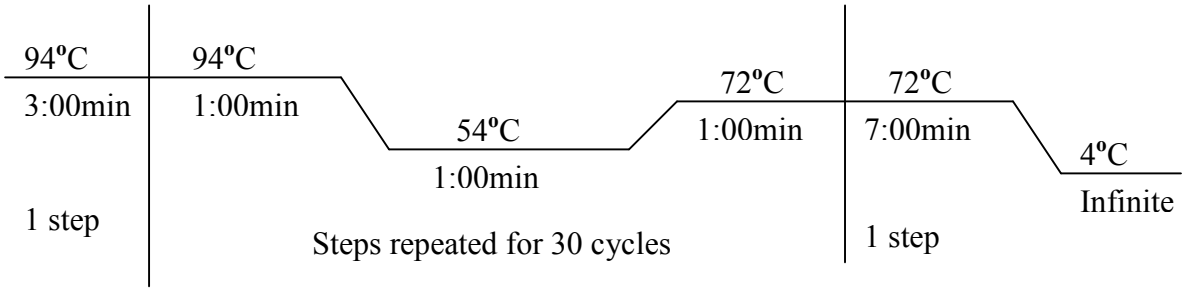
The standard PCR cycle used the following parameters



P.vivax:

A 1640 bp length of the AMA - 1 gene of *P.vivax* was also amplified (Fig 2.3) using the primers designed, based on the flanking regions of the gene. Forward and reverse primers used are (5'GCG GGA TCC ATG AAT AAA ATA TAC TAC ATA ATC 3', and 5' GCG TCT AGA TTG TCC TCG CCC CAG AAG 3') having an additional GCG overhang and Bam HI and Xba I sites at their respective ends. 100 ng of parasite DNA was taken as target for each amplification reaction, which utilized standard components for a 50-µl-reaction volume.

The PCR programme used for amplification:



A standard PCR reaction (50 µl volume) was set up for both *P.falciparum* and *P. vivax* AMA-1 using respective templates and primers as per the details given below -

Template DNA	-	3 µl (100 ng)
dNTP mix	-	4 µl
Primers	-	4 µl (100ng/µl)
Taq Buffer	-	5 µl
Taq polymerase	-	1 µl
Nuclease free H_2O	-	29 µl

Agarose Gel Electrophoresis for resolving DNA fragments

Separation of DNA fragments was done by electrophoresis through agarose gels at 70 volts in TBE buffer. 0.5 µg of Ethidium Bromide was added to the molten agarose just before pouring. The DNA fragments were viewed in a Syngene UV gel documentation system.

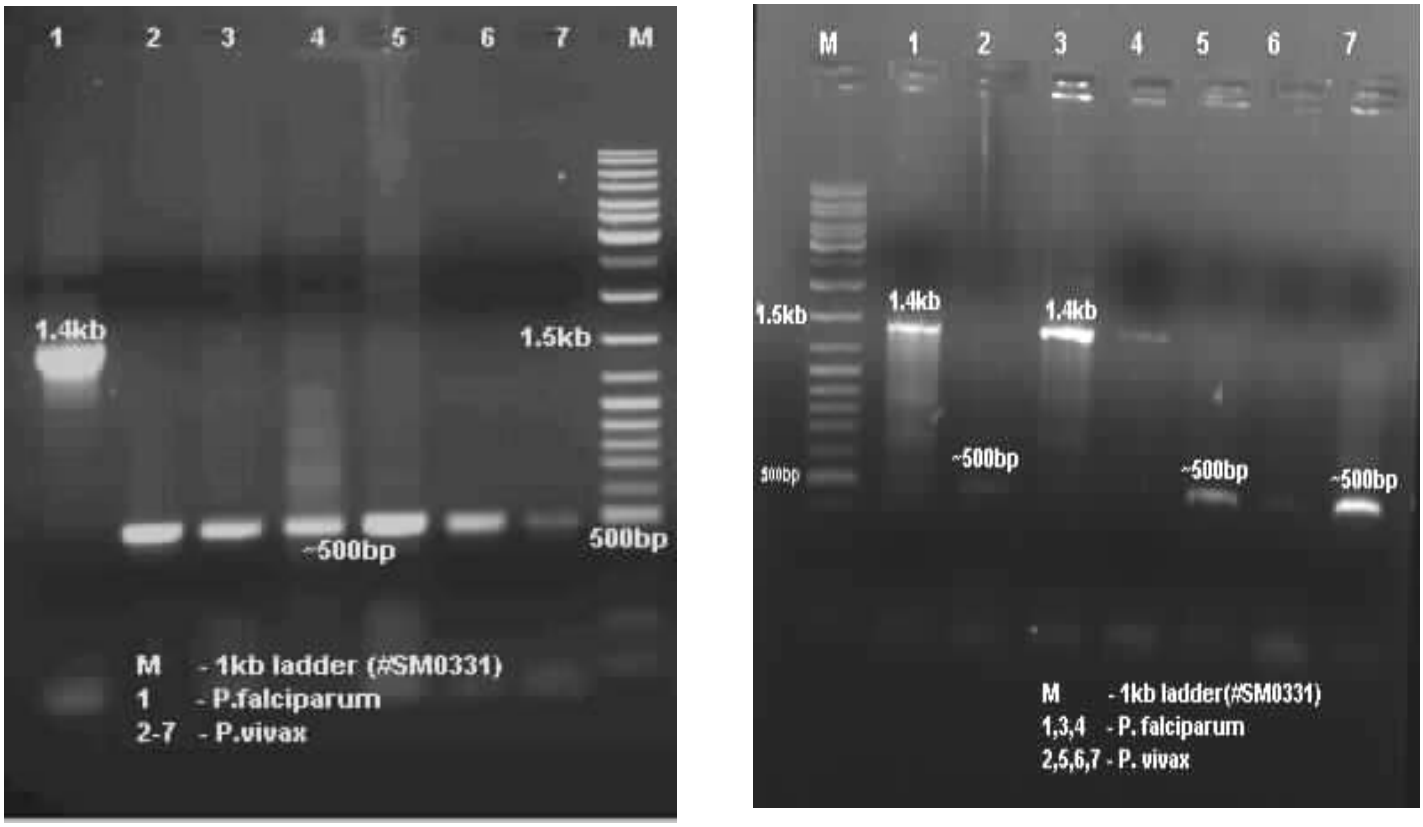


Figure 2.1 Diagnostic PCR showing the differences between *P.falciparum* and *P.vivax.*

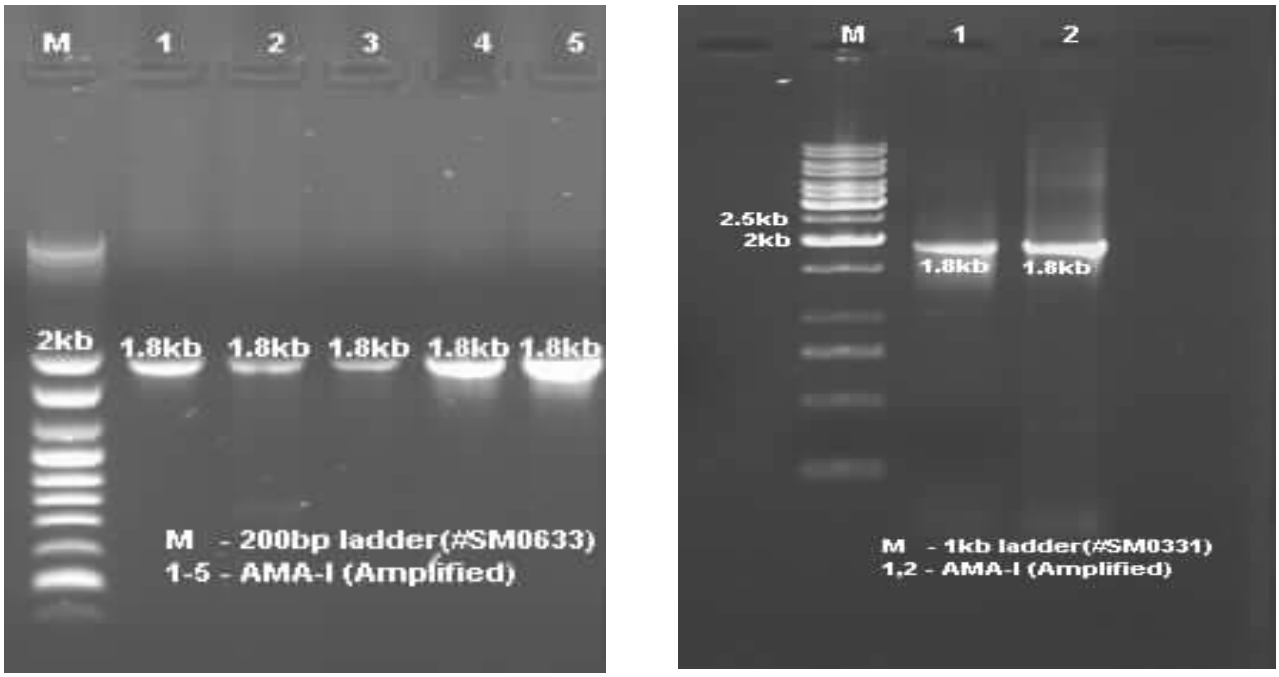


Figure 2.2 Amplification of AMA-1 gene of *Plasmodium falciparum* from Indian Isolates

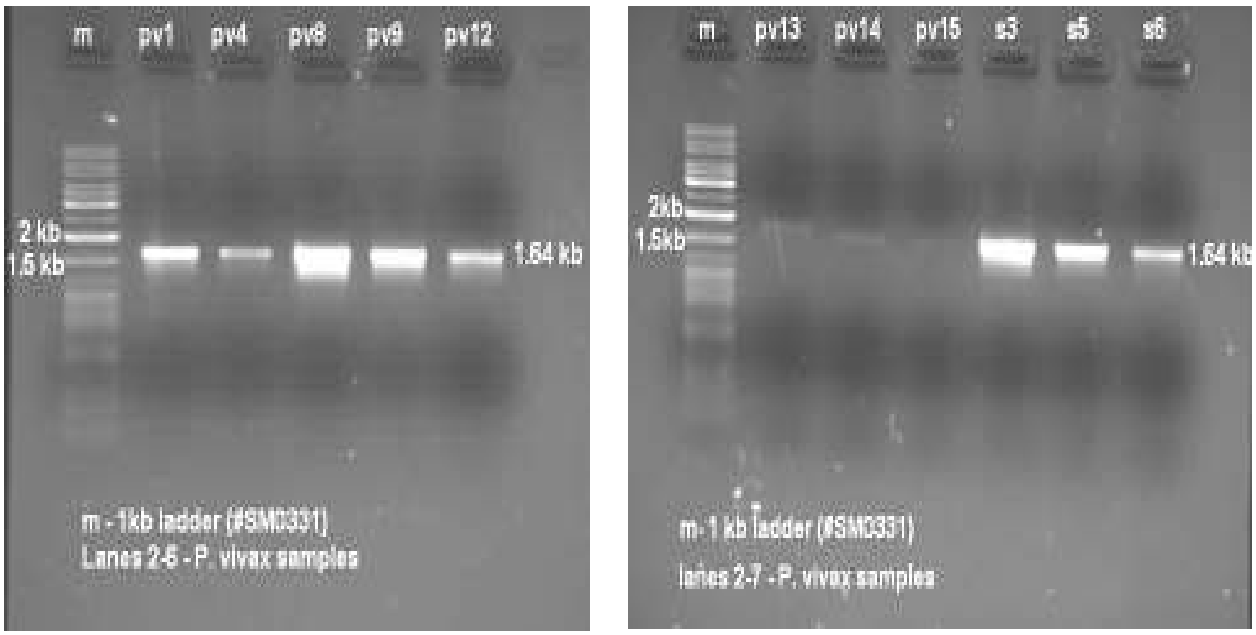


Figure 2.3 Amplification of AMA-1 gene of *Plasmodium vivax* from Indian Isolates.

Cloning of AMA - 1 gene of *P.falciparum* into pRSET A vector (Fig 2.4)

1) BamHI (MBI Fermentas)
2) Hind III (MBI Fermentas)
3) Buffer for Restriction Digestion: Y^+ Tango (MBI Fermentas)
4) T4 DNA Ligase (New England BioLabs)
5) T4 DNA Ligase Buffer (New England BioLabs)

Plasmid Extraction Procedure

1) Solution I: 50 mM Glucose
   10 mM EDTA (pH 8.0)
   25 mM Tris-Cl (pH 8.0)
2) Solution II: 0.2 N NaOH
   1 % SDS
3) Solution III: 5 M Potassium acetate (pH 5.2)
4) Tris saturated Phenol
5) Chloroform:Isoamyl alcohol
6) Absolute alcohol
7) RNAse
8) 1X TE buffer (pH 8.0)

Cells were pelleted down from overnight cultures and re-suspended in Ice-cold solution I. To this freshly prepared solution II was added and incubated on ice for a couple of minutes with intermittent mixing. Gentle vortexing was done by inverting the tubes after adding Ice-cold solution III. The lysed material was then spun at 12000 rpm for 5 min at 4 ^oC. A conventional phenol : chloroform extraction was then performed, and the DNA in the aqueous phase was precipitated by addition of chilled ethanol in the presence of 0.2 M NaCl. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer (Sambrook *et al*., 1989).

Restriction digestion of vector and insert

The vector was double digested with BamHI and Hind III enzymes as per the reaction mix given below and was purified using Qiagen Gel Extraction Kit. The insert being a PCR product was purified using PCR purification QIAGEN kit and double digested with BamHI and Hind III enzymes (Sambrook *et al*., 1989). After this, digested insert was gel eluted and quantified for setting up a ligation.

Reaction for restriction digestion of vector

Template DNA	- 2 µl (500 ng – 1µg)
Restriction Enzyme	- 2 µl (5 units each of BamHI and Hind III)
Buffer (10x)	- 2 µl
Sterile Millipore water	- Volume to make 20 µl

Reaction was left at 37 ^{o}C for 1.5 hours.

Reaction for restriction digestion of insert

Template DNA	- 8 µl (1µg)
Restriction Enzyme	- 2 µl (5 units each of BamHI and Hind III)
Buffer (10x)	- 2 µl
Sterile Millipore water	- Volume to make 20µl

Reaction was left at 37 ^{o}C for 1.5 hours.

Ligation

The quantities of both vector and insert were calculated by measuring O.D. at 260 nm. Based on the quantity calculated, a reaction mix was prepared as follows:

Vector	-	1 part
Insert	-	3 parts
T4 DNA Ligase	-	1 unit
Ligase Buffer (10x)	-	1 X (2µl for 20µl reaction)
Sterile Millipore Water	-	Volume to make 20µl

The reaction mixture was left at 16 – 22 ^{o}C for 6 – 16 hours depending on the specification mentioned by T4 DNA ligase enzyme supplier and used for transformation.

Transformation of *E. coli* with pRSET A

Preparation of competent cells:

1) 0.1 M $CaCl_2$
2) 50% Glycerol in 100 mM $CaCl_2$
3) LB medium

Cells were pelleted out from a culture grown to 0.4 – 0.6 OD, re-suspended in 10 ml of 0.1 M $CaCl_2$ and allowed to stand on ice for 30 – 40 min. The cells were spun at 5000 rpm for 5 min and re-suspended in 2 ml of ice cold 0.1 M $CaCl_2$. These were incubated on ice for 60 – 90 min. To this, Glycerol was added and aliquoted with immediate snap freezing.

Approximately 100 ng of the plasmid DNA was added to the competent cells and incubated on ice for 45 min. The microfuge tubes were rapidly transferred to a 42^0C water bath for a heat shock for 90 sec. These tubes were replaced on ice to which 600 µl fresh autoclaved LB was added in the hood and incubated at 37^0C for 1 hr. This was plated on to LB agar plates with appropriate antibiotics and incubated over night at 37^0C.

The colonies were screened for the insert by plasmid extraction, and double digestion was done to confirm the recombinant construct. (Fig 2.4)

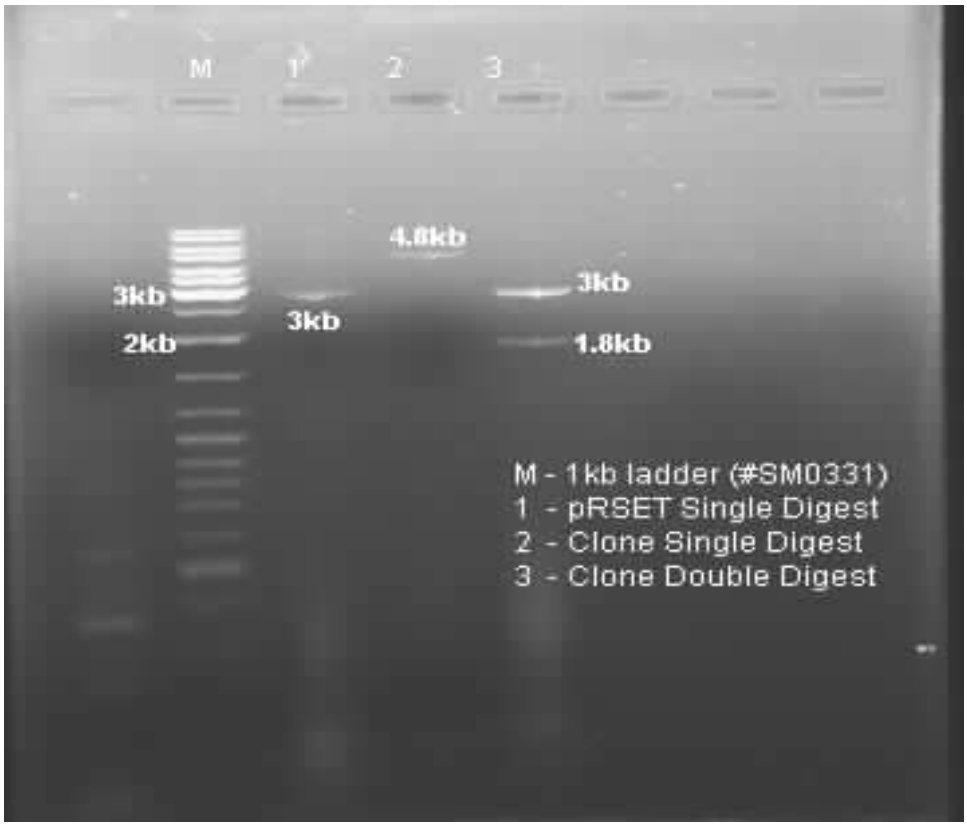


Figure 2.4 Restriction digestion of the AMA- 1 construct in pRSET vector.

DNA Sequencing

For *falciparum*: Sequencing of both, the clones and amplicons, were done at CDFD, Hyderabad.

For *vivax*: Three set of amplicons from different PCR reactions were eluted and amplicons were send for sequencing to Shankarnetralaya and Oscimum Scientific for commercial sequencing. The sequences were then submitted to the Genbank database. The Genbank accession numbers are EF025187 to EF025197.

Alignments of the sequences and polymorphism studies:

The sequences obtained by sequencing were then analysed using various available bioinformatics tools like Genetool, Peptool, Clustal W etc. and the polymorphism of AMA - 1 for both *P. falciparum* and *P. vivax* were studied, at the nucleotide as well as the protein level, and the Indian isolates were compared with the respective reported standard strains. (Appendix 1 and Appendix 2).

Subcloning of the AMA-1 gene from the pRSET A vector into the pET 21C vector:

The AMA-1 gene was subcloned into the pET 21C vector, as it is a better expression vector. The vector was extracted using the standard plasmid extraction alkaline lysis method and subjected to simultaneous digestion as per the following reaction:

Reaction for restriction digestion of vector

Vector (pET21C)	-	1 µl (500ng – 1µg)
Hind III	-	1 µl (5 units)
Bam H1	-	1 µl (5 units)
Buffer Y+ Tango (10x)	-	2 µl
Sterile Millipore water	-	Volume to make 10µl

Reaction was left at 37 ^{o}C for 1.5 hours.

Reaction for restriction digestion of insert

Clone (pRSET + Insert)	-	1 µl (1µg)
Hind III	-	1 µl (5 units)
Bam H1	-	1 µl (5 units)
Buffer (10x)	-	2 µl
Sterile Millipore water	-	Volume to make 10µl

Reaction was left at 37 ^{o}C for 1.5 hours.

The pET vector was found to be of 5.4 kb. After digestion, the pRSET clone gave two bands of 3 kb and 1.8 kb respectively. The 1.8 kb fragment ie.double digested insert and the vector were cut out from the gel and purified using the QIAGEN GEL EXTRACTION KIT.

The vector and the insert were then ligated and transformed into the host and checked for the clone. A double digestion was performed to confirm the presence of the insert in the clone (Fig. 2.5) and proceeded with expression studies. A PCR amplification of the clone using AMA - 1 primers were also done for confirmation.

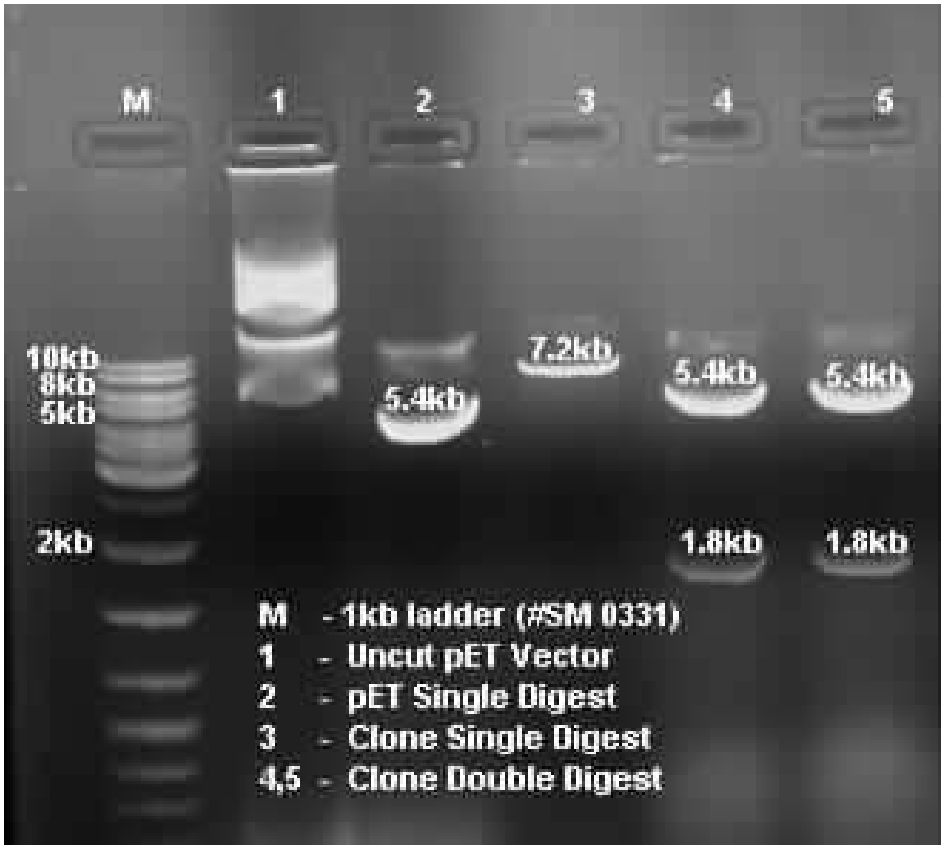


Figure 2.5. Restriction digestion of the AMA- 1 construct in pET21C vector.

Cloning and Expression Studies of AMA-1 gene of *P. falciparum* in *E.coli.*

The clones were sequenced using the T7 forward primer, and the orientation of insert and the reading frame of the insert with respect to the vector were checked from the sequence information, using the GeneTool software. After confirming that the insert was in proper orientation and reading frame, expression studies were carried out in *E.coli*.

Transformation of clones into BL21 host:

To express the protein, the clones were transformed into *E.coli* BL21(DE3) pLysS and BL21(DE3) host system. The individual vectors were also transformed into *E.coli* BL21(DE3) pLysS and BL21(DE3) cells for controls.

BL21 is the most widely used host background for protein expression and has the advantage of being deficient in both *lon* and *ompT* proteases. BL21 (DE3) strain is lysogenic for a prophage that contains an IPTG inducible T7 RNA polymerase. BL21(DE3) pLysS is a derivative of *E. coli* B and are chemically competent cells used for tight control of protein expression in T7 RNA polymerase based systems and results in superior isolation of intact recombinant proteins (Pan *et. al.* 2000).

E.coli BL21(DE3) pLysS transformants containing the clone were streaked into LB plates containing 35 μg/ml chloramphenicol and 100 μg/ml ampicillin. Choramphenicol selects for the maintenance of the pLysS required for the T7 lysozyme expression and ampicillin selects for the vector.

Co-transformation of the clone with the RIG plasmid for expression of the AT rich AMA - 1 gene

1) RIG plasmid (Novagen)
2) BL21(DE3) *E.coli* strain
3) Chloramphenicol
4) Ampicillin

Overexpression of parasite genes is important for providing sufficient amounts of recombinant protein for immunological applications, such as the production of recombinant antigens for the generation of research antibodies. The overexpression of parasite genes in *Escherichia coli,* in particular those of *Plasmodium falciparum*, has often been a challenge because of the codon bias of these organisms. *P. falciparum* has an extremely AT-rich genome of about 80%, which has in many instances made heterologous expression of *Plasmodium* genes in *E. coli* very difficult.

The RIG-plasmid is a useful tool which allows for the overexpression of several AT-rich parasite genes in *E. coli* (Baca *et. al.* 2000). The RIG-plasmid is compatible with most commercially available vectors and *E. coli* expression strains. The RIG-plasmid is a simple and convenient tool to test when performing initial overexpression experiments of AT-rich genes. The RIG-plasmid, was co-transformed into *E. coli* along with the expression vector containing a parasite gene (AMA-1).

Induction of protein expression in the bacterial system:

1) IPTG (Isopropyl thiogalactoside)
2) 2X SDS sample buffer
3) Chloramphenicol
4) Ampicillin

E. coli BL21 transformants were inoculated in LB broth + chloramphenicol (35 µg/ml) + ampicillin (100 µg/ml). A 12 hour primary culture was then used for secondary

inoculation of 25 ml LB broth + chloramphenicol (35 µg/ml) + ampicillin (100 µg/ml). The secondary culture was incubated at 37^0C for 3 hrs to get an O.D of 0.4 - 0.6. 1 ml aliquot of cells were removed, prior to IPTG induction and centrifuged. The pellet was dissolved in 100 µl of the 2X SDS sample buffer and stored as –20 ^{0}C to give the zero hour sample.

IPTG was then added to a final concentration of 1mM in LB broth inoculated with *E.coli* BL21 transformants i.e. the vectors and clones. Samples were then collected at 1 hour intervals upto the 6^{th} hour. SDS – PAGE was run to analyze the expression level of the proteins.

SDS-PAGE (Polyacrylamide gel electrophoresis for separation of proteins)

SDS – polyacrylamide gel electrophoresis was carried out using the Bio-Rad vertical slab gel apparatus for SDS-PAGE with discontinuous buffer system.

1) Acrylamide mixture
   - Acrylamide – 29.2%
   - N, N' – methylenebisacrylamide – 0.8%
   - Distilled water – 100ml
2) Tris-glycine electrophoresis buffer
3) TEMED (N,N,N',N' – tetramethylethylenediamine)
4) Ammonium persulphate
5) Resolving gel (10%) – 10 ml
   - 30% acrylamide - 3.3 ml
   - 1.5 M Tris (pH – 8.8) - 2.5 ml
   - Water - 4.0 ml
   - 10% APS - 100 µl
   - TEMED - 20 µl
   - 10%SDS - 0.1ml

6) Stacking gel (5%) – 5 ml

30% acrylamide	- 830 µl
1.0 M Tris (pH – 6.8)	- 630 µl
Water	- 3.4 ml
10% APS	- 50 µl
TEMED	- 8 µl
10% SDS	- 50 µl

7) Staining solution (100 ml)

Coomasie Brilliant Blue	- 0.20g
Methanol	- 45 ml
Glacial Acetic Acid	- 10 ml
Water	- 45 ml

8) Destaining solution (100 ml)

Glacial Acetic Acid	- 10 ml
Methanol	- 45 ml
Water	- 45 ml

The glass plates were washed and cleaned to make them grease free. The electrophoresis apparatus was then assembled with the glass plates with spacers and checked for leakage. The resolving gel was prepared and loaded in the space between glass plates. Sufficient space was left for the stacking gel solution. The space was filled with water, and the acrylamide was allowed to solidify for 30 mins. Water was removed carefully and stacking gel was prepared and loaded on top of the resolving gel. Immediately the comb was inserted and the gel was allowed to solidify for 30 mins. After polymerization is complete, the comb was removed carefully. The gel was mounted in the electrophoresis apparatus. Tris glycine electrophoresis buffer was added to the top and bottom reservoirs. The samples were heated to 100˚C for 5-6 mins to denature the proteins. 15 µl of the samples were loaded into the wells. A high molecular weight protein marker was also loaded along with the samples. The electrophoretic apparatus was connected to an electric power supply and a voltage of 70V was applied. The gel was then run till the

bromophenol blue dye reached the bottom of the resolving gel. The glass plates were then removed. Gel was scooped out and the edge marked for orientation of the gel. The gel was stained with Coomasie brillant blue staining solution by placing it in a slowly rotating platform. The gel was then destained, by placing it in the destaining solution for 4 - 8 hours. The gel was then photographed to view the bands.

Western Transfer

1) Transfer apparatus
2) Filter paper
3) Nitrocellulose membrane (Schleider and Schulle)
4) SDS polyacrylamide gel containing separated proteins (along with a pre-stained marker)
5) Transfer buffer (Tank blotting)

8 pieces of filter paper and a piece of membrane to the same size as the gel were cut. Membrane was incubated for 10 mins in tank blotting transfer buffer. Filter paper and membrane were soaked in tank blotting transfer buffer. Avoiding air bubbles, 4 sheets of filter paper on the fiber pad were placed, followed by the gel, the membrane, 4 sheets of filter paper and finally the other fiber pad. Transfer was carried out for 2 hours at 110 mA. After transfer, the orientation of the gel on the membrane was marked.

Immunodetection with Anti-His antibodies / Anti-His AP conjugates:

1) Western blot
2) TBS buffer
3) TBS- Tween buffer
4) Anti His Antibody (QIAGEN, USA)
5) Anti-mouse secondary antibody alkaline phosphatase (AP) conjugate (Bangalore GENEI)
6) Blocking buffer: 3% BSA in TBS buffer
7) Staining solutions for alkaline phosphatase - BCIP/NBT (Substrate for alkaline phosphatase (Bangalore GENEI)

The membrane was washed twice for 10 mins, each time with TBS buffer at room temperature and incubated for 2 hrs in blocking buffer at room temperature. Again the membrane was washed twice for 10 min each time in TBS-Tween buffer at room temperature. It was then, incubated in Anti His antibody (1/1500 dilution of antibody in blocking buffer) at room temperature for 1 hour. The membrane was washed twice for 10 min each time in TBS-Tween buffer at room temperature. Again it was washed for 10 min in TBS buffer at room temperature. Then, incubated with the secondary antibody solution (1/2000 dilution in 3% BSA in TBS) for 1 h at room temperature. It was then washed 4 times for 10 min each time in TBS-Tween buffer at room temperature and stained with BCIP/NBT (Substrate for Alkaline phosphatase, Bangalore GENEI) solution for 15 mins. the chromogenic reaction was stopped by rinsing the membrane twice with water. The membrane was dried and photographed.

Reverse transcriptase PCR (RT-PCR) to check the expression upto transcription:

Total RNA was extracted from the induced *E. coli* BL21pLys S cultures transformed with just the vector and the recombinant molecules using the standard protocol.

1) Protoplasting buffer
2) Gram negative lysis buffer
3) Formaldehyde gel loading buffer
4) Formaldehyde gel running buffer
5) DEPC water
6) EDTA
7) Ethanol
8) Lysozyme
9) Saturated NaCl

10 ml of 16 hr old culture was taken and pelleted by centrifuging at 10,000 rpm for 10 mins. The cells were resuspended in 10 ml of protoplasting buffer . To it, 80 µl of lysozyme was added and it was incubated in ice for 5 mins. The collected protoplast was resuspended in gram negative lysis buffer and 15µl of DEPC water was added to it. To this 250 µl of saturated NaCl was added and after centrifuging at 12,000 for 15 mins. the supernatant was collected and to it, ice cold ethanol was added and kept for overnight precipitation at –20^{o}C. The pellet was redissolved in DEPC water and the extracted RNA was stored at –70^{o}C.

The RNA was quantified and to 30 µg of RNA 5X gel running buffer was added and the sample was incubated at 65^{o}C for 15 mins and then chilled in ice. To this 1µ of EtBr and 2µl of gel loading buffer was added. The sample was loaded along with RNA ladder and run at 80-100 V.

After visualizing the total RNA, then the RNA sample was subjected to reverse transcription using the Qiagen Sensiscript RT Kit. A PCR reaction was setup using the *Plasmodium falciparum* AMA - 1 primers using the standardized conditions as given. On running the agarose gel, an amplification of 1.8 kb fragment of AMA - 1 was found in both the pRSET clone as well as the pET clone. To confirm the absence of DNA contamination in the RNA preparation, a PCR reaction was set up with the RNA sample and no amplification was seen, removing the doubt of any DNA contamination.

Designing of synthetic peptides for the epitopes:

The reported epitopes found in AMA - 1 were compared in the Indian isolates, and a B cell epitope in domain II (E1) showing considerable variation was selected (Fig 2.5) and the synthetic peptides were synthesized. The synthesized variants were labeled as E1a, E1b, E1c, E1d and E1e respectively, which differed from one another in one or two amino acids. These 5 peptides were made available by CDC, Atlanta.

The sequences of the designed peptides were as follows:

E1a :– DGNCEDIPHVNEFPAIDL (Standard 3D7)

E1b : DGNCEDIPHVNEFSANDL

E1c :- DGNCEDIPHGNEFPANDL

E1d :- DGNWEDIPHVNEFSANDL

E1e :- DGNWEDIPHVNEFSAIDL

Study area, Human subjects, and sera collection:

The study region selected was Rajasthan, which lies in the western part of India. 1 – 5 ml of parasitized blood samples were collected randomly at different stages from different areas in these regions, which were known to be endemic for *P. falciparum* malaria. Blood samples were collected from 25 patients in the age group of 20 to 40 years, with informed consent. Sera was separated from blood samples at the site and stored at -20^{o} C till analysis.

Serum separation from the *P.falciparum* infected blood samples:

After blood collection, the blood was allowed to clot for 30 – 60 min at 37^{o} C. The tube was flicked several times to dislodge the blood clot. This clot was then kept at 4 ^{o} C for overnight to allow it to contract. The serum was then separated from the clot by centrifugation at 10,000 rpm for 10 min at 4 ^{o} C. The serum was stored at –20 ^{o} C till the analysis.

ELISA (Enzyme Linked Immunosorbant Assay)

For analyzing human sera

1) 96 well Microtitre Plates (Grenier, Germany)
2) Peptides
3) 1 X PBS (Phosphate Buffered Saline)
4) Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6)
5) Wash Buffer: PBS-Tween 20
6) Blocking buffer: 5 % casein in PBS –Tween 20
7) Conjugate: goat anti-human IgG peroxidase (Bangalore Genei)
8) Substrate: TMB/H_2O_2 (Bangalore Genei)
9) Stop Solution: 2N H_2SO_4

Serum samples from 25 patients were analyzed by enzyme-linked immunosorbent assay (ELISA) (Harlow *et al*., 1988) for total IgG titres to E1a, E1b, E1c, E1d, & E1e. Micro titer plates were coated with the appropriate antigen (150 ng of peptide per well, diluted in 100 µl of 1X coating buffer) and incubated at 4^{o}C overnight. Wells were washed thrice with 1X PBS - Tween 20 (0.2 %), blocked with the blocking buffer and incubated at 37^{o}C for 2 hrs. After washing with the wash buffer three times, serum diluted (1/2000) in blocking buffer was added (100 µl/well) and incubated at 37^{0} C for 2 hrs. After washing thrice with wash buffer, 100 µl of secondary antibody diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^{o} C for 2 hrs. Again after washing, 100 µl of substrate solution was added in dark and left at room temperature for 20 mins, for the

color to develop. 100 µl of stop solution was added and the absorbance was read at 450 nm using ELISA Reader STATFAX 2100.

Threshold of positivity was an OD value of 0. 0914, 0.1088, 0.102, 0.195, and 0.397 for E1a, E1b, E1c, E1d and E1e respectively, based on the mean plus SD of the reactivity of sera from 14 healthy controls who were not exposed to malaria before. All the assays were performed in duplicate. The intra assay standard error, which was calculated, was not significantly different.

Vectors and Bacterial strains used

1) pRSET A (Invitrogen USA)
2) pET 21c (Invitrogen USA)
3) *E.coli* DH5α (IMTECH, Chandigarh, India)
4) *E. coli* BL21DE3 (Invitrogen USA)
5) *E. coli* BL21 pLysS (Invitrogen USA)
6) RIG plasmid (Novagen, USA)

Various kits used for this study

1) Qiagen Gel Extraction Kit (Qiagen USA)
2) Qiagen PCR Purification Kit (Qiagen USA)
3) Qiagen Anti – His Antibody Kit (Qiagen USA)
4) Qiagen Sensiscript Reverse Transcriptase Kit (Qiagen USA)

Medium for bacterial culture

1) LB (Luria Bertani Medium) – 1000 ml (pH - 7.2)

   Bactotryptone - 10.0 g,

   Bacto-yeast extract - 5.0 g

   NaCl - 10.0 g

2) Plating agar:

   18.0 g of bactoagar were added to LB medium prior to autoclaving.

Composition of various buffers used in the study

1) TE pH (8.0)

   10 mM Tris-Cl (pH 8.4)

   1mM EDTA (pH 8.0)

2) Tris Borate EDTA (TBE) 10 X Buffer (1000 ml)

   Tris base 108 g

   Boric acid 55.0 g

   0.5 M EDTA (pH 8.0) 40 ml.

3) Tris – Acetate Buffer (50X)

   Tris base - 242 gm

   Glacial Acetic Acid - 57.1 ml

   0.5M EDTA (pH 8.0) - 100 ml

4) DNA gel loading buffer (6X)

   0.25 % Xylene cyanol,

   0.25 % Bromophenol blue

   30 % glycerol in water

5) Phosphate Buffered Saline (1X) pH 7.2

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.4 mM KH_2PO_4

6) Coating Buffer For ELISA (10 X)

0.025 M Na_2CO_3

0.025 M $NaHCO_3$

Distilled water 100 ml

7) Protoplasting buffer for RNA extraction

15 mM Tris Cl (pH – 8.00)

450 mM sucrose

8 mM EDTA

8) Gram negative lysis buffer

10 mM Tris Cl (pH- 8.00)

10 mM NaCl

1mM Sodium citrate

1.5% SDS

9) RNA gel running buffer

0.1 M MOPS (pH – 7.00)

40 mM sodium acetate

5 mM EDTA (pH – 8.00)

10) Formaldehyde gel loading buffer for RNA

50% glycerol

1mM EDTA

0.25% Bromo phenol blue

0.25% Xylene cyanol

11) Transfer buffer (Tank blotting) for Western Blotting: (pH – 8.3)

25 mM Tris base

150 mM glycine

20% methanol

12) Tris-glycine electrophoresis buffer:

25 mM Tris

250 mM Glycine

0.1% SDS

Buffers for restriction endonucleases, T4 DNA Ligase and Taq DNA polymerase :

The various buffers used were provided by the manufacturers for these enzymes and used as per the instructions provided.

Antibiotics used

1) Tetracycline (Hi Media)
2) Ampicillin (Ameresco)
3) Chloramphenicol (Hi Media)

Chapter 3

Polymorphism studies in the Apical Membrane Antigen of P.vivax from Indian Isolates

Introduction

Plasmodium vivax is the most common and most widely distributed of four human malaria species (*P. falciparum*, *malariae*, *ovale*, and *vivax),* with an estimated burden of 70–80 million cases annually (Sina, B., 2002). Although it causes a less severe disease than *Plasmodium falciparum*, being rarely lethal, *P. vivax* affects the working capacity of the population and the lack of efficient drug distribution favors the onset of drug resistant strains (Wilairatana, P. *et. al.* 1999, Imwong, M. *et. al*. 2001). *P. vivax* causes up to 65% of malaria in India and is becoming increasingly resistant to malarial drugs. Moreover recent reports suggest that *P.vivax* is also responsible for causing severe malaria in parts of India. (Kochar *et. al.* 2005).

Symptoms of *P. vivax* malaria are similar to those of other types of malaria and include cyclical fever and chills, headache, weakness, vomiting, and diarrhea. The most common complication is enlargement of the spleen. *P.vivax* malaria is rarely fatal, but relapses often occur months to years after treatment because some of the parasites can become dormant in the liver. Special medication can be taken to kill the dormant parasites.

There is an intense global effort to develop an effective vaccine in addition to the malaria control measures currently in use. Several vaccine candidate antigens have been identified against different stages of the two main human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*, and are being developed as a part of a subunit vaccine (Miller *et. al.* 1998, Richie *et. al* . 2002). A major concern in malaria vaccine development is the polymorphism observed among different *Plasmodium* isolates in different geographical areas across the globe. Several *in vitro* and epidemiological studies have demonstrated that natural variations can abrogate immune recognition (Qari *et. al.*

1998, De La Cruz *et. al*. 1988). Thus, studies of sequence and antigenic diversity of malaria vaccine candidate antigens becomes a subject of considerable importance.

AMA – 1 of ***Plasmodium vivax***: The Apical Membrane Antigen (AMA-1) is a leading vaccine candidate antigen against asexual blood stages of malaria parasite. Apical membrane antigen (AMA-1) is one of the proteins involved in merozoite invasion of erythrocytes (Thomas *et .al* 1984, Deans *et. al* 1988). The coding region of *P. vivax* AMA-1 is 1686 bp in length with a 56% A+T content, which is not only lower than that of the *P. falciparum* (70%)and *Plasmodium chabaudi* (69%), but also lower than that of the *P. fragile* and *P. knowlesi* (60%). There is no repetitive element in the sequence. The translated protein sequence is 562 amino acids long, containing 19 cysteines. All 19 cysteines are conserved in *P. fragile* and *P.knowlesi* and 16 are conserved in *P. falciparum*. The protein also contains two hydrophobic regions a 13 amino acid putative signal sequence at the N-terminal and a 24 amino acid potential transmembrane domain towards C-terminus.

As a promising blood stage malaria vaccine candidate, AMA-1 has been cloned and sequenced from a number of *Plasmodium* species including *Plasmodium falciparum* (Peterson *et. al* 1989), *Plasmodium knowlesi* (Waters *et.al*. 1990), *Plasmodium fragile* (Peterson *et. al* 1990), and *Plasmodium chabaudi* (Marshall *et. al* 1989). *P.vivax* AMA-1 is more closely related to the AMA-1 from *P. fragile* and *P. knowlesi* than from *P. chabaudi* or *P. falciparum*. The nucleotide sequence comparison of the *P. vivax* AMA-1 to that of *P.knowlesi*, *P.fragile*, *P. chabaudi* and *P. falciparum* shows 86%, 85%, 60%, and 54% identity at the nucleotide level and the amino acid sequences gave 86%, 83%, 57%, and 52% identity respectively. Limited sequence variation of the AMA - 1 gene has been reported among the *falciparum* strains (Thomas *et. al.* 1990) as well as two of the known *P. knowlesi* strains (Waters *et. al*. 1991). However there are no extensive polymorphism studies done on the sequence of AMA-1 gene of *P.vivax*, a species that causes substantial human morbidity. The study of polymorphism is not only important in establishing the antigenic repertoire of isolates from malaria endemic regions but also in elucidating the mechanisms by which antigenic diversity may be generated.

One of the first studies reported on *P.vivax* AMA – 1 was by Cheng *et. al.* 1994, where they reported the complete sequences of the *P. vivax* AMA-1 gene amplified from two patients, one from Philipines and the other from Brisbane who had visited Indonesia, Malaysia and Thailand. The two gene sequences differed only by 12 single nucleotide substitutions resulting in 9 amino acid changes. Majority of these changes 8/12 occurred in a region from 300 – 700 bp resulting in 6 amino acid substitutions. This region was then probed with additional 6 Asian pacific isolates and the amino acid sequence comparison showed additional 8 non synonymous amino acid substitutions. Thus, they also reported sequence variations in one polymorphic region of AMA-1 from 6 Asia Pacific isolates.

Figtree *et. al*. (2000) sequenced the polymorphic regions of the AMA-1 gene corresponding to base pairs 320 –738 from 219 isolates from different parts like Africa, China, India, Indonesia, Philippines, Papua New Guinea, Solomon Islands and Thailand. The sequences were analysed to examine the population diversity both within and between geographical areas. From the 219 sequences analyzed, 69 haplotypes were identified with 22 polymorphic sites. Analysis of the differences between the sequences gave an average pairwise difference of 7.16 for the comparison of all sequences obtained with a corresponding nucleotide diversity per base pair was 0.0174 _+ 0.0090. Only 4 previously unreported polymorphic nucleotides were found, and three new substitutions were found at previously reported polymorphic regions.

Han *et. al.* (2002) examined the genetic variation in the region encompassing the apical membrane antigen – 1 (PvAMA-1) of parasites by DNA seqencing of the 22 re-emerging *P.vivax* isolates from Korea. This study showed that the 22 isolates belong to previously reported SKO1 (9 isolates) and SKOII (13 isolates) haplotypes. The present results strongly suggested that the reemergence of *P.vivax* in South Korea originated from East Asia and this was a significant study being the first report on PvAMA-1 genes of the re-emerging *P. vivax* in South Korea.

Another study from re-emerging Korean isolates by Chung *et. al.* (2003) investigated the polymorphism of PvAMA-1 in 30 Korean Isolates corresponding to nucleotides 324 - 735. Two genotypes (SKA and SKG) were identified on the basis of amino acid substitution. They confirmed that these were similar to Chinese isolates and the reemergence originated in East Asia.

MAbs reactive with AMA-1 and capable of inhibiting erythrocyte invasion in vitro recognize disulfide bond-dependent conformational epitopes (Deans *et. al.* 1987, Thomas *et. al*. 1984) and only the properly folded protein confers protection (Anders *et.al.* 1998, Crewther *et. al*. 1996, and Deans *et. al.* 1988). Therefore a eukaryotic expression system will most readily produce AMA-1 material directly qualified for vaccine evaluation. Kocken *et. al.* (1999) described for the first time high-level secreted expression (over 50 mg/liter) of the *Plasmodium vivax* AMA-1 (PV66/AMA-1) ectodomain by using the methylotrophic yeast *Pichia pastoris*. To prevent nonnative glycosylation, a conservatively mutagenized PV66/AMA-1 gene (PV66Δglyc) lacking N-glycosylation sites was also developed. Expression of the PV66Δglyc ectodomain yielded similar levels of a homogeneous product that was nonglycosylated and was readily purified by ion-exchange and gel filtration chromatographies. Recombinant PV66Δglyc43–487 was reactive with conformation-dependent monoclonal antibodies. With the SBAS2 adjuvant, Pichia-expressed PV66Δglyc43–487 was highly immunogenic in five rhesus monkeys, inducing immunoglobulin G enzyme-linked immunosorbent assay titers in excess of 1:200,000. This group of monkeys had a weak trend showing lower cumulative parasite loads following a *Plasmodium cynomolgi* infection than in the control group. This was the first report of PV66/AMA-1 protein production in any system.

To check whether the diversity in sequences reported in AMA-1 were a result of immune selection, a recent study was carried out by Rodriguez *et. al.* 2005, where they evaluated the naturally acquired antibody response to the *Plasmodium vivax* apical membrane antigen 1 (PvAMA-1), a leading vaccine candidate against malaria. The gene encoding the PvAMA-1 ectodomain region (amino acids 43–487) was cloned by PCR using genomic DNA from a Brazilian individual with patent *P. vivax* infection. A recombinant

protein representing the PvAMA-1 ectodomain was expressed in *Escherichia coli* and refolded. By ELISA, this recombinant protein reacted with 85 and 48.5% of the IgG or IgM antibodies, respectively, from Brazilian individuals with patent *P. vivax* malaria. IgG1 was the predominant subclass of IgG. This study clearly provided further evidence that PvAMA-1 is highly immunogenic during natural infection in humans and displays limited polymorphism in Brazil.

To date, little is known about the extent of sequence variation in the *Plasmodium vivax AMA-1* gene (*Pvama-1*) among Indian isolates. Since *P. vivax* accounts for >50% of malaria cases in India, it is essential to know the *Pvama-1* gene variability in these countries to sustain it as a vaccine candidate. The extent of polymorphism in *Pvama-1* gene among Indian isolates is studied and reported here which can be of immense importance to the malaria vaccine initiative, against *Plasmodium vivax* .

Globally, a relatively small proportion of malaria vaccine development funding goes toward *P. vivax* vaccine candidates, even though much less is known about this form of the malaria parasite. However, 1999 saw the establishment of an Asian *P. vivax* network to create, improve, and expand research on the parasite. Because of the frequency of concurrent *P. vivax* and *P. falciparum* infection, scientists hope to eventually develop a combined vaccine that will prevent and/or lessen the severity of both these types of malaria.

Results and Discussion:

The gene encoding the AMA - 1 is highly conserved among *Plasmodium* species of rhodents, monkeys and humans. This suggests that AMA –1 is essential to the biology of the parasite and that functional constraints limit the diversity of the gene. However, in all the cases where the sequence from different isolates of the same species have been examined, there are many regions of the gene where many apparently unlinked single base substitutions occur. However, the genetic diversity of the AMA –1 gene has not been studied extensively.

Polymorphism studies to detect the sequence diversity in the AMA-1 gene of *P.vivax* is very significant for malaria vaccine design. The limited information on genetic diversity of the *P.vivax* AMA - 1 gene can be judged by the fact that only 2 full length sequences are available and compared (Cheng *et. al*., 1994). Out of the two sequences one PH-84 was from Morong, Philipines, and the other PVQ was from Brisbane, (the latter had visited Indonesia, Malaysia and Thailand). Kocken *et. al*. in 1999 published the 1335 bp sequence of Sal 1 isolate of *P.vivax*. Most of the other studies in *P. vivax* have concentrated only on the hyper variable region in first half of the gene, i.e. from 300 bp to 750 bp. (Figtree *et. al.,* 2000., Han *et. al.* 2002., Chung *et. al.* 2003). The sequence diversity of the AMA - 1 gene of *P.vivax* has been studied to a very limited extent in an endemic country like India where *P.vivax* is responsible for causing 50% of the infections. In view of this fact, it is also significant to note that, there are no reports of the complete gene sequences of AMA-1 of *P.vivax* from India. This study aims to classify the polymorphism found in the nearly complete *P.vivax* gene from 11 Indian isolates.

The AMA - 1 (Apical Membrane Antigen – 1) gene from *P.vivax* was amplified from 11 Indian Isolates living in the western part of the country in Rajasthan. Out of the 11 samples studied, three samples S-3, S-5, and S-6 were of patients with severe malaria. A single band of 1640 bp (corresponding to 1-1640 bp of the PH-84 sequence) was obtained after PCR amplification and the amplicons were sequenced twice using automated sequencing at Shankaranethralaya, Chennai and Oscimum Biosolutions , Hyderabad. The results were analyzed and compared with the corresponding previously reported sequences of the prototypic alleles PH-84, PVQ (Cheng *et .al.*, 1994) and Sal 1 (Kocken *et.al.,* 1999).

Analysis and comparison of the Indian isolates at the nucleotide level showed that point mutations occur at 44 positions along the length of the gene as against the 23 changes reported previously (Cheng *et. al*., 1994, Kocken *et. al.,* 1999). Out of these nucleotide substitutions, 5 result in synonymous amino acid changes and 39 are non-synonymous amino acid changes. 16 out of these 48 mutations occur at the first base of the codon, 16 at the second base and 12 at the third base of the codon, resulting in significant amino acid changes in the AMA-1 protein. So, our study shows nearly 2 times more variation as compared to previous reports. The changes of PvAMA-1 at nucleotide level are depicted in Table 3.1

At the protein level, our analysis showed a total of 32 amino acid changes as against only 21 reported previously (Cheng *et.al* 1994, Figtree *et. al*. 2000, Eun-Taek Han *et. al.* 2002., Joon-Yong Chung *et.al.* 2003). Out of the 21, 17 changes were conserved, while variations at positions 133, 172, 191 and 218 were not found in our study with Indian isolates. 15 new changes were found in our study at positions 11, 52, 63, 66, 69, 277, 472, 368, 382, 438, 445, 519, 530, 538 and 540 (Table 3. 2).

The AMA-1 protein of *P.vivax* has an ectodomain region comprising of Amino acids 43 – 487 (Pizarro *et. al.* 2005). Study of variations in the ectodomain shows that 27 out of 32 amino acid changes lies in this region, making it highly polymorphic (Table 3.2). This could have significant effect on the immunological properties of the antigen.

PAIRWISE PERCENT IDENTITY WITH PH - 84:

The greatest number of nucleotide difference was seen in S5 isolate that accounts for 1.4 % variability with the prototype. The isolate showing maximum similarity with the prototype was PV6 with 99.4% identity. (Table 3. 3). At the protein level, maximum variability was found in PV2 isolate, which showed 3.2 % variations with the prototype PH – 84. The isolate, which was closest to the prototype showing maximum similarity was PV8 with 99% identity. (Table 3. 4).

Table 3.1: Analysis and comparison of the Pv AMA-1in Indian isolates at the nucleotide level

S.NO	Position of base change	Codon change position	PH-84	PVQ	Sal1	PV1	PV2	PV3	PV4	PV6	PV8	PV9	PV12	S3	S5	S6	Codon change	A.mino Acid change
1	31	1	A	A	-	A	A	A	A	G	A	A	A	A	A	A	AGC-GGC	Ser-Gly
2	154	1	A	A	G	A	A	A	A	A	A	A	A	A	A	A	AGT-GGT	Gly-Ser
3	188	2	A	A	A	A	G	A	A	A	A	A	A	A	A	A	GAC-GGC	Asp-Gly
4	197	2	G	G	G	G	A	G	G	G	G	G	G	G	G	G	AGA-AAA	Arg-Lys
5	206	2	G	G	G	G	A	G	G	G	G	G	G	G	G	G	AGT-AAT	Ser-Asn
6	306	3	C	C	T	C	C	C	C	C	C	C	C	C	T	T	TCA-TCT	Val-Val
7	318	3	C	C	T	C	C	C	C	C	C	C	C	C	T	T	TCC-TCT	Ser-Ser
8	319	1	G	G	G	G	G	G	G	G	A	G	G	G	G	G	GCT-AAT	Ala-Asn Ala-Asp
9	320	2	C	A	A	C	C	C	C	C	A	C	C	C	A	A	GCT-AAT GCT-GAC	Ala-Asn Ala-Asp
10	321	3	T	T	C	T	T	T	T	T	T	T	T	T	C	C	GCT-GAC	Ala-Asn Ala-Asp
11	335	2	C	C	G	G	A	A	C	A	C	A	C	C	A	A	ACC-AGA ACC-AAA	Thr-Arg Thr-Lys
12	336	3	C	C	A	A	C	A	C	A	C	A	C	C	A	A	ACC-AGA ACC-AAA	Thr-Arg Thr-Lys
13	359	2	G	G	A	G	G	G	G	G	G	G	G	G	G	G	AGG-AAG	Arg-Lys
14	390	3	G	A	T	T	T	T	T	T	T	T	T	T	T	T	AAA-AAT	Lys-Asn

15	394	1	G	G	A	G	G	G	G	G	G	G	G	G	A	A	GAT-AAT	Asp-Asn
16	411	1	A	A	G	A	A	A	A	A	A	A	A	A	A	A	CCC-CCA	Pro-Pro
17	418	1	T	T	T	T	T	T	A	T	T	T	A	T	A	A	TTA-ATA	Leu-Ile
18	422	2	C	A	C	C	C	C	C	C	C	C	C	A	C	C	GCG-GAG	Ala-Glu
19	434	2	A	C	A	A	C	A	C	A	C	A	C	C	C	C	GAA-GCA	Glu-Ala
20	565	1	A	G	G	A	A	A	A	A	G	A	A	A	G	G	AAT-GAA	Asn-Glu
21	567	3	T	A	A	A	A	A	A	A	A	A	A	T	A	A	AAT-GAA AAT-AAA	Asn-Glu Asn-Lys
22	568	1	A	A	A	A	A	A	G	A	A	A	G	A	A	A	AAA-GAA	Lys-Glu
23	577	1	C	T	C	C	C	C	C	C	C	C	C	C	C	C	CAC-TAC	His-Tyr
24	628	1	T	C	C	T	T	T	T	T	T	T	T	C	T	C	TCA-CCA	Ser-Pro
25	680	2	A	A	A	A	A	A	T	A	A	A	T	T	A	A	GAA-GTA	Glu-Val
26	682	1	A	A	A	A	A	A	G	A	A	A	G	G	A	A	AGC-GAT	Ser-Asp
27	683	2	G	G	G	G	G	G	A	G	G	G	A	A	G	G	AGC-GAT	Ser-Asp
28	684	3	C	C	C	C	C	C	T	C	C	C	T	T	C	C	AGC-GAT	Ser-Asp
29	829	1	A	A	G	A	A	A	A	A	A	A	A	A	A	A	AAG-GAG	Glu-Lys
30	849	3	T	C	C	C	C	C	C	C	C	C	C	C	C	C	AAT-AAC	Asp-Asp
31	1056	3	T	G	G	G	G	G	G	G	T	G	G	G	G	T	AAT-AAG	Asp-Lys
32	1103	2	A	A	A	A	A	A	A	A	A	A	A	A	T	T	AAA-ATA	Lys- Ile

33	1145	2	T	T	T	T	T	T	A	T	T	T	A	T	T	T	GTA-GAG	Val-Glu
34	1146	3	A	A	A	A	A	A	G	A	A	A	G	A	A	A	GTA-GAG	Val-Glu
35	1151	2	C	G	T	G	G	G	C	G	C	G	C	C	C	C	CCG-CGG	Pro-Arg
36	1155	3	G	T	G	T	T	T	G	T	G	T	G	G	G	G	GAG-GAT	Glu-Asp
37	1313	2	A	A	G	A	-	A	A	A	A	A	A	G	A	G	CAC-CGC	His-Arg
38	1333	1	A	A	A	A	-	A	A	A	A	A	A	G	A	A	AAC-GAC	Asn-Asp
39	1414	1	G	G	A	G	G	G	G	G	G	G	G	G	G	G	GAT-AAT	Asn-Asp
40	1556	2	T	T	-	G	-	T	T	T	T	T	T	T	T	T	ATG-AGG	Met-Arg
41	1588	1	A	A	-	T	-	A	A	A	A	A	A	G	A	A	ACC-TCC ACC-GCC	Thr-Ser Thr-Ala
42	1612	1	C	C	-	C	-	C	C	C	C	C	A	C	C	C	CCC-ACC	Pro-Thr
43	1618	1	G	G	-	G	-	G	G	G	G	G	G	G	G	A	GCC-AGC	Ala-Ser
44	1619	2	C	C	-	C	-	C	C	C	C	C	C	C	C	G	GCC-AGC	Ala-Ser

Table 3. 2: Amino acid changes of the Pv AMA-1 in Indian Isolates

S.No	Position of Amino Acid	PH-84 (A)	PVQ (B)	Sal 1 (C)	PV1	PV2	PV3	PV4	PV6	PV8	PV9	PV12	S3	S5	S6	No. of Indian Isolates having Aminoacids A,B,C – similar to PH- 84, PVQ, Sal1 * - different from prototypes.
1	11	S	S	-	S	S	S	S	G	S	S	S	S	S	S	10 (A,B), 1*
2	52	S	S	G	S	S	S	S	S	S	S	S	S	S	S	11(A,B)
3	63	D	D	D	D	G	D	D	D	D	D	D	D	D	D	10 (A,B, C), 1*
4	66	R	R	R	R	K	R	R	R	R	R	R	R	R	R	10 (A,B, C), 1*
5	69	S	S	S	S	N	S	S	S	S	S	S	S	S	S	10 (A,B,C), 1*
6	107	A	D	D	A	A	A	A	A	N	A	A	A	D	D	8A, 2 (B,C) 1*
7	112	T	T	R	R	N	K	T	K	T	K	T	T	K	K	4 (A,B), 1C 6*
8	120	R	R	K	R	R	R	R	R	R	R	R	R	R	R	11(A,B)
9	130	K	K	N	N	N	N	N	N	N	N	N	N	N	N	11C
10	132	D	D	N	D	D	D	D	D	D	D	D	D	N	N	9 (A,B), 2C
11	140	L	L	L	L	L	L	I	L	L	L	I	L	I	I	7 (A,B,C), 4*
12	141	A	E	A	A	A	A	A	A	A	A	A	E	A	A	10 (A,C), 1B
13	145	E	A	E	E	A	E	A	E	A	E	A	A	A	A	4 (A, C), 7B
14	189	N	E	E	K	K	K	K	K	E	K	K	N	E	E	1A, 3 (B, C) 7*

15	190	K	K	K	K	K	K	E	K	K	K	E	K	K	K	9 (A,B,C), 2*
16	193	H	Y	Y	H	H	H	H	H	H	H	H	H	H	H	11A
17	210	S	P	P	S	S	S	S	S	S	S	S	P	S	P	9A, 2 (B,C)
18	227	E	E	E	E	E	E	V	E	E	E	V	V	E	E	8 (A,B,C) 3*
19	228	S	S	S	S	S	S	D	S	S	S	D	D	S	S	8 (A,B,C), 3*
20	277	K	K	E	K	K	K	K	K	K	K	K	K	K	K	11(A,B)
21	352	N	K	K	K	K	K	K	K	N	K	K	K	K	N	2 A, 9 (B,C)
22	368	K	K	K	K	K	K	K	K	K	K	K	K	I	I	9 (A,B,C), 2*
23	382	V	V	V	V	V	V	E	V	V	V	E	V	V	V	9 (A,B,C), 2*
24	384	P	R	L	R	R	R	P	R	P	R	P	P	P	P	6 A, 5 B
25	385	E	D	E	D	D	D	E	D	E	D	E	E	E	E	6 (A,C), 5B
26	438	H	H	R	H	-	H	H	H	H	H	H	R	H	R	8 (A,B), 2C
27	445	N	N	N	N	-	N	N	N	N	N	N	D	N	N	9 (A,B,C), 1*
28	472	D	D	N	D	-	D	D	D	D	D	D	D	D	D	10(A,B)
29	519	M	M	-	R	-	M	M	M	M	M	M	M	M	M	9 (A,B), 1*
30	530	T	T	-	S	-	T	T	T	T	T	T	A	T	T	8 (A,B), 2*
31	538	P	P	-	P	-	P	P	P	P	P	T	P	P	P	9 (A,B), 1*
32.	540	A	A	-	A	-	A	A	A	A	A	A	A	A	T	9 (A,B) 1*

* Number of Indian isolates showing amino acid residues altogether different from PH-84 , PVQ & Sal 1

Table 3.3 Pairwise percent identity of Pv AMA-1 of Indian isolates with PH-84 at nucleotide level

S.No.	Sample	%identity with PH - 84
1	PV1	99.2
2	PV2	99
3	PV3	99
4	PV4	98.7
5	PV6	99.4
6	PV8	99.2
7	PV9	98.7
8	PV12	98.7
9	S3	98.8
10	S5	98.6
11	S6	98.9

Table 3. 4 : Pairwise percent identity of Pv AMA-1 of Indian isolates with PH - 84 at protein level.

S.No.	Sample	Pairwise percent identity with PH - 84
1.	PV1	98.5
2.	PV2	96.8
3.	PV3	98.8
4.	PV4	98.1
5.	PV6	98.5
6.	PV8	99
7.	PV9	98.8
8.	PV12	98.05
9.	S3	98.25
10.	S5	97.95
11.	S6	97.75

Cheng *et. al*. did a sequence comparison of the near full length AMA-1 gene from the two *P.vivax* patients which showed limited polymorphism . These gene sequences differed by 12 single bases, resulting in 9 amino acid substitutions. The majority of these changes 8/12 occurred in the region from 300 – 700 bp resulting in 6 amino acid substitution*s.* This region was then studied in additional 6 Asian pacific isolates, and 8 more nonsynonymous amino acid substitutions were reported. Thus, based on these sequence variations, this polymorphic region of AMA-1 was classified as the hypervariable region.

Analysis of the Pv AMA-1 hypervaribale region, from 300 – 750 bp, by Figtree *et. al.* (2000) in isolates from ten different geographic locations, reported sequence variations at 22 nucleotide positions. Figtree *et. al*., 2000., based on the 219 sequences analyzed, identified 69 haplotypes with 22 polymorphic sites, resulting in 17 amino acid changes in the hypervariable region of PvAMA-1. So, based on these studies, a total of 18 amino acid changes were reported in the hypervariable region of the AMA-1 protien. (Cheng *et. al,* 1994 , Figtree *et.al*. 2001, Kocken *et. al.,* 1999). Among the different *P. vivax* isolates, the Pv AMA-1 gene exhibits sequence diversity and has been used as a useful marker for typing *P. vivax* populations.

Analysis of this region in our Indian isolates shows that a total of 14 changes out of the 18 previously reported changes are conserved. However, 4 changes found previously were not found in the 11 isolates studied from India. Even in the 14 conserved changes some differences were observed. At position 107, a new amino acid change is observed in isolate PV8 where it is N instead of a dimorphic change reported previously from A to D. Similarly at position 112, 4 Indian isolates contain T and show similarity with prototypes PH-84 and PVQ , whereas one isolate has R shows similarity with Sal I. However, 5 isolates show amino acid K at this position and one isolate shows a change from T to N. At amino acid position 120, all Indian strains have R and are similar to the prototype PH - 84 and PVQ, but different from Sal 1 which has K at this position. Similarly at position 130, all the Indian isolates are similar to Sal 1 and have N whereas the other two prototypes PH-84 and PVQ have K at this position. The four amino acid positions where

all the Indian isolates are similar to all three prototypes and show no variation are 133, 172, 191, and 218.

On comparing the 69 haplotypes with our 11 Indian samples, we found that only 5 isolates confer within the previously reported 69 haplotypes (Table 3.5). All the 5 isolates match with three of the previously reported 12 Indian haplotypes (Figtree *et. al.,* 2000). From the remaining 6 sequences 5 new haplotypes have been identified in this study (Table 3.5). These haplotypes differ in the amino acid positions 112, 145, and 189 to generate these 5 new combinations not reported in any other study (Table 3.5).

The structure of PvAMA-1 is stabilized by 19 cysteine residues, which are conserved as per previous reports. Our study also confirmed this fact as it was found that all 19 cysteine residues were totally conserved in the 11 Indian isolates studied.

Table 3. 5: Haplotypes found in the Indian *P.vivax* isolates

Sample	**Position of the Aminoacid**																	**Haplotypes Identified**
	112	120	130	132	133	140	141	145	172	189	190	191	193	210	218	227	228	
PV1	R	R	N	D	D	L	A	E	A	K	K	T	H	S	V	E	S	**New**
PV2	N	R	N	D	D	L	A	A	A	K	K	T	H	S	V	E	S	**New**
PV3	K	R	N	D	D	L	A	E	A	K	K	T	H	S	V	E	S	**New**
PV4	T	R	N	D	D	I	A	A	A	K	E	T	H	S	V	V	D	ABM
PV6	K	R	N	D	D	L	A	E	A	K	K	T	H	S	V	E	S	**New**
PV8	T	R	N	D	D	L	A	A	A	E	K	T	H	S	V	E	S	**New**
PV9	K	R	N	D	D	L	A	E	A	K	K	T	H	S	V	E	S	**New**
PV12	T	R	N	D	D	I	A	A	A	K	E	T	H	S	V	V	D	ABM
S3	T	R	N	D	D	L	E	A	A	N	K	T	H	P	V	V	D	ACP
S5	K	R	N	N	D	I	A	A	A	E	K	T	H	S	V	E	S	AAZ
S6	K	R	N	N	D	I	A	A	A	E	K	T	H	P	V	E	S	AAY

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Unique features of the three severe malaria cases were analysed to detect if any differences were observed in the genetic profile of these isolates, as compared to reported non-severe cases. Based on our study, it was found that there was no marked difference within the hypervariable region of *P. vivax* AMA-1. However, at positions 445 and 530, one isolate S3, showed variations from N to D and T to A respectively. S5 and S6 showed a unique variation at position 368 where K was substituted with I. Also, S6 at amino acid position 540 showed a substitution from A to T. The other severe malaria cases showed conserved amino acids at these positions with one of the prototypes. So the significance of these changes need to be further elucidated in severe malaria cases.

In conclusion, we have studied the genetic diversity of the 1640 bp region of Pv AMA-1 from Indian isolates, and found much higher variation than reported previously. Moreover, the 5 new haplotypes identified in this study, are previously unreported in any other study, making this outcome very significant. Our results, though are preliminary findings, indicate that still much more extensive study needs to be done on the polymorphism of this important vaccine candidate, and geographic distribution of different haplotypes would be very significant in malaria vaccine design.

Chapter 4

Investigation of Genetic Diversity of the Apical Membrane Antigen of *P. falciparum* in Isolates from India.

Introduction:

Variation among malaria parasites has been assumed to be a major reason why immunity to infection with a particular species of malaria parasite develops after repeated infections over a number of years. Recent progress in the characterization of malaria antigens has provided support for this assumption by revealing extensive polymorphisms and associated antigenic diversity in many different parasite proteins. The most detailed information available concerns antigens expressed by the asexual blood stages of *P. falciparum* the parasite that causes the most severe form of human malaria. (Anders R.F. *et. al.* 1994).

There are two aspects of antigenic diversity among malaria parasites. One aspect concerns polymorphisms in allelic genes, giving rise to the expression of structurally and antigenically distinct forms of a particular protein in different parasites. Because there are large number of different polymorphic genes that are not linked, and there is recombination and reassortment of these genes during meiosis, there is potential for a very large number of different genotypes within one species of *Plasmodium* (Kemp *et. al.,* 1986). A second aspect of diversity is antigenic variation whereby a clonal population of parasites can change its antigenic phenotype.

Asexual blood stage antigens of *P. falciparum* can be classified into those exhibiting minor polymorphisms involving point mutations and those in which there are major polymorphisms often involving changes in repetitive sequences.

Apical Membrane antigen (AMA-1), a likely candidate for inclusion in a malaria vaccine, is a protein in which diversity has been generated by a series of point mutations. Studies have been carried out to study the polymorphism present in the apical membrane antigen 1 both at the genetic level and at the protein level.

These polymorphism studies serve two major functions :

i. To study the effect on function
ii. to study the effect on immunogenecity of the antigen

A long term goal of such studies related to vaccine development is, to identify naturally immunogenic determinants of key vaccine antigens and to assess how the parasite genetic diversity relates to natural immunity. A step in accomplishing this goal is the characterization of the genetic diversity using isolates of same species from different geographic regions and at the same time isolates of different species of *Plasmodium*.

One of the earliest studies carried out by Thomas *et.al* (1990) concluded that proline residues and cysteine residues are highly conserved in analogous molecules of AMA-1 present across various species of the malarial parasite. None of the cysteine residues undergo any form of substitution across different strains of *Plasmodium falciparum*, and also across different species of *Plasmodium*. These residues are important for the functional conformation of the molecule and hence are conserved. They also described a hot region of variation to be present between residues 160-210 and much of the remaining variability is present in clusters distributed throughout the molecule. The overall variation between the 5 strains that they studied was found to be just 4 % at both the genetic and protein level.

An extension of the above studies was done by Oliveria *et.al (*1996) where, the predicted amino acid sequence of the four field isolates were aligned and compared with five laboratory strain sequences. No significant difference was found in field isolates that are subject to host immune pressures. The overall sequence identity at DNA level for the four

field isolates parasite genes was greater than 98%. Identity and similarity at amino acid level between field and laboratory derived strains ranges from 94. 85 – 97.74 % and 96.78 – 99.03 % respectively. Thus, this study suggested a remarkable conservation of the AMA-1 molecule. The study and analysis of the sequence of this antigen allowed the division of the molecule into 12 blocks. Fig. 5. Blocks 1,3,5,7,9 and 11 are conserved regions of the protein, representing 43% of the whole molecule. Blocks 2,6,8,10, and 12 are semi-conserved regions with clusters of mutations scattered among stretches of conserved amino acids. 20 % of all the mutations that are found in the AMA-1 molecule are located in block 4, a stretch of 46 amino acids located between the first two-conserved cysteine residues. Block 11 contains the amino acid sequence considered the trans-membrane domain of the molecule. It is an extremely conserved region in *Plasmodium falciparum* AMA-1 sequence. Block 12 spans 55 amino acids and composes the cytoplasmic domain of the protein. A few mutations are found in this area, but as this block is not directly exposed to protective host immune responses, this area is not involved in parasite evasion mechanisms **.** So a conclusion was drawn based on these studies that AMA-1 is a very conserved molecule among the various antigens studied.

Following these, Marshall *et. al*. (1996) carried out studies on field isolates and they reported that AMA-1 occurs as distinct allelic variants that differ by a number of point mutation. They reported a greater degree of variation than previously known. They also defined the region between the first and eighth cysteine residues of the mature AMA-1 protein (148-337 amino acids) as the hypervariable region or HVR as 24 of the 53 positions falling within this region. Sequence differences in the HVR defined residues 138 – 307 allow the AMA-1 sequence to be grouped into several families. Computer analysis revealed a series of restriction enzyme sites that were used to group AMA-1 alleles into 4 major families (Table 4.5).

Based on these data, Eisen *et. al* (1999) reported a *Plasmodium falciparum* AMA-1 gene generated by intragenic recombination. The identification of an AMA-1 intragenic recombination presented an additional mechanism of generation of sequence diversity. The intragenic recombination event occurred in domain I between nucleotide positions

524 – 555, a region that is completely conserved in other *Plasmodium falciparum* sequences. This was the first report of an apparent intragenic recombination in AMA-1.

In another significant study Verra and Hughes (2000) tested whether the host immune system exerts positive diversifying selection on AMA-1, by comparing the ratio of synonymous and non – synonymous mutations in AMA-1. They concluded that AMA-1 polymorphism is maintained by balancing selection related to avoidance of immune recognition by the vertebrate host. Hughes *et. al.* in 1995 analysed the numbers of synonymous and nonsynonymous nucleotide substitutions per site in eight polymorphic genes of *P. falciparum* and suggested that the sporozoite and the merozoite surface proteins are under positive selection, presumable exerted by host immune system. A strong diversifying selection on domains of the *Plasmodium falciparum* AMA-1 gene was also reported by Polley and Conway in the year 2003**.** Escalante *et. al* (2001) also showed definitive evidence for positive natural selection in the genes encoding AMA - 1, CSP, LSA-1, MSA-1 and Pfs48/45 again by comparison of synonymous and nonsynonymous substitutions in these genes.

About the domains of AMA-1, earlier study by Hodder *et. al* (1996) reported that distribution of mutations within the sequence of AMA-1 is not uniform. Domain I is the most diverse containing 58 % of all sites where amino acid substitutions take place. He also predicted that these amino acid substitutions might define conformational disulphide bond dependent epitopes that are recognized by protective immune responses.

However, the most comprehensive and recent publication on polymorphism studies is by Escalante *et. al*. in the year 2001 where they have investigated the genetic diversity in AMA-1 gene in natural populations of western Kenya and compared it with parasite populations from other geographic regions. They also concluded that the cytoplasmic tail appears to be more polymorphic than the extracellular and transmembrane regions. They also reported that the number of non-synonymous substitutions is nearly three times the number of synonymous substitutions. They also proved extensive polymorphism in the B and T – cell epitopes of AMA-1. They established that polymorphism observed in 7 of

the 9 epitopes is maintained by natural selection. The vaccine related significance of all the above mentioned polymorphism need to be further investigated.

Alfred Cortes *et. al* in the year 2002 sequenced the most variable region of AMA - 1 (domain I) from 168 isolates in Papua New Guinea in both symptomatic and asymptomatic lesions . Neutrality tests applied to these sequences provided strong evidence of selective pressure operating on the sequence of *AMA - 1* domain I, consistent with AMA - 1 being a target of protective immunity. Similarly, a peculiar pattern of geographical diversity and the particular substitutions found were suggestive of strong constraints acting on the evolution of AMA - 1 at the population level, probably as a result of immune pressure. In addition, a strong imbalance between symptomatic and asymptomatic infections was detected in the frequency of particular residues at certain polymorphic positions, pointing to AMA - 1 as being one of the determinants of the morbidity associated with a particular strain. The information yielded by this study has implications for the design and assessment of AMA – 1 based vaccines and provides additional data supporting the importance of AMA - 1 as a malaria vaccine candidate.

Results and Discussion

The AMA - 1 (Apical Membrane Antigen – 1) gene from *P. falciparum* was amplified from 10 Indian Isolates (R1-R5 from Rajasthan; S1-S5 from Siliguri, West Bengal) cloned, and both the amplicons and clones were sequenced using automated sequencing at CDFD, Hyderabad and the results were analyzed, and compared with the corresponding previously reported sequences of two prototypic alleles FC27 and 3D7.

DIVERSITY OF THE ANTIGEN AT GENETIC LEVEL:

The polymorphism study of the *Plasmodium falciparum* AMA - 1 gene at nucleotide level from the 10 Indian isolates shows that, point mutations occur at 161 positions along the length of the gene. Out of these, 30 are synonymous mutations and 131 are non-

synonymous changes. 55 mutations occur at the first base of codon, 50 at the second base and 56 at the third base of the codon. The variation of Pf AMA-1 among Indian isolates is depicted in Table 4.1.

Significant among these are variations found at positions 82, 88, 135, 146, 290, 523, 559, 561, 599, 616, 799, 1213, 1632, 1741, and 1767 where 80% of the Indian isolates are showing the same change against the prototype. Out of these, 82, 88, 135,146, and 1215 are new changes found in this study while others are previously reported changes. Another significant change includes at positions 960 and 1009 whyich are responsible for cysteine conversions to tryptophan and cysteine to glycine/serine at amino acid positions 320, at position 337 respectively.

Table 4. 1. Differences of Pf AMA-1 of Indian Isolates with prototype at nucleotide level

S.No	Position of base change	Codon change position	3D7	FC27	R1	R2	R3	R4	R5	S1	S2	S3	S4	S5	Codon change	Amino Acid change
1.	44	2	T	T	T	T	T	T	T	T	C	T	T	T	TTT - TCC	Phe - Ser
2.	45	3	T	T	T	T	T	T	T	T	C	T	T	T	TTT - TCC	Phe - Ser
3.	46	1	A	A	A	A	A	A	A	A	A	A	A	T	ACA - TCA	Thr - Ser
4.	56	2	T	T	G	T	T	T	T	T	G	T	T	G	ATA - AGA	Ile - Arg
5.	75	3	G	G	G	G	G	G	G	G	T	G	G	G	CAG - CAT	Gln - His
6.	76	1	A	A	T	A	A	A	A	A	T	A	A	T	AAT - TCT	Asn - Ser
7.	77	2	A	A	C	A	A	A	A	C	C	A	A	C	AAT - TCT	Asn - Ser
8.	82	1	T	T	T	T	G	G	G	G	G	G	G	G	TGG - GGG	Trp - Gly
9.	88	1	C	C	C	C	G	G	G	G	G	G	G	G	CAT - GAT	His - Asp
10.	102	3	T	A	T	T	T	T	T	T	T	T	T	T	AAT - AAA	Asn - Lys
11.	116	2	G	A	G	A	A	A	A	A	A	A	A	A	CGT - CAT	Arg - His
12.	135	3	G	G	G	G	C	C	C	C	C	C	C	C	AGG - AGC	Arg - Ser
13.	146	2	A	A	A	A	C	C	C	C	C	C	C	C	AAA - ACA	Lys - Thr
14.	154	1	G	C	G	G	G	G	G	G	G	G	G	G	GAA - CAA	Glu - Gln
15.	290	2	T	T	T	T	A	A	A	A	A	A	A	A	ATT - AAT	Ile - Asn
16.	523	1	T	T	G	G	G	G	G	G	G	G	G	G	TAT - GAT	Tyr - Asp
17.	559	1	G	G	G	A	A	A	A	A	A	A	A	A	GAA - AAT	Glu - Asn
18.	561	3	A	A	A	T	T	T	T	T	T	T	T	T	GAA - AAT	Glu - Asn
19.	589	1	G	G	C	G	G	G	G	G	G	G	G	G	GAA - CAA	Glu - Gln
20.	591	3	A	A	A	T	T	T	T	T	T	T	T	T	GAA - GAT	Glu - Asp
21.	599	2	A	A	A	G	G	G	G	G	G	G	G	G	CAT - CGT	His - Arg
22.	616	1	A	A	G	G	G	G	G	G	G	G	G	G	AAA - GAA	Lys - Glu
23.	674	2	T	T	A	T	T	T	T	T	T	T	T	T	ATT -AAT	Ile - Asn
24.	688	1	A	A	G	A	A	A	A	A	A	A	A	A	AAA - GAA	Lys - Glu
25.	727	1	A	A	A	A	C	A	A	A	A	A	A	A	AAA - CAA	Lys - Gln
26.	747	3	A	A	A	A	T	A	A	A	A	A	A	A	ATA -ATT	Ile -Ile
27.	799	1	G	G	C	C	C	C	C	C	C	C	C	C	GAA - CAA	Glu - Gln
28.	812	2	A	A	A	A	A	A	A	A	A	A	C	A	AAC - ACC	Asn - Thr

29.	817	1	A	A	A	A	C	A	A	A	A	A	A	A	ATG - CTG	Met - Leu
30.	818	2	T	T	T	T	T	T	T	T	G	T	T	T	ATG - AGG	Met - Arg
31.	834	3	A	A	A	A	A	A	A	A	A	A	C	A	CCA - CCC	Pro - Pro
32.	845	2	T	T	T	T	T	A	A	A	A	A	A	A	ATA - AAA	Ile - Lys
33.	848	2	C	C	C	T	C	C	C	C	C	C	C	C	TCA - TTA	Ser - Leu
34.	853	1	C	C	C	G	C	C	C	C	C	C	C	C	CAA - GAA	Gln - Glu
35.	856	1	A	A	A	A	C	A	A	A	A	A	A	A	AAC - CAC	Asn - His
36.	860	2	A	A	A	A	A	A	A	A	A	A	G	G	TAT - TGT	Tyr - Cys
37.	864	3	A	A	A	A	A	A	A	A	A	A	C	A	ACA - ACC	Thr - Thr
38.	876	3	G	A	A	G	G	A	A	A	A	A	G	A	AAG - AAA	Lys - Lys
39.	890	2	A	A	A	A	A	A	A	A	A	A	A	G	AAC - AGC	Asn - Ser
40.	898	1	A	A	A	A	A	A	A	G	A	A	A	G	AAA - GAA	Lys - Glu
41.	921	3	A	A	A	A	A	A	A	C	A	A	A	A	TTA - TTC	Leu -Phe
42.	922	1	C	C	G	C	C	C	C	C	C	C	C	C	CAG - GAG	Glu -Gln
43.	923	2	A	A	A	A	C	A	C	A	A	A	C	A	CAG - CCG	Glu -Pro
44.	927	3	T	T	T	C	T	T	T	T	T	T	T	T	AAT -AAC	Asn -Asn
45.	943	1	T	T	T	T	T	T	T	G	T	T	T	T	TGG - GGG	Trp -Gly
46.	950	2	A	A	A	A	A	A	A	A	A	A	G	A	GAT - GGT	Asp - Gly
46.	960	3	T	T	T	G	T	T	T	T	G	T	G	G	TGT - TGG	Cys - Trp
48.	963	3	A	A	A	A	A	A	A	A	A	A	G	A	GAA - GAG	Glu - Glu
49.	969	3	A	A	A	A	A	A	A	A	C	A	A	C	ATA - ATC	Ile - Ile
50.	972	3	A	A	C	A	A	A	A	A	A	A	C	C	CCA - CCC	Pro - Pro
51.	977	2	T	T	T	T	T	T	G	T	T	T	G	T	GTA - GGG	Val - Gly
52.	978	3	A	A	A	A	A	G	G	A	A	G	G	A	GTA - GTG	Val - Val
53.	979	1	A	A	A	A	A	G	A	A	A	A	A	A	AAT - GAT	Asn - Asp
54.	988	1	C	T	T	T	C	T	C	T	T	T	C	T	CCA - TCA	Pro - Ser
55.	995	2	T	T	T	A	T	A	A	A	T	A	T	A	ATT - AAT	Iso - Asn
56.	998	2	A	A	A	A	A	A	A	A	A	A	A	T	GAT - GTT	Asp - Val
57.	1004	2	T	T	T	A	T	T	T	T	T	T	T	T	TTT - TAT	Phe - Tyr
58.	1008	3	A	A	A	A	A	A	A	G	G	A	A	A	GAA - GAG	Glu - Glu
59.	1009	1	T	T	T	T	T	T	T	T	T	T	G	A	TGT – GGT AGT	Cys - Gly Ser

60.	1018	1	T	T	T	T	T	T	T	T	T	T	T	A	TTA - ATA	Leu - Ile
61.	1030	1	T	T	T	T	T	T	T	C	T	T	T	C	TTG - CTG	Leu - Leu
62.	1033	1	A	A	A	A	A	A	A	A	A	A	G	G	AGT - GGT	Ser - Gly
63.	1035	3	T	T	T	C	T	T	T	T	T	T	T	T	AGT - AGC	Ser - Ser
64.	1046	2	A	A	A	A	C	A	A	A	A	A	A	A	CAA - CCA	Gln - Pro
65.	1047	3	A	A	C	A	A	A	C	A	C	C	A	A	CAA - CAC	Gln - His
66.	1055	2	A	A	C	A	A	A	A	A	A	A	A	A	CAA - CCA	Gln - Pro
67.	1061	2	A	A	A	A	A	A	A	A	A	A	G	A	GAA - GGA	Glu - Gly
68.	1062	3	A	A	A	C	A	A	A	C	A	A	A	C	GAA - GAC	Glu - Asp
69.	1064	2	A	A	A	A	A	A	A	A	A	A	A	C	CAA - CCA	Gln - Pro
70.	1065	3	A	A	C	A	A	A	A	A	A	A	C	C	CAA - CAC	Gln - His
71.	1071	3	A	A	A	T	A	A	A	A	A	A	A	A	TTA - TTT	Leu - Phe
72.	1072	1	A	A	A	T	A	A	A	A	A	A	A	C	ACA - TCA	Thr - Ser
73.	1074	3	A	A	A	G	G	A	A	A	A	A	G	A	ACA - ACG	Thr - Thr
74.	1079	2	A	A	A	A	A	A	A	A	A	A	G	A	TAT - TGT	Tyr - Cys
75.	1087	1	A	A	A	C	A	A	A	A	A	A	A	A	ATT - CTT	Ile - Leu
76.	1088	2	T	T	T	T	T	T	T	T	T	T	T	A	ATT - AAT	Ile - Asn
77.	1093	1	G	G	G	G	G	G	G	G	G	G	G	A	GAA - AGA	Glu - Arg
78.	1094	2	A	A	A	G	A	A	A	A	A	A	G	G	GAA - AGA	Glu - Arg
79.	1095	3	A	A	A	C	A	A	A	A	A	A	A	A	GAA - AGA	Glu - Arg
80.	1100	2	T	T	T	T	T	T	T	T	T	T	C	T	TTC - TCC	Phe - Ser
81.	1102	1	A	A	A	A	A	A	C	A	A	A	A	A	AAA - CAA	Lys - Gln
82.	1103	2	A	A	A	A	A	A	A	A	A	A	G	A	AAA - AGA	Lys - Arg
83.	1107	3	T	T	T	C	T	T	T	T	T	T	T	T	AAT - AAC	Asn - Asn
84.	1112	2	A	A	A	C	A	C	A	A	C	A	G	A	AAC - ACC AGC	Asn - Thr Ser
85.	1140	3	T	T	T	C	T	T	T	T	T	T	T	C	CTT - CTC	Leu - Leu
86.	1144	1	A	A	A	A	A	A	C	A	A	A	A	C	ACT - CCT	Thr - Pro
87.	1149	3	T	T	T	T	T	G	T	T	T	T	T	T	GGT - GGG	Gly - Gly
88.	1157	2	A	A	A	G	A	A	A	A	A	A	A	A	AAA - AGA	Lys - Arg
89.	1183	1	A	A	A	C	A	A	A	A	A	A	A	A	AAG - CAG	Lys - Gln
90.	1188	3	T	T	T	A	T	T	T	T	T	T	T	T	GGT - GGA	Gly - Gly

91.	1194	3	T	T	T	G	T	T	T	T	T	T	T	T	AAT - AAG	Asn - Lys
92.	1195	1	T	T	T	T	G	T	T	T	T	T	T	T	TGG - GGG	Trp - Gly
93.	1207	1	A	A	A	A	A	A	A	A	A	A	C	A	AAC - CCC	Asn - Pro
94.	1208	2	A	A	A	A	A	C	A	A	A	A	C	A	AAC - CCC ACC	Asn - Pro Thr
95.	1210	1	A	A	A	A	A	G	A	A	A	A	A	A	ACA - GCA	Thr - Ala
96.	1211	2	C	C	G	C	G	C	G	G	G	G	G	G	ACA - AGA	Thr - Arg
97.	1213	1	G	G	A	G	A	G	A	A	A	A	A	A	GAA - AAA	Glu - Lys
98.	1215	3	A	A	A	C	A	A	A	A	A	A	A	A	GAA - GAC	Glu - Asp
99.	1216	1	A	A	A	A	A	A	A	C	A	A	A	A	ACA - CCA	Thr - Pro
100.	1218	3	A	A	A	A	A	C	A	A	A	A	A	A	ACA - ACC	Thr - Thr
101.	1219	1	C	C	C	C	C	C	C	C	C	C	A	C	CAA - AAA	Gln - Lys
102.	1245	3	A	A	A	C	C	A	A	A	A	A	A	A	AAA - AAC	Lys - Asn
103.	1261	1	A	A	A	G	A	A	A	A	A	A	A	A	AAC - GAC	Asn - Asp
104.	1280	2	C	C	C	G	C	C	C	C	C	C	C	C	GCT - GGT	Ala - Gly
105.	1291	1	T	T	T	G	T	T	T	T	T	T	T	T	TTG - GTG	Leu - Val
106.	1299	3	T	T	T	T	T	C	T	T	T	T	T	T	CAT - CAC	His - His
107.	1315	1	A	C	C	C	C	C	C	C	C	C	C	C	AAC - CAC	Asn - His
108.	1342	1	G	A	G	G	G	G	G	G	G	G	G	G	GAT - AAT	Asp - Asn
109.	1352	2	T	T	A	T	T	T	T	T	T	T	T	T	ATG - AAG	Met - Lys
110.	1377	3	A	A	A	T	A	A	A	A	A	A	A	A	AAA - AAT	Lys - Asn
111.	1381.	1	A	A	A	A	A	T	A	A	A	A	A	A	ATT - TTT	Ile - Phe
112.	1382	2	T	T	T	C	T	T	T	T	T	T	T	T	ATT -ACT	Ile -Thr
113.	1400	2	A	A	A	G	A	A	A	A	A	A	A	A	GAT - GGT	Asp - Gly
114.	1410	3	G	G	G	C	G	G	G	G	G	G	G	G	GGG - GGC	Gly - Gly
115.	1431	3	A	A	A	G	A	A	A	A	A	A	A	A	CCA - CCG	Pro - Pro
116.	1454	2	A	A	T	A	A	A	A	A	A	A	A	A	AAA - ATA	Lys - Ile
117.	1478	2	A	A	C	C	C	A	C	A	C	A	A	C	GAC - GCC	Asp - Ala
118.	1488	3	G	T	T	G	T	T	T	T	T	T	T	T	ATG - ATT	Met - Ile
119.	1497	3	T	T	T	G	T	T	T	T	T	T	T	T	AAT - AAG	Asn - Lys
120.	1507	1	C	A	C	C	A	A	A	A	A	A	A	A	CGT - AAT	Arg - Asn
121.	1508	2	G	A	A	G	A	A	A	A	A	A	A	A	CGT - AAT	Arg - Asn

122.	1548	3	A	A	A	C	A	A	A	A	A	A	A	A	GTA - GTC	Val - Val
123.	1590	3	T	T	T	C	T	T	T	T	T	T	T	T	GAT - GAC	Asp - Asp
124.	1610	2	A	A	A	C	A	A	A	A	A	A	A	A	GAA - GCA	Glu - Ala
125.	1621	1	A	A	A	T	A	A	A	A	A	A	A	A	ACT - TCT	Thr - Ser
126.	1632	3	A	A	C	C	C	C	C	C	C	C	C	C	AAA - ACC	Lys - Thr
127.	1657	1	G	G	G	T	G	G	G	C	G	G	G	G	GCT - TCT	Ala - Ser
128.	1665	3	C	C	C	G	C	C	C	-	C	C	C	C	GTC - GTG	Val - Val
129.	1677	3	A	A	A	G	A	A	A	-	A	A	A	A	GCA - GCG	Ala - Ala
130.	1678	1	A	A	A	G	A	A	A	-	A	A	A	A	ACT - GCT	Thr - Ala
131.	1693	1	T	T	C	T	T	T	T	-	T	T	T	T	TAT - CAT	Tyr - His
132.	1716	3	T	T	T	C	T	T	T	-	T	T	T	T	AAT - AAC	Asn - Asn
133.	1717	1	G	G	G	C	G	G	G	-	G	G	G	G	GCT - CCC	Ala - Pro
134.	1719	3	T	T	T	C	T	T	T	-	T	T	T	T	GCT - GCC	Ala - Ala
135.	1722	3	A	A	A	C	A	A	A	-	A	A	A	A	GAA - GAC	Glu - Asp
136.	1724	2	A	A	A	C	A	A	A	-	A	A	A	A	AAA - ACA	Lys - Thr
137.	1727	2	A	A	A	G	A	A	A	-	A	A	A	A	TAT - TGT	Tyr - Cys
138.	1740	3	T	T	T	C	T	T	T	-	T	T	T	T	GAT - GAC	Asp - Asp
139	1741	1	G	G	C	C	C	C	C	-	C	C	C	C	GAA - CAA	Glu - Gln
140.	1746	3	A	A	A	C	A	A	A	-	A	A	A	A	CCA - CCC	Pro - Pro
141.	1750	1	G	C	G	G	G	G	G	-	G	G	G	G	GAT - CAT	Asp - His
142.	1757	2	G	G	G	C	G	G	G	-	G	G	G	G	GGG - GCG	Gly - Ala
143.	1767	3	T	T	A	A	A	A	A	-	A	A	A	A	AAT - AAA	Asn - Lys
144.	1773	3	A	A	A	C	A	A	A	-	A	A	A	A	AGA - AGC	Arg - Ser
145.	1786	1	T	T	T	G	T	T	T	-	T	T	T	G	TTA - GGA	Leu - Gly
146.	1787	2	T	T	T	G	T	T	T	-	T	T	T	G	TTA - GGA	Leu - Gly
147.	1789	1	G	G	G	A	G	G	G	-	G	G	G	G	GAT- AAT	Asp -Asn
148.	1794	3	T	T	T	C	T	T	T	-	T	T	T	T	CCT - CCC	Pro - Pro
149.	1808	2	G	G	G	C	G	G	G	-	G	G	G	C	TGG - TCC TCG	Trp - Ser Ser
150.	1809	2	G	G	G	C	G	G	G	-	G	G	G	G	TGG - TCC TCG	Trp – Ser - Ser
151.	1811	2	G	G	G	C	G	G	G	-	G	G	G	G	GGG - GCC	Gly - Ala

152.	1812	3	G	G	G	G	G	G	G	-	G	G	G	G	GGG - GCC	Gly - Ala
153.	1818	3	A	A	A	G	A	-	G	-	A	A	-	A	GAA - GAG	Glu - Glu
154.	1819	1	A	A	A	G	A	-	-	-	A	-	-	A	AAA - GGA	Lys - Gly
155.	1820	2	A	A	A	G	A	-	-	-	A	-	-	A	AAA - GGA	Lys - Gly
156.	1828	1	T	T	T	C	T	-	-	-	T	-	-	T	TCA - CCA	Ser - Pro
157.	1840	1	A	A	T	A	-	-	-	-	A	-	-	C	ACC - TCC CCC	Thr - Ser Pro
158.	1842	3	A	A	A	T	-	-	-	-	A	-	-	A	CCA - CCT	Pro - Pro
159.	1844	2	T	T	T	C	-	-	-	-	T	-	-	T	GTT - GCC	Val - Ala
160.	1845	3	T	T	T	C	-	-	-	-	T	-	-	T	GTT - GCC	Val - Ala
161.	1846	1	C	C	C	G	-	-	-	-	C	-	-	C	CTG - GTG	Leu - Val

VARIATION AT AMINO ACID LEVEL:

At the amino acid level, a total of 99 amino acid changes were seen across the length of the protein as against the 54 reported previously (Table 4.2). Domain wise split up of this variation in Indian isolates suggests that, Domain II was the most polymorphic with 31 out of 99 amino acid substitutions located in this domain. Most of the other literature reported so far give Domain I as the most polymorphic which also includes the hypervariable region or HVR from 160-320 amino acids (Oliveira *et.al*. 1996, Marshall *et.al.*1996). However, in Domain II , previously reported changes were only 7 . We find that among the 10 Indian Isolates 31 out of 99 amino acid substitutions are found in domain II making it more polymorphic than domain I and III. Domain I has 19 changes and domain III has 14 amino acid changes which are in accordance with the literature reported so far. The domain-wise difference in the ten Indian isolates is also given in (Table 4. 2).

Bai *et. al*. (2005) had reported 5 highly polymorphic residues, 187, 197, 200, 230 and 243 which form a cluster in the structure of AMA-1 at the top left of the molecule which is the exposed and most accessible to antibodies reacting with parasite surface. Study of these residues in Indian isolates shows that all Indian isolates show a change from prototype at positions 187, 197 and 200. 9 out of the 10 isolates show similar pattern of animo acids at these positions, whereas it is the same as prototype in position 230 and 243. They also reported conservative dimorphism at position 395 (either R or K). However in our study one variant R2 shows Q in that position.

One significant amino acid change found in our study is at position 97 of the AMA-1 protein. Howell *et. al* (2001) had reported that the N-terminal truncation of the 83 kDa protein occurs between Ser 96 and Isoleucine 97 and that this is also the beginning of Domain I of ectodomain. Our study shows a change at position 97 from I to N in 8 isolates which could affect the proteolytic processing and in turn distribution of AMA-1 on surface. The effect of this change needs to be investigated.

COMPARISON WITH OTHER PREVIOUSLY REPORTED INDIAN ISOLATES:

Escalante *et . al*. in the year 2001 reported the AMA –1 sequences from 5 Indian isolates collected from MRC, Delhi. Comparison of our 10 isolates with previously reported Indian isolates shows that out of the 99 changes seen in this study, 30 are old previously reported in other Indian isolates or elsewhere (Table 4.2). 69 changes are totally new changes observed only in this study. In addition to these, certain changes reported previously by Escalante *et.al* are not found in our study, at positions 50, 162,167,172, 187,190,196, 200,201, 242, 243, 263, 296, 302, 395, 435 and 512. The overall difference found on comparison with previously reported isolates is found to be about 4 - 5%.

Significant among these are changes at positions 187 and 263. At 187, all the isolates reported previously show K whereas all our 10 isolates show N in this position, whereas in the prototype 3D7 it is E. The dimorphism at this position reported by Bai *et. al.* is not confirmed as this comparison shows the presence of three different amino acids at this positon. At position 263, responsible for the disulphide bond structure of AMA-1 one previously reported isolate having accession no. AY0164031 shows a change from cysteine to tryptophan. Though this change was not observed in our study, we find Cys to tryptophan variation at position 320. A cystein to glycine and serine change was also found at position 337. These data clearly indicate that significant variation is seen in the Indian isolates that need to be properly investigated.

Table 4. 2. Differences of Pf AMA-1 of Indian Isolates with prototype at protein level

S.NO.	POSITION OF AMINO ACID	Domain	3D7	FC27	R1	R2	R3	R4	R5	S1	S2	S3	S4	S5	Whether reported previousl in indian isolates
1.	15	PRO	F	F	F	F	F	F	F	F	S	F	F	F	NO
2.	16	PRO	T	T	T	T	T	T	T	T	T	T	T	S	NO
3.	19	PRO	I	I	R	I	I	I	I	I	R	I	I	R	NO
4.	25	PRO	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	NO
5.	26	PRO	N	N	S	N	N	N	N	T	S	N	N	S	NO
6.	28	PRO	W	W	W	W	G	G	G	G	G	G	G	G	NO
7	30	PRO	H	H	H	H	D	D	D	D	D	D	D	D	NO
8.	34	PRO	N	K	N	N	N	N	N	N	N	N	N	N	YES
9.	39	PRO	R	H	R	H	H	H	H	H	H	H	H	H	YES
10.	45	PRO	R	R	R	R	S	S	S	S	S	S	S	S	NO
11.	49	PRO	K	K	K	K	T	T	T	T	T	T	T	T	NO
12.	52	PRO	E	Q	E	E	E	E	E	E	E	E	E	E	YES
13.	97	DOM I	I	I	I	I	N	N	N	N	N	N	N	N	NO
14.	175	DOM I	Y	Y	D	D	D	D	D	D	D	D	D	D	YES
15.	187	DOM I	E	E	E	N	N	N	N	N	N	N	N	N	YES
16.	197	DOM I	E	E	Q	D	D	D	D	D	D	D	D	D	YES
17.	200	DOM I	H	H	H	R	R	R	R	R	R	R	R	R	YES
18.	206	DOM I	K	K	E	E	E	E	E	E	E	E	E	E	YES
19.	225	DOM I	I	I	N	I	I	I	I	I	I	I	I	I	YES
20.	230	DOM I	K	K	E	K	K	K	K	K	K	K	K	K	YES
21.	243	DOM I	K	K	K	K	Q	K	K	K	K	K	K	K	NO
22.	267	DOM I	E	E	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	YES
23.	271	DOM I	N	N	N	N	N	N	N	N	N	N	T	N	NO

24.	273	DOM I	M	M	M	M	L	M	R	M	M	M	M	M	
25.	282	DOM I	I	I	I	I	I	K	K	K	K	K	K	K	YES
26.	283	DOM I	S	S	S	L	S	S	S	S	S	S	S	S	YES
27.	285	DOM I	Q	Q	Q	E	Q	Q	Q	Q	Q	Q	Q	Q	YES
28.	286	DOM I	N	N	N	N	H	N	N	N	N	N	N	N	NO
29.	287	DOM I	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	C	C	NO
30.	297	DOM I	N	N	N	N	N	N	N	N	N	N	N	S	NO
31.	300	DOM I	K	K	K	K	K	K	K	E	K	K	K	E	YES
32.	307	LOOP	L	L	L	L	L	L	L	F	L	L	L	L	NO
33.	308	LOOP	Q	Q	E	Q	P	Q	P	Q	Q	Q	P	Q	YES
34.	315	LOOP	W	W	W	W	W	W	W	G	W	W	W	W	NO
35.	317	LOOP	D	D	D	D	D	D	D	D	D	D	G	D	NO
36.	320	DOM II	C	C	C	W	C	C	C	C	W	C	W	W	NO
37.	326	DOM II	V	V	V	V	V	V	G	V	V	V	G	V	NO
38.	327	DOM II	N	N	N	N	N	D	N	N	N	N	N	N	NO
39.	330	DOM II	P	S	S	S	P	S	P	S	S	S	P	S	YES
40.	332	DOM II	I	I	I	N	I	N	N	N	I	N	I	N	YES
41.	335	DOM II	F	F	F	Y	F	F	F	F	F	F	F	F	NO
42.	337	DOM II	C	C	C	C	C	C	C	C	C	C	G	S	NO
43.	340	DOM II	L	L	L	L	L	L	L	L	L	L	L	I	NO
44.	345	DOM II	S	S	S	S	S	S	S	S	S	S	G	G	NO
45.	349	DOM II	Q	Q	H	Q	P	Q	H	Q	H	H	Q	Q	NO
46.	354	DOM II	E	E	E	D	E	E	E	D	E	E	G	D	NO
47.	355	DOM II	Q	Q	H	Q	Q	Q	Q	Q	Q	Q	H	P	NO
48.	357	DOM II	L	L	L	F	L	L	L	L	L	L	L	L	NO
49.	358	DOM II	T	T	T	S	T	T	T	T	T	T	T	P	NO
50.	360	DOM II	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	C	Y	NO
51.	363	DOM II	I	I	I	L	I	I	I	I	I	I	I	N	NO
52.	365	DOM II	E	E	E	G	E	E	E	E	E	E	G	R	NO
53.	367	DOM II	F	F	F	F	F	F	F	F	F	F	S	F	NO

54.	368	DOM II	K	K	K	K	K	K	Q	K	K	K	R	K	NO
55.	371	DOM II	N	N	N	T	N	T	N	N	T	N	S	N	NO
56.	382	DOM II	T	T	T	T	T	T	P	T	T	T	T	P	NO
57.	386	DOM II	K	K	K	R	K	K	K	K	K	K	K	K	NO
58.	395	DOM II	K	K	K	Q	K	K	K	K	K	K	K	K	YES
59.	398	DOM II	N	N	N	K	N	N	N	N	N	N	N	N	NO
60.	399	DOM II	W	W	W	W	G	W	W	W	W	W	W	W	NO
61.	403	DOM II	N	N	N	N	N	T	N	N	N	N	P	N	NO
62.	404	DOM II	T	T	R	T	R	A	R	R	R	R	R	R	YES
63.	405	DOM II	E	E	K	D	K	E	K	K	K	K	K	K	YES
64.	406	DOM II	T	T	T	T	T	T	T	P	T	T	T	T	NO
65.	407	DOM II	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	K	Q	NO
66.	415	DOM II	K	K	K	N	N	K	K	K	K	K	K	K	NO
67.	421	LOOP	N	N	N	D	N	N	N	N	N	N	N	N	NO
68.	427	LOOP	A	A	A	G	A	A	A	A	A	A	A	A	NO
69.	431	LOOP	L	L	L	V	L	L	L	L	L	L	L	L	NO
70.	439	DOM III	N	H	H	H	H	H	H	H	H	H	H	H	YES
71.	448	DOM III	D	N	D	D	D	D	D	D	D	D	D	D	YES
72.	451	DOM III	M	M	K	M	M	M	M	M	M	M	M	M	YES
73.	459	DOM III	K	K	K	N	K	K	K	K	K	K	K	K	NO
74.	461	DOM III	I	I	I	T	I	F	I	I	I	I	I	I	NO
75.	467	DOM III	D	D	D	G	D	D	D	D	D	D	D	D	NO
76.	485	DOM III	K	K	I	K	K	K	K	K	K	K	K	K	YES
77.	493	DOM III	D	D	A	A	A	D	A	D	A	D	D	A	YES
78.	496	DOM III	M	I	I	I	M	I	I	I	I	I	I	I	YES
79.	499	DOM III	N	N	M	K	M	M	M	M	M	M	M	M	NO
80.	503	DOM III	R	N	H	R	N	N	N	N	N	N	N	N	YES
81.	537	DOM III	E	E	E	A	E	E	E	E	E	E	E	E	NO
82.	541	DOM III	T	T	T	S	T	T	T	T	T	T	T	T	NO
83.	544	DOM III	K	K	N	N	N	N	N	N	N	N	N	N	YES

84.	553	TRANSMEMB	A	A	A	S	A	A	A	-	A	A	A	A	NO
85.	560	TRANSMEMB	T	T	T	A	T	T	T	-	T	T	T	T	NO
86.	565	TRANSMEMB	Y	Y	H	Y	Y	Y	Y	-	Y	Y	Y	Y	NO
87.	573	CYTOPLASMIC	A	A	A	P	A	A	A	-	A	A	A	A	NO
88.	574	CYTOPLASMIC	E	E	E	D	E	E	E	-	E	E	E	E	NO
89.	575	CYTOPLASMIC	K	K	K	T	K	K	K	-	K	K	K	K	NO
90.	576	CYTOPLASMIC	Y	Y	Y	C	Y	Y	Y	-	Y	Y	Y	Y	NO
91.	581	CYTOPLASMIC	E	E	Q	Q	Q	Q	Q	-	Q	Q	Q	Q	YES
92.	584	CYTOPLASMIC	D	H	D	D	D	D	D	-	D	D	D	D	YES
93.	586	CYTOPLASMIC	G	G	G	A	G	G	G	-	G	G	G	G	NO
94.	589	CYTOPLASMIC	N	N	K	K	K	K	K	-	K	K	K	K	YES
95.	591	CYTOPLASMIC	R	R	R	S	R	R	R	-	R	R	R	R	NO
96.	596	CYTOPLASMIC	L	L	L	G	L	L	L	-	L	L	L	G	NO
97.	597	CYTOPLASMIC	D	D	D	N	D	D	D	-	D	D	D	D	NO
98.	603	CYTOPLASMIC	W	W	W	S	W	W	W	-	W	W	W	S	NO
99.	604	CYTOPLASMIC	G	G	G	A	G	G	G	-	G	G	G	G	NO

PAIRWISE PERCENT IDENTITY WITH 3D7:

The greatest number of nucleotide difference was seen in R2 isolate that accounts for 4.4% variability with the prototype. The isolate showing maximum similarity with the prototype was R1 with only 20 amino acid changes and 96.1% identity (Table 4. 3 and 4. 4). R2, S4, and S5 isolates show relatively higher variation and they also show change in 2 of the Cystiene residues conserved making their immunological characterization very very significant.

Table 4. 3: Pairwise percent identity of Pf AMA-1 with 3D7 at nucleotide level

S.No.	Sample	No.of nucleotide diff. with 3D7	%identity with 3D7
1.	R1	28	98.5%
2.	R2	79	95.6%
3.	R3	33	98.2%
4.	R4	32	98.2%
5.	R5	33	98.2%
6.	S1	33	98%
7.	S2	38	97.9%
8.	S3	28	98.5%
9.	S4	46	97.4%
10.	S5	54	97%

Table 4. 4 : Pairwise percent identity of Pf AMA-1 with 3D7 at protein level

S.No.	Sample	No. of amino acid diff. with 3D7	Pairwise percent identity
1.	R1	24	96.1%
2.	R2	55	91.2%
3.	R3	28	95.35%
4.	R4	26	95.6%
5.	R5	20	95.35%
6.	S1	26	95.05%
7.	S2	25	94.9%
8.	S3	20	96%
9.	S4	33	93.6%
10.	S5	36	93.1%

GROUPING OF AMA - 1 ALLELES INTO REPORTED FAMILIES:

Marshall *et. al*. (1996) had grouped the AMA - 1 alleles into four major family groups based on a series of restriction enzyme sites. Out of the 10 Indian isolates nine fall into Group IV family, whereas one allele does not fit into these family groups, indicating that this classification scheme for rapid genotyping of AMA - 1 alleles needs further validation (Table 4. 5).

Table 4. 5. Restriction site analysis of the Pf AMA-1 isolates and their grouping

	Restriction enzyme Dra I	SspI	Mae I	MsrI	Sau3A1	Group
	TTT/AAA	AAT/ATT	/GTNAC	T/TAA	/GATC	
	495	520	569	583	586	
3D7	-	+	+	-	-	I
FC27	-	+	+	-	-	I
R1	-	-	+	-	+	NEW
R2	-	-	+	-	-	IV
R3	-	-	+	-	-	IV
R4	-	-	+	-	-	IV
R5	-	-	+	-	-	IV
S1	-	-	+	-	-	IV
S2	-	-	+	-	-	IV
S3	-	-	+	-	-	IV
S4	-	-	+	-	-	IV
S5	-	-	+	-	-	IV

REPORT OF INTRAGENIC RECOMBINATION:

Eisen *et. al.* (1999) had reported an intragenic recombination event between two alleles one belonging to family I and family II respectively. However in these ten Indian isolates no such recombination event was observed.

COMPARISON OF 16 CYSTEINE RESIDUES:

The structure of AMA-1 protein is stabilized by 8 disulphide bonds formed between 16 conserved cysteine residues. Analysis for the ten Indian isolates for these 16 cysteine residues indicates variations at two positions – 320 (where cysteine is replaced by tryptophan in 4 isolates) and 337 (where cysteine is replaced by glycine and serine in one isolate each (Table 4. 6). This difference could be very significant as the complete reported structure of AMA-1 is based on the conserved 16 cysteine residues.

Table 4. 6. Comparison of reported cysteine residues in Indian isolates with 3D7

S.No.	Position of cysteine residue	3D7	FC27	R1	R2	R3	R4	R5	S1	S2	S3	S4	S5
1.	149	C	C	C	C	C	C	C	C	C	C	C	C
2.	217	C	C	C	C	C	C	C	C	C	C	C	C
3.	247	C	C	C	C	C	C	C	C	C	C	C	C
4.	263	C	C	C	C	C	C	C	C	C	C	C	C
5.	275	C	C	C	C	C	C	C	C	C	C	C	C
6.	302	C	C	C	C	C	C	C	C	C	C	C	C
7.	320	C	C	C	W	C	C	C	C	W	C	W	W
8.	337	C	C	C	C	C	C	C	C	C	C	G	S
9.	409	C	C	C	C	C	C	C	C	C	C	C	C
10.	418	C	C	C	C	C	C	C	C	C	C	C	C
11.	443	C	C	C	C	C	C	C	C	C	C	C	C
12.	490	C	C	C	C	C	C	C	C	C	C	C	C
13.	492	C	C	C	C	C	C	C	C	C	C	C	C
14.	502	C	C	C	C	C	C	C	C	C	C	C	C
15.	507	C	C	C	C	C	C	C	C	C	C	C	C
16.	509	C	C	C	C	C	C	C	C	C	C	C	C

VARIATION IN REPORTED B and T CELL EPITOPES:

The 9 epitopes (B as well as T) reported previously by Escalante *et. al*., in 2001 were studied for their variations in 10 Indian isolates and all the epitopes showed variation at one or more amino acid positions. Some of these variations were previously identified ones and few changes were new reported only in this study. So, the diversity of the antigen at the epitopes was much higher than found previously. This could greatly affect the immunological characteristics of AMA-1 and have implications in the vaccine candidate potential of AMA-1. (Table 4. 7).

Table 4. 7. Variations in reported B and T- cell epitopes of the AMA - 1 protein of *P. falciparum*

S.No	Residues	Epitope	3D7 Sequence reported	Sample	Indian Isolates
1.	14-35	T cell epitope	EFTYMINFGRGQNYWEHPYQNS	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	EFTYMINFGRGQNYWEHPYQKS DFLYMRNFGRGQSYWEHPYQNS EFTYMINFGRGQNYWEHPYQNS EFTYMINFGRGQNYGEDPYQNS EFTYMINFGRGQNYGEDPYQNS EFTYMINFGRGQNYGEDPYQNS EFTYMINFGRGQTYGEDPYQNS ESTYMRNFGRGHSYGEDPYQNS EFTYMINFGRGQNYGEDPYQNS EFTYMINFGRGQNYGEDPYQNS EFSYMRNFGRGQSYGEDPYQNS
2.	41-57	B cell epitope	INEHREHPKEYEYPLHQ	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	INEHREHPKEYQYPLHQ INEHREHPKEYEYPLHQ INEHREHPKEYEYPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYE YPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYEYPLHQ INEHSEHPTEYEYPLHQ

3.	259-271	T cell epitope	GPRYCNKDESKRN	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	GPRYCNKDESKRN GPRYRNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRN GPRYCNKDQSKRT GPRYCNKDQSKRN
4.	279 – 288	B&T cell epitope	AKDISFQNYT	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	AKDISFQNYT AKDISFQNYT AKDILFENYT AKDKSFQNYT AKDKSFQNYT AKDKSFQNYT AKDKSFQNYT AKDKSFQNYT AKDKSFQNYT AKDKSFQNCT AKDKSFQNCT
5.	317 – 334	B cell epitope	DGNCEDIPHVNEFPAIDL	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	DGNCEDIPHVNEFSAIDL DGNCEDIPHVNEFSAIDL DGNWEDIPHVNEFSANDL DGNCEDIPHVNEFPAIDL DGNCEDIPPVDEFSANDL DGNCEDIPHGNEFPANDL DGNCEDIPHVNEFSANDL DGNWEDIPHVNEFSAIDL DGNCEDIPHVNEFSANDL GGNWEDIPHGNEFPAIDL DGNWEDIPHVNEFSANDL
6.	321 – 338	B cell epitope	EDIPHVNEFPAIDLFECN	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	EDIPHVNEFSAIDLFECN EDIPHVNEFSAIDLFECN EDIPHVNEFSANDLYECN EDIPHVNEFPANDLFECN EDIPPVDEFSANDLFECN EDIPHGNEFPANDLFECN EDIPHVNEFSANDLFECN ENIPHVNEFSAIDLFECN EDIPHVNEFSANDLFECN EDIPHGNEFSAIDLFEGN EDIPHVNEFSANVLFESN

7.	348 – 366	T cell epitope	DQPKQYEQHLTDYEKIKEG	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	DQPKQYEQHLTDYEKIKEG DHPKPYDHHLTDYEKIKEG DQLKQYEQHFTDYEKLKGG DPPKQYEQHLTDYEKIKEG DQPKQYEQHLTDYEKIKEG DHPKQYEQHLTDYEKIKEG DPPKQYDQHLTDYEKIKEG DHPKQYEQHLTDYEKIKEG DHPKQYEQHLTDYEKIKEG DQPKQYGHHLTDCEKIKGG DQPKQYDPHLPDYEKNKRG
8.	444 – 461	T cell epitope	SLYKDEIMKEIERESKRI	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	SLYKNEIMKEIERESKRI SLYKDEIKKEIERESKRI SLYKDEIMKAIERVSNRT SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRF SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRI SLYKDEIMKEIERESKRI
9.	571 – 588	B&T cell epitope	GNAEKYDKMDEPQDYGKS	FC27 R1 R2 R3 R4 R5 S1 S2 S3 S4 S5	GNAEKYDKMDEPQHYGKS GNAEKYDKMDQPQDYGKS GNPDTCDKMDQPQDYATS GNAEKYDKMDQPQDYGKS GNAEKYDKMDQPQDYGKS GNAEKYDKMDQPQDYGKS ---------- GNAEKYDKMDQPQDYGKS GNAEKYDKMDQPQDYGKS GNAEKYDKMDQPQDYGKS GNAEKYDKMDQPQDYGKS

Large font size: previously reported position of variation
Underlined: New variation found in Indian isolates.

To summarize, the sequence diversity of the AMA-1 gene in *P. falciparum* confines only to point mutations which is responsible for nearly 4 - 8% variation among the various alleles. This is relatively a conserved molecule as compared to the other antigens of the malarial parasite. This conforms with the reported literature, but then in our study the split up of this variation has been different and domain II has been found to be more polymorphic than reported previously. So, the role of domain II needs to be elucidated to understand the effect of this polymorphism on the immunogenecity of the parasite. Moreover, even this 4 – 8 % variation is significant in strain specific immunity against the parasite and differences in epitopes could give differences in the immunological properties of the antigen and need to be studied in greater detail to be included in vaccine.

Such studies to characterize these variations would require the expression of protein in bulk and to achieve that a variant allele was cloned and expression studies of full length gene was carried out in prokaryotic system.

Chapter 5

Studies on expression of AMA – 1 protein of *P. falciparum* in *E.coli* from field isolates

Introduction:

The apical membrane antigen (AMA-1) has emerged as a promising vaccine candidate against malaria. Advanced evaluation of its protective efficacy and immunological properties requires the production of highly purified and correctly folded protein. Overexpression of parasite genes is important for providing sufficient amounts of recombinant protein for biophysical studies, such as X-ray crystallography and NMR, as well as for immunological applications, such as vaccine production and the production of recombinant antigens for the generation of research antibodies. These studies will ultimately involve expression of recombinant proteins in heterologous systems. Therefore, the choice of the expression system for malarial antigens is important. Bacterial expression systems as that of *E.coli*, Yeast and Baculovirus systems have been used to express the Apical Membrane Antigen.

Dutta *et.al*. (2002) described the process for the expression, fermentation, refolding and purification of the recombinant ectodomain of 3D7 AMA-1 (from amino acid 83 – 531) of *P.falciparum* produced in *E.coli*. A synthetic gene containing the *E.coli* codon bias was cloned into a modified pET 32 vector and the recombinant protein was produced using a redox modified *E.coli* strain Origami (DE3).

To study the effect of AMA-1 allelic diversity on the ability of a recombinant AMA-1 to offer protective immunity, two allelic forms of AMA-1 (FVO & 3D7) were expressed in *Pichia pastoris* at sufficient economy scale to be used for clinical vaccine studies. (Kennedy *et. al*., 2002). Synthetic AMA-1 sequences encoding amino acids 25 – 546 were subcloned into *P.pastoris* expression plasmid pPIC9k. Clones with optimal expression were selected for large scale production by fermentation and recombinant protein was expressed.

Lalitha *et. al* (2004) investigated the antibody responses directed against the individual subdomains of AMA - 1. They constructed 6 different genes that expresses each of these domains separately – D1, DII, DIII separately, and in combination DI+II , DII+III, DI+III by amplifying from a synthetic *E.coli* codon optimized AMA-1 ectodomain gene of the 3D7 isolate and ligated as per the combinations required. These clones were expressed and the purified protein were used to immunize rabbits to raise construct specific antibodies. They deduced that DI + II induced a significant amount of growth inhibitory antibodies active in growth and invasion assay.

Giersing *et. al.* (2005) investigated the role of post-translational modification in modulating the functional immune responses to recombinant AMA - 1. Expression in heterologous systems may introduce posttranslational modifications, which are not present in authentic parasite protein. Subsequent alterations of antigen integrity and/or stability compared to native molecule are likely to detrimentally affect the functionality of the molecule. For this purpose, they used synthetic genes of two separate alleles of PfAMA - 1 in which the native N-glycosylation sites have been mutated, and these were expressed using the *E.coli* and *P.pastoris* expression systems.

Long before this, Kocken *et. al.* (1998) previously had investigated the heterologous trans-species expression of *P. falciparum* AMA - 1 in rodent parasite *P. berghei.* They showed that, the AMA-1 protein is expressed in a conformationally and functionally relevant form and that depending on the time of expression it is differentially routed within the developing parasite and that it is highly immunogenic within the context of a rodent malaria parasite. Hodder *et.al* (1996) had cloned the *P. chabaudi* AMA-1 sequences in Baculovirus system and expressed them in Sf9 insect cells.

However, all the studies done so far on *P. falciparum* AMA-1 have used the synthetic gene constructs of AMA-1 for expression studies and the constructs were pre-modified by normalizing their AT content according to published values for *E.coli* and *P. pastoris* codon bias.. To study all the allelic variants of AMA-1 found in endemic regions

recombinant protein expression is a must, as generating synthetic gene constructs is totally impractical. Hence, the expression of full length AMA-1 is important for the immunological characterization of the protein.

Partial success was achieved by Lalitha *et.al* (2005) when they expressed partial sequences of AMA-1 in *E.coli* expression system from field isolates. In order to examine the strain specificity of antibodies elicited to AMA-1 two diverse allelic variants of domain I + II of AMA-1 from Indian *P.falciparum* isolates were cloned and expressed in *E.coli*. The proteins were purified and the purified proteins were analysed by a conformation specific monoclonal Ab and hyper immune sera for further characterization.

The aim of our present study is to carry it further and attempt to express full length AMA-1 allelic variants from field isolates. To this effect, expression studies of AMA-1 gene in *E.coli* were carried out. The expressed allelic variants could then be studied for their immunological properties like immunogenecity and protective efficacy.

Results and Discussion

The AMA - 1 gene of 1.8 kb was cloned into the pRSET vector, sequenced, and after confirming the orientation and reading frame, was subjected to expression studies to get protein expression. The pRSET vector and the recombinant clone were transformed into the expression host *E. coli* BL21(DE3) pLysS and induced with 1mM of IPTG. The collected samples were run on SDS-PAGE gel along with a high molecular weight protein marker. On comparison with the vector, there was a very faint induction, and at around 72 kDa a faint induced band was observed in the clone as compared to the vector control.

The induction kinetics were then altered and various concentrations of IPTG like 0.5, 1.0, 1.5, 2.0, 3.0,.4.0, 5.0, and 8.0 mM were tried to improve induction and time duration of collection of samples was also increased from 6 hrs to a maximum of 12 hrs. This also

did not give the desired result and persisted with a faint band in the clone as compared to the control.

To improve the expression, another host known for very high level expression the *E.coli* BL21(DE3) was transformed with the same pRSET clone and vector. Induction was performed at three concentrations of IPTG – 0.5, 1.0, and 3 mM . On running the PAGE, similar gel pictures as with the host *E.coli* BL21(DE3) pLysS were obtained, inferring that host did not alter the expression of protein too much.

As the next step, since most of the *Plasmodium* antigens were expressed using the pET series of vectors, (Dutta *et.al*. 2002) the insert was subcloned into pET 21C vector. The intention was to try expression with the changed vector. Transformation was done in both *E.coli* BL21(DE3) and *E.coli* BL21(DE3) plysS host. After induction, again a very faint band was observed, indicating that both the pET and pRSET vectors gave similar levels of induction and expression.

The overexpression of parasite genes in *Escherichia coli*, in particular those of *Plasmodium falciparum*, has often been a challenge because of the codon bias of these organisms. *P. falciparum* has an extremely AT-rich genome of about 80%, which has in many instances made heterologous expression of *Plasmodium* genes in *E. coli* very difficult. Certain codons which are preferentially used by *P. falciparum* are rarely used by *E. coli.* These rare codons have been shown to greatly diminish expression levels of recombinant protein in *E. coli* because of translational stalling.

To circumvent these problems, the RIG plasmid approach was chosen. The RIG-plasmid is a simple and convenient tool to test when performing initial overexpression experiments of AT-rich genes. The AMA - 1 gene of *P.falciparum,* being an AT rich gene could be overexpressed with co-transformation of the clone with the RIG plasmid. RIG carries a chloramphenicol-resistance gene. The RIG-plasmid also carries the genes that encode three tRNAs - Arg, Ile, Gly. These genes direct the consitutive expression of tRNAs that recognize the codons AGA/AGG (Arg, R), ATA (Ile, I), and GGA (Gly,G).

The increased levels of these three tRNAs may help *E. coli* better translate parasite mRNAs that are rich in these specific codons, and thus may increase the yields of recombinant parasite proteins like AMA-1 (Baca *et. al*., 2000).

In order to get expression of the AMA - 1 gene the RIG-plasmid, was co-transformed along with the expression vector containing a parasite gene AMA - 1 (clone) into *E.coli* BL21(DE3) host. *E.coli* BL21(DE3) pLysS could not be used as host because the chloramphenicol selection for RIG would not be possible as Chloramphenicol is also the selectable marker for pLysS and transformation with RIG could not be confirmed. This was done with the assumption that, the time and expense of redesigning parasite genes into the optimal codon bias of *E. coli* may now be circumvented by use of the RIG-plasmid. After co-transformation, the host was induced with 1mM IPTG for 6 hrs and the collected samples were run on SDS – PAGE gel. However, the expression level of the AMA - 1 gene was not affected, and inspite of using the RIG plasmid a very faint band was observed indicating minimal expression (Fig. 5.1).

These results gave insight to the fact that a minimal basal level of expression is only seen in all the cases studied so far, based on the faint band obtained in each of the cases. To confirm this, a Western Blot was done and the His tagged protein was detected using Anti – His antibody and chromogenic substrate BCIP/NBT (Substrate for Alkaline phosphatase, Bangalore GENEI). The Western Blot when treated with chromogenic substrate BCIP/NBT (Substrate for Alkaline Phosphatase, Bangalore GENEI) did not yield the desired band, indicating that there was no expression of AMA - 1 in both the vectors, or the minimal expression in both the vectors was probably undetectable in the Western blot.

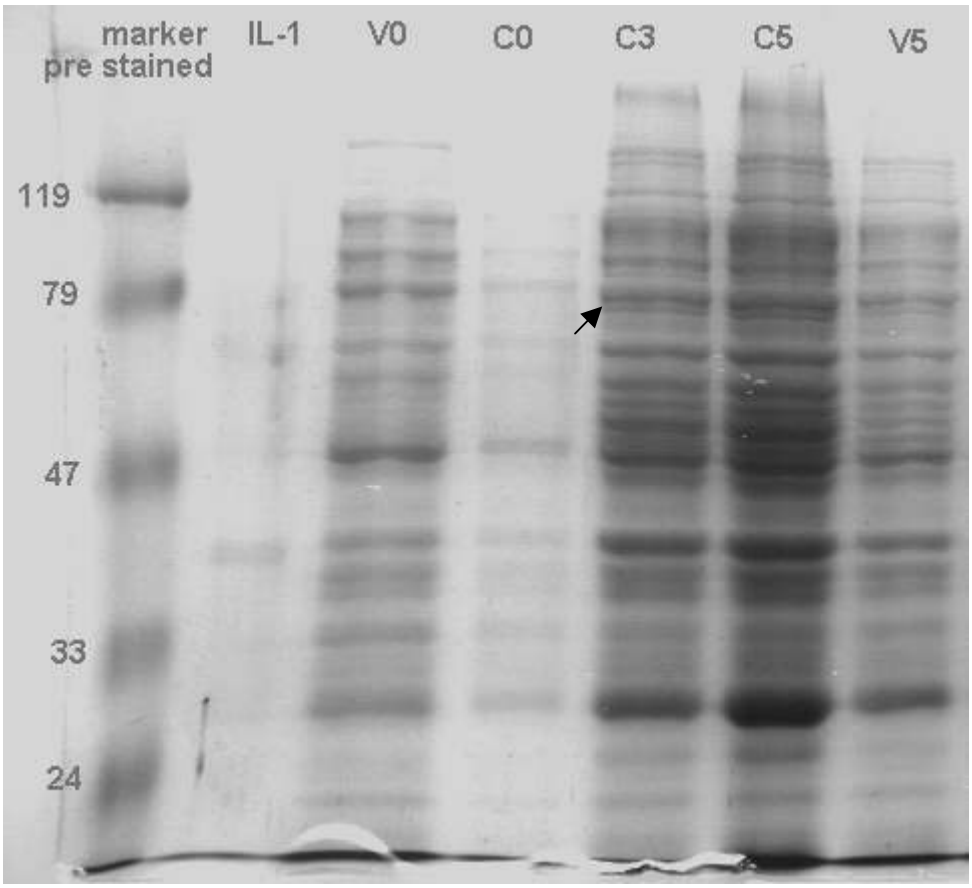


Figure 5.1 SDS PAGE picture of BL21 (DE3) co-transformed with RIG plasmid and induced with 1mM IPTG.

Il-1 – Control

V0 - Vector (0 hr)
V5 - Vector (5 hr)

C0 - Clone (0 hr)
C3 - Clone (3 hr)
C5 - Clone (5 hr)

Most of the expression studies done so far have used a synthetic gene with *E.coli* codon bias for expression of AMA - 1 and hence, we feel this problem needs to be circumvented to get complete expression of the full protein. Absence of glycans in bacterial systems may also affect the posttranslational modification of the protein and lead to protein instability, which could also affect the expression profile. However studies show expression and proper folding of this protein for immunological analysis, negating absence of glycans as a reason for non-expression of protein. So, the predominant factor for non-expression should be codon bias by *E.coli*.

To test this hypothesis further, we did a RT-PCR to check if transcription was fine and it was not affected. Total RNA was extracted from the induced *E.coli* BL21pLys S cultures transformed with just the vector and the recombinant molecules using the standard protocol. After visualizing the total RNA, then the RNA sample was subjected to reverse transcription using the Qiagen Sensiscript RT Kit. A PCR reaction was setup using the *Plasmodium falciparum* AMA - 1 primers using the standardized conditions as given . On running the agarose gel, an amplification of 1.8 kb fragment of AMA - 1 was found in both the pRSET clone as well as the pET clone (Fig.5.2). This indicated the presence of mRNA and its subsequent conversion to cDNA, which then acted as a template for PCR amplification with the AMA-1 primers.

To confirm the absence of DNA contamination in the RNA preparation, a PCR reaction was set up with the RNA sample and no amplification was seen, removing the doubt of any DNA contamination. No amplification was seen in this case (Fig.5.2), which confirmed that there was no considerable DNA contamination, which could have amplified otherwise.

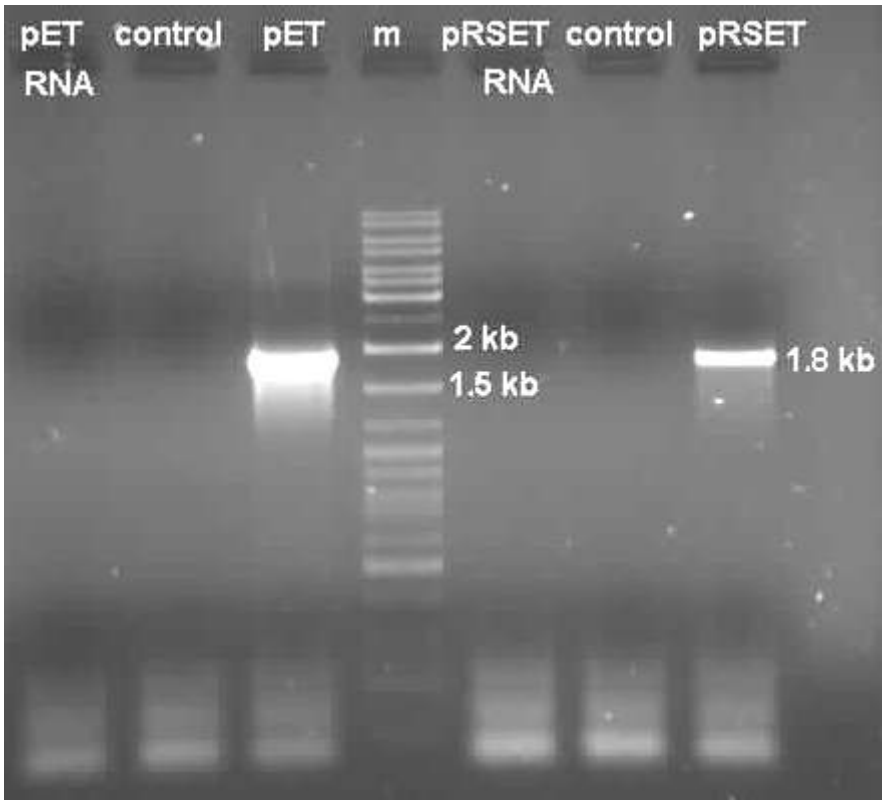


Figure 5.2 RT – PCR using AMA – 1 primers for both the pRSET and pET clones.

To reaffirm and confirm this fact that codon bias was the reason for non-expression of AMA-1 protein from the field isolates of India, in the *E.coli* expression system, a theoretical study was carried out to compare the codons of *E.coli* and codons found in our protein. *E.coli* shows codon bias against 5 amino acids represented by 7 codons (Table 5. 1) (Novy *et.al.* 2001).

Table 5. 1: Codon bias found in *E.coli*.

S. No.	Amino Acid	*E.coli* codon bias against
1.	Arginine	AGG, AGA, CGA
2.	Glycine	GGA
3.	Isoleucine	AUA
4.	Leucine	CUA
5.	Proline	CCC

Study of our protein shows a total of 48 codons for which *E.coli* showed codon bias throughout the length of the protein. The individual distribution for each amino acid is given in Table 5. 2.

Table 5. 2: Distribution of codons for which *E.coli* shows bias in AMA - 1

S. No.	Amino Acid.	No. of codons showing bias by *E.coli*					Total
		Prosequence	Domain I	Domain II	Domain III	Ectodomain (I+II+III)	Along the protein
1	Arginine	1	8	2	5	15	19
2.	Glycine	3	5	1	0	6	12
3.	Isoleucine	2	5	1	2	8	11
4.	Leucine	1	1	1	0	2	3
5.	Proline	0	0	1	1	2	3

So, these results clearly show that the codon bias by *E.coli* for these five amino acids is responsible for non-expression of protein in *E.coli* system. Use of RIG plasmid also did not favour expression because it neutralizes the codon bias for only 3 of the 5 amino acids. These results substantiated our stand that codon bias was affecting the protein expression in *E.coli.*

There are no reports as yet of the expression of complete protein from field isolates in *E.coli* due to codon bias. So, we suggest this can be circumvented by using a modified host strain like Rosetta, which codes for more t-RNAs and would help in better expression of the protein.

Chapter 6

Evaluation of antibody profiles in naturally infected individuals, to peptides based on different AMA-1 epitope variants

Introduction:

Malaria continues to be a major cause of mortality and morbidity in the most heavily populated areas of the world. In the past few years, due to the emergence of drug resistance in the malarial parasite, and insecticide resistance in vector, development of a vaccine against malaria is a must for improving public health in the tropical world. The complex life cycle of the malarial parasite and the extensive variability among strains of *Plasmodium* species dictate that, an effective malaria vaccine will require inducing protective antibodies as well as effector T lymphocytes, specific for variants of multiple antigens expressed at different stages of life cycle. This means that both antibody dependent and antibody-independent effector mechanisms are involved in protective immunity to malaria.

The existence of protective antibodies against erythrocytic stage malaria parasites was originally demonstrated by passive transfer of immunoglobulin *in-vitro*. (Cohen *et. al.,* 1961). One of the candidate vaccine antigen, that plays an important role in erythrocyte invasion is the Apical Membrane Antigen of *Plasmodium*. It is synthesized very late in schizogony and is processed at the time of schizont rupture and merozoite release, into merozoite surface polypeptides that are readily shed. (Deans *et.al.,* 1984). The ultimate test of a putative protective antigen is to establish whether vaccination with antigen can confer protection on susceptible individuals.

To study the immune response against AMA-1, first study was carried out by Deans, *et. al.* 1988 where rhesus monkeys were immunized with purified 66 kDa AMA-1 of *P.knowlesi* , and this produced antibodies that inhibited merozoite invasion of red cells *in-vitro.* This was followed by a study by Collins *et. al*. 1994 where Saimiri monkeys

were immunized with recombinant *P. fragile* AMA - 1 expressed in baculovirus system. Protection obtained in this case was similar to *P.knowlesi*. Rechallenge of the immunized animals with *P. falciparum*, resulted in delayed parasite counts suggestive of protection. This study exhibited and proved some amount of heterologous protection.

Lal *et. al* in 1996 mapped the immunodominant T-cell determinants of AMA-1 and proved that it may be possible to induce T-cell memory that can recognize different variant forms of the parasite. This information was based on the study of natural immune responses against the AMA-1 vaccine antigen using synthetic peptides and it also conclusively pointed out that some of the potent proliferative T epitopes were found to be localized in the highly conserved regions of AMA-1, suggesting these regions as highly immunogenic for the protein.

Crewther *et. al.,* 1996, investigated the impact of nature of diversity in the *P.chabaudi* homolog of AMA-1, by challenging it with a heterologous strain of *P.chabaudi*. Results showed that the variant had little effect on heterologous challenge, but provide significant protection against the homologous DS parasite, indicating that protective immune response involves recognition of strain specific epitopes.

A number of other experimental findings also suggested that AMA-1 may elicit a protective immune response against infections by the parasite in various animal models. Immunological studies conducted with *Plasmodium knowlesi* AMA-1 demonstrated that invasion inhibiting antibodies could be induced by the native AMA-1 of this parasite. Vaccination trials in rhesus monkeys demonstrated effective immunity against *Plasmodium knowlesi* challenge (Deans *et. al.* 1982., Thomas *et .al*. 1984). Later vaccination trials in rhesus monkeys demonstrated effective immunity against a *P.knowlesi* challenge *(*Deans *et. al.,* 1988*).* Results from recent vaccine trials in a rhesus monkey model with *Plasmodium fragile* (Collins *et. al*. 1994) and in a rodent model with *Plasmodium chaubadi* (Crewther *et.al*, 1996, Anders *et.al*. 1998) lent further support for the potential of AMA-1 as a vaccine candidate. Immunization of mice with recombinant proteins of *Plasmodium chabaudi* containing only the ectodomain of AMA-1 gave

complete protection against subsequent challenge with *Plasmodium chabaudi* (Anders *et. al*. 1998). Similar work was done with *Plasmodium yoelli* on mice and protective immunity was observed (Narum *et. al.* 2000). In yet another significant study immunization of rabbits was done with two allelic forms of AMA – 1, refolded *Plasmodium falciparum* AMA-1 singly and in combination, to study the effect of allelic diversity on AMA-1 to protect against human infection by different *Plasmodium falciparum* strains. This paper provided experimental evidence that AMA-1 allelic diversity is a result of immune pressure (Kennedy *et. al*. 2002**).** Similarly, various studies were carried out to understand the mechanism of immunity due to AMA-1, by studying the responses to different B and T cell epitopes, in different endemic areas.

Analysis of populations from areas of high malarial transmission in Senegal was done by Thomas *et. al*, 1994 where they demonstrated a very high prevalence (94-100%) of naturally acquired IgG responses to AMA-1. A statistically significant age-related change in antibody levels to AMA-1 was also observed in this study. Kocken *et.al* (1998) investigated heterologous (trans-species) expression and immunogenecity of human malaria *Plasmodium falciparum* AMA-1 and it was found that trans species expressed AMA-1 was highly immunogenic in mice, resulting in a response against a functionally critical domain of the molecule. A limited number of major T cell sites, both immunodominant and cryptic were identified in conserved and variable region of the protein by Amante *et.al.,* in 1997.

Xu *et. al*. (2000) for the first time showed that immunity to AMA - 1 was Antibody dependent T-cell mediated immunity, by proving that CD4+ T-cell depletion and not γδ T cell depletion can cause a significant drop in antiparasite immunity in either immunized normal or immunized B cell KO mice.

Udhayakumar *et. al.* (2001), in an attempt to characterize malaria antigen specific responses to synthetic peptides representing various regions of the protein, in adults naturally exposed to malaria in western Kenya, found that although the T-cell proliferative responses to AMA-1 were transient and short lived, the antibody levels were

relatively stable. They concluded that natural exposure to malaria does not induce long lasting T-cell memory responses to AMA-1 epitopes, even in areas of intense malaria transmission.

The purification, characterization and immunogenecity of the refolded ectodomain of *Plasmodium falciparum* AMA-1 expressed in *E.coli*. was done by Dutta. *et al*. in 2002 and the *in-vitro* parasite invasion data with antibodies raised against this recombinant AMA-1/E reaffirmed the potential of AMA - 1 as an important vaccine candidate. A recent study by Dutta *et. al* in 2003 proved that, at low concentrations, IgG antibodies against correctly folded recombinant Pf AMA - 1 act by disrupting proteolytic processing of AMA-1. These antibodies essentially prevented the circumferential redistribution of AMA-1. Other significant studies in this direction include the one by Howell *et al.* in the year 2001 where they identified a epitope mapped to position between Ile 97 – Tyr 425 which proved that antibody binding domains I and II would interfere with AMA-1 function. A recent addition to these studies came by Nair *et al.* in the year 2002 when they proposed that domain III also has epitopes for antibody recognition especially the disulphide bond stabilized core.

Lalitha *et al*. in 2004 studied the immune and antibody response against individual domains of AMA - 1 by constructing six different genes that express each of the domains separately (D I, D II, or D III) or in combination with another domain (D I+II, D II+III, or D I+III). They demonstrated that D I+II induced a significant amount of the growth-inhibitory antibodies active in the growth and invasion assay. Healer *et al*., (2005) proved that **a**llelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum* . This study is the first to use *P. falciparum* allelic exchange to examine the relationship between genetic diversity and susceptibility to protective antibodies.

In connection with this, a more detailed study by Cortes.*et.al*. in 2005 characterized the antibody responses against AMA - 1 in 262 individuals from Papua New Guinea exposed to malaria by using different allelic forms of the full AMA - 1 ectodomain and some

individual subdomains. The majority of individuals had very high levels of antibodies against AMA - 1. The prevalence and titer of these antibodies increased with age. Although antibodies against conserved regions of the molecule were predominant in the majority of individuals, most plasma samples also contained antibodies directed against polymorphic regions of the antigen. In a few individuals, predominantly from younger age groups, the majority of antibodies against AMA - 1 were directed against polymorphic epitopes. The D10 allelic form of AMA - 1 apparently contains most if not all of the epitopes present in the other allelic forms tested, which might argue for its inclusion in future AMA – 1 based vaccines to be tested. Some important epitopes in AMA - 1 involved residues located in domain II or III but depended on more than one domain**.** In this attempt, Pan Weiquing *et. al*., in 2004 created a *Plasmodium falciparum* chimeric protein Pfcp - 2.9 having the C-terminal region of Msp-1 and the domain III region of AMA - 1. This Pfcp - 2.9 was found to be highly immunogenic in rabbits as well as rhesus monkeys. It induced both, the Msp1-19 antibodies as well as AMA - 1(III) antibodies and was able to completely inhibit the parasite growth *in vitro*.

In order to investigate the role of posttranslational modification like N-glycosylation, O glycosylation, etc. in modulating the functional immune responses to AMA - 1 these sites were mutated and recombinant AMA-1 expressed in *E.coli* and *P. pastoris* systems. After this study, they concluded that both *E.coli* and *P.pastoris* derived antigens were immunologically and functionally equivalent and are unaffected by the posttranslational modification resulting from expression in these two systems. This actually broadened the choice of expression systems for AMA - 1 antigen production and allows factors such as yield and cost to be the determining criteria.

However, the ultimate test of a malaria vaccine will be determined in field trials under natural conditions of parasite exposure**.** Early clinical trials with AMA-1 show protective immunity in humans (Saul *et. al*. 2005).

The most recent study on invasion inhibiting antibody was published by Coley *et. al.* (2006), where they described an anti-AMA-1 monoclonal antibody (MAb 1F9) that

inhibits the invasion of *Plasmodium falciparum* parasites *in vitro*. They showed that, both reactivity of MAb 1F9 with AMA-1 and MAb 1F9-mediated invasion inhibition were strain specific. They identified a single polymorphic residue in domain I of AMA-1 that is critical for MAb 1F9 binding. The identities of all other polymorphic residues investigated in this domain had little effect on the binding of the antibody. Examination of the *P. falciparum* AMA-1 crystal structure localized this residue to a surface-exposed α-helix at the apex of the polypeptide. This description of a polymorphic inhibitory epitope on AMA-1 adds supporting evidence to the hypothesis that immune pressure is responsible for the polymorphisms seen in this molecule.

All these studies, using animal models and studying the natural immune responses in adults exposed to malaria, clearly indicate the important role of AMA - 1 in offering protective immunity and justifies it as an important vaccine candidate. However, no such study has been carried out in an endemic country like India, where the host responses and the antigenic variation would both be crucial factors for vaccine design. With this aim, Ab responses to AMA-1 were studied in natural Indian population exposed to malaria.

Results and Discussion:

The blood stage vaccine candidate AMA-1 of *P. falciparum* has been studied extensively, and various naturally immunogenic B-cell and T-cell recognition sites or epitopes have been reported. The genetic diversity of these epitopes have also been studied, and it has been found that all the 9 epitopes reported in AMA-1 of *Plasmodium falciparum* are polymorphic and show significant degree of variation. (Escalante, *et. al.* 2001, Lal *et. al.* 1996 and Udhayakumar *et. al*. 2001).

The study of these 9 epitopes reveals that only 3 of the 9 are B – cell epitopes while remaining epitopes are all either T – cell or T and B cell epitopes. Out of these three B cell epitopes, one is in the prosequence region of protein (Aa 41-57), while the other two

are in Domain II of the ectodomain (Amino acids 317-334 and 321-338). The B – cell recognition region from 317 to 334 was chosen for this study (Fig. 6.1).

This region has been reported to show considerable variation at 5 amino acid positions. (Escalante *et. al.* 2001). Polymorphism study of this region in our 10 Indian isolates reveals higher degree of variation . We find variations at two more amino acid positions within this stretch, in addition to the 5 positions reported previously. (Escalante *et. al.* 2001). One of the two new variation is found in Cysteine responsible for maintaining the disulphide bond structure of the protein. So, these 7 /17 amino acids which show variation and this could be very significant in the immunological activity of this linear B – cell epitope (Fig. 6.1). Hence, in an attempt to confirm the effect of variation on immunogenecity, the present study aims to investigate the humoral response to synthetic peptides designed for variants of this epitope, in adults living in areas of India where malaria has been endemic. Details of variation in this epitope and the synthetic peptides designed for these variants are given in Table 6.1.

Table 6.1 Peptides designed against the B-cell epitope variants of AMA-1 from Indian isolates.

Name of the peptide	Sequence in Indian isolates (amino acid 317 – 334)	Indian isolate showing the change	No of isolates having the change
E1a	DGNCEDI PHVNEFPAIDL	R3	1
E1b	DGNCEDIPHVNEFSANDL	S1,S3	2
E1c	DGNCEDIPHGNEFPANDL	R5	1
E1d	DGNWEDIPHVNEFSANDL	R2,S5	2
E1e	DGNWEDIPHVNEFSAIDL	S2	1

Sequence of prototype: 3D7: DGNCEDI PHVNEFPAIDL

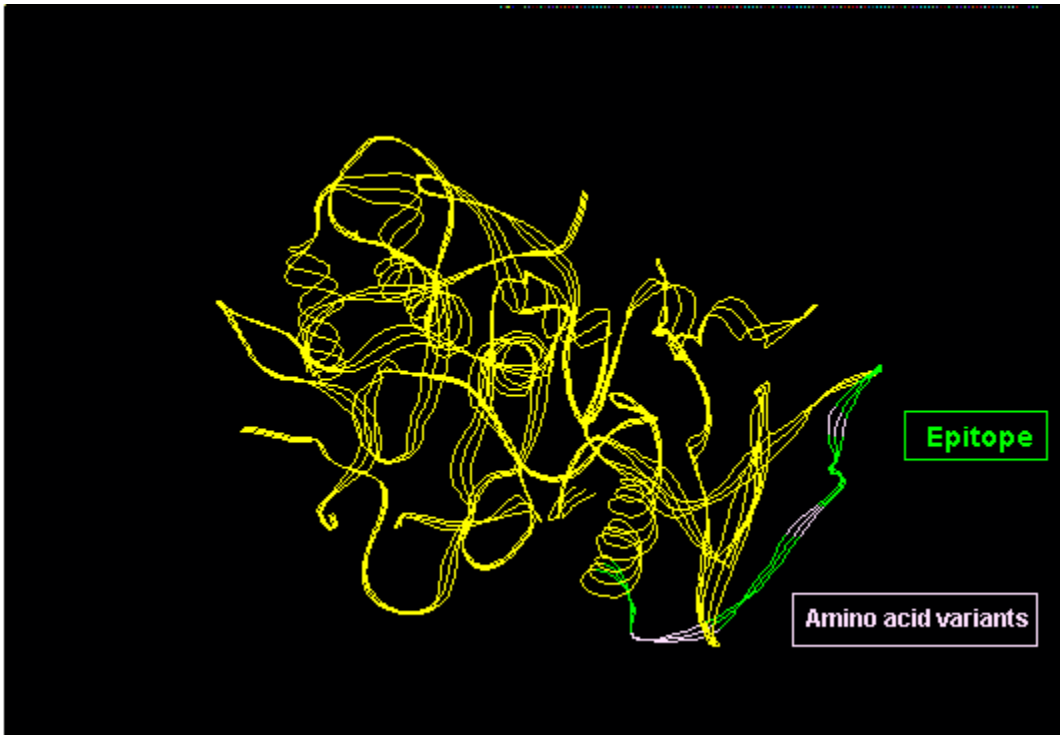


Figure 6.1. The structural distribution of E1 peptide corresponding to *P. falciparum* AMA-1 derived from data based on solution structure of the same (Bai *et.al.* 2005)

E1 : DGNXEDIPHXNEFXXXDL

E1a: - - - C - - - - - V - - - PAI - -

E1b: - - - C - - - - - V- - - SAN - -

E1c: - - - C - - - - - G - - - PAN - -

E1d: - - - W- - - - - V - - - SAN - -

E1e: - - - W- - - - - V - - - SAI - -

The analysis of naturally acquired serum IgG responses by ELISA to the epitope variants found in Indian isolates, demonstrated a high prevalence of serum IgG response against peptides studied. Threshold of positivity was an OD value of 0. 0914, 0.1088, 0.102, 0.195, and 0.397 for E1a, E1b, E1c, E1d and E1e respectively, based on the mean plus SD of the reactivity of sera from 14 healthy controls who were not exposed to malaria before. Out of the 25 malaria patients sera tested, only 4 (16%) responded to all 5 variants. No sera sample was found to be negative for all the peptides tested, indicating that all patients responded to atleast one of the epitope variant studied.

Antibody response against the 5 epitope variants studied in 25 patients sera, indicates that 3 variants E1a, E1b, E1c showed higher frequency of response as compared to E1d and E1e. The individual response frequencies against E1a, E1b, E1c , E1d, and E1e were found to be 88% (22/25), 76% (19/25), 100% (25/25), 56% (14/25) and 52% (13/25) respectively (Fig 6.2 – 6.6 and Table 6. 2).

E1c shows the highest response among the peptides, with all the 25 individuals in study , showing good antibody titre to this peptide. This suggest that, E1c is either the most immunogenic among peptide variants studied, or the most predominant allele exposed to Indian population under consideration or it has the best consensus conformational similarity to all the others. Analysis of E1c at amino acid level shows a change at position 326, from Valine to Glycine, a change not found in any of the other peptides studied. This could contribute to the increased immunogenicity of E1c.

Two peptide variants E1d and E1e show significantly lower antibody response among the peptides studied. This low binding could probably indicate that this allele is less predominant or could be these amino acids that are substituted are probably contact residues. Analysis of amino acid combination of this variant shows that they differ from other peptides at four positions, 320, 330, 331, 332. However, E1b and these two peptides have common amino acids in positions 330, 331 and 332 and differ by only one amino acid i.e. 320, where cysteine is replaced by tryptophan in E1d andE1e. Even this

one amino acid change, contributes to decrease in immunogenecity of E1d and E1e by nearly 20%, indicating the importance of Cysteine at this position.

Our data suggest that there is cross reactivity among different variants, represented by different peptides corresponding to E1a, E1b, E1c, E1d and E1e (Fig 6.2 – 6.6). There is also a possibility that the sera collected could be from patients with a mixed parasite population with respect to these alleles. These results however should be proved conclusively by carrying out animal studies.

Table 6. 2: ELISA Result Analysis of 25 Malaria Patients Sera

Peptides	**E1a**	**E1b**	**E1c**	**E1d**	**E1e**
Negative Cutoff	0.0914	0.1088	0.102	0.195	0.397
PS1	+	+	+	+	+
PS2	+	+	+	+	+
PS3	+	+	+	+	+
PS4	-	+	+	+	+
PS5	-	-	+	+	+
PS6	+	+	+	+	-
PS7	+	+	+	+	-
PS8	+	+	+	+	-
PS9	+	+	+	+	-
PS10	+	-	+	+	+
PS11	+	+	+	-	-
PS12	+	+	+	-	-
PS13	+	-	+	+	+
PS14	+	+	+	-	-
PS15	+	+	+	-	-
PS16	+	+	+	-	-
PS17	+	-	+	+	+
PS18	+	+	+	-	-
PS19	+	+	+	-	-
PS20	-	-	+	+	-
PS21	+	+	+	-	+
PS22	+	+	+	-	+
PS23	+	-	+	-	+
PS24	+	+	+	-	+
PS25	+	+	+	+	+
Total No (+)	22	19	25	14	13
Total No (-)	3	6	0	11	12
Response frequency (%)	88	76	100	56	52

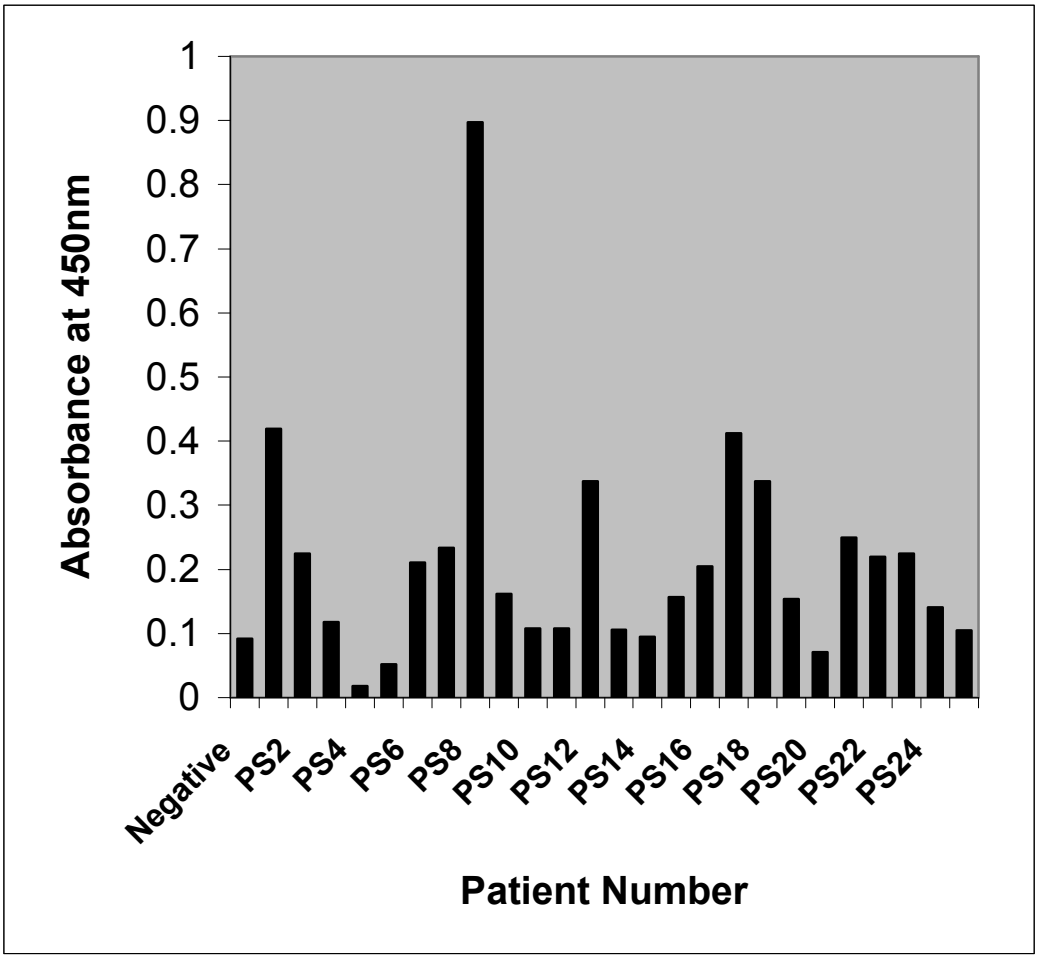


Figure. 6.2 Antibody Profile against E1a

Legends:

Negative : Negative Cutoff Value

PS 1 - PS 25 : Patients Sera Samples

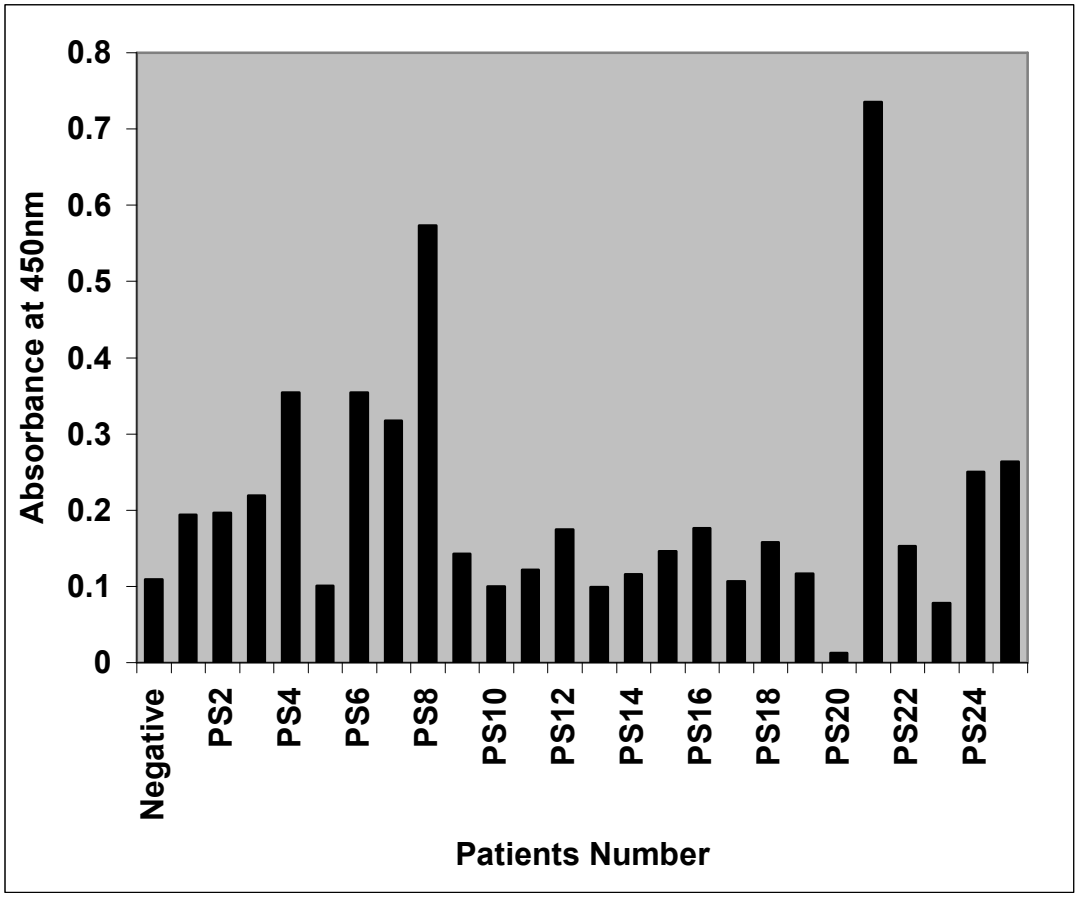


Figure 6.3 Antibody Profile against E1b

Legends:

Negative : Negative Cutoff Value

PS 1- PS 25 : Patients Sera Samples

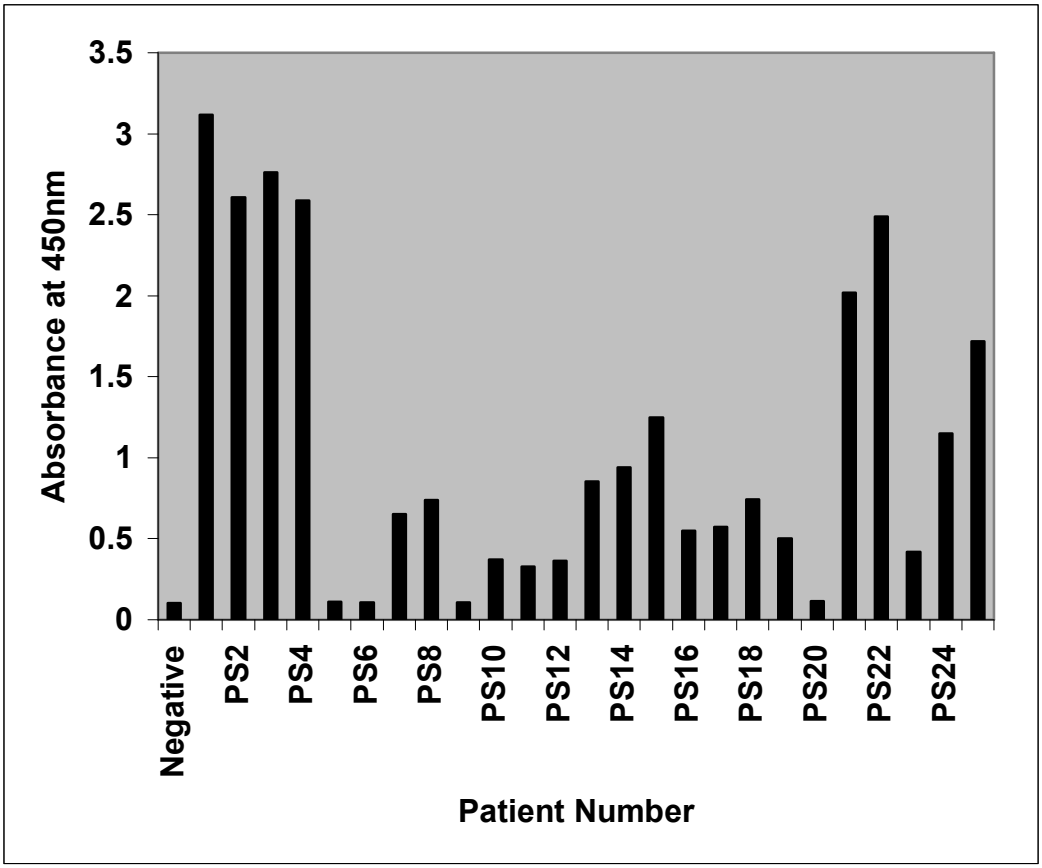


Figure 6.4 Antibody Profile against E1c

Legends:

Negative : Negative Cutoff Value

PS 1 – PS 25 : Patients Sera Samples

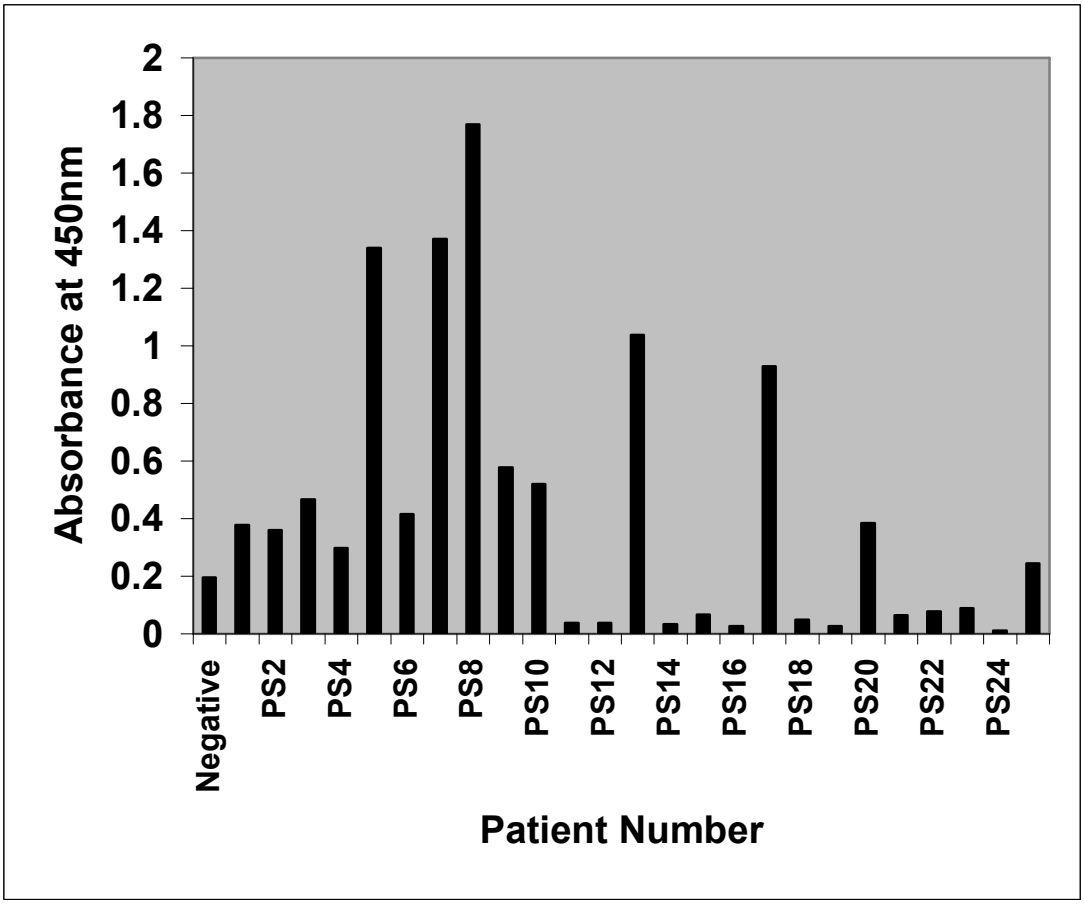


Figure 6.5 Antibody Profile against E1d

Legends:

Negative : Negative Cutoff Value

PS 1- PS 25 : Patients Sera Samples

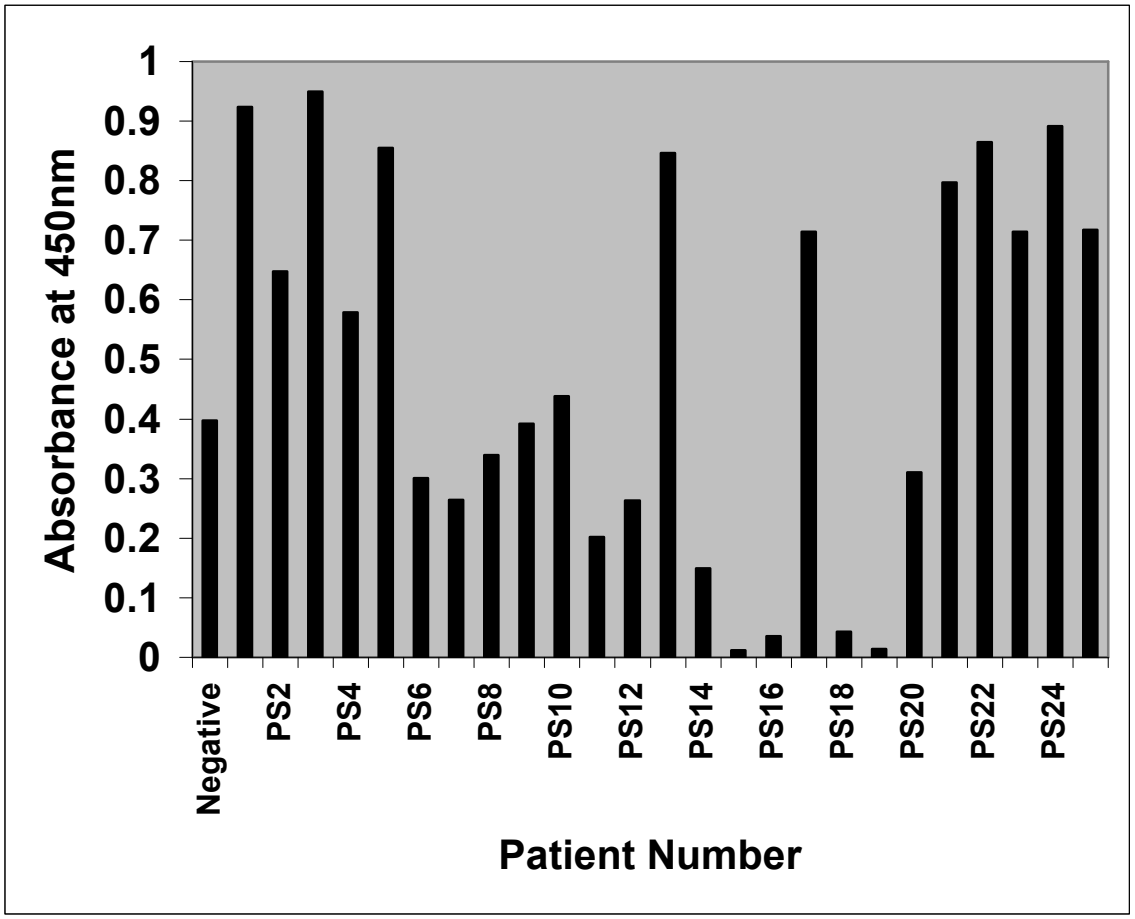


Figure 6.6 Antibody Profile against E1e

Legends:

Negative : Negative Cutoff Value

PS 1 – PS 25 : Patients Sera Samples

Chapter 7

Conclusions

1. We have investigated the sequence diversity of the Apical Membrane Antigen, in both *Plasmodium falciparum* and *Plasmodium vivax* from Indian field isolates, and showed considerable variations as compared to sequences previously reported in literature so far. Our study on *P. falciparum* identifies 99 amino acid substitutions as against the 54 reported previously. Moreover, we have found variation in two of the 16 conserved Cysteine residues, which could be very significant in the antigenic property of this protein. Our study implicates Domain II to be equally polymorphic as domain I as against the studies reported previously which implicated domain I as the most hypervariable region of Pf AMA-1. Much higher variation has also been found in the reported epitopes of this protein making the study immunologically significant.

2. This is first kind of study done in India, for *P.vivax* for the nearly full length of AMA-1 gene. Our study finds that the analysis of genetic diversity of the 1640 bp region of Pv AMA-1 from Indian isolates shows much higher variation than reported previously. At the protein level, our analysis showed a total of 28 amino acid changes as against only 16 reported previously. 11 new changes were found in our study at positions 11, 63, 66, 69, 368, 382, 438, 445, 519, 530, 538 and 540. Moreover, 5 new haplotypes have been identified in this study, which were not previously reported in any other study, making this outcome very significant.

3. No reports exists as on date, on the expression of full-length AMA-1 protein from field isolates. So, cloning of the AMA-1 gene into expression vectors and expression studies of the clone were carried out. Various differentiating parameters were tried like,
    - Two different expression vectors,
    - Multiple expression systems,
    - Co transformation with the RIG plasmid etc.

However, the expression of the full-length protein in the *E.coli* prokaryotic system was not successful.

4. Due to non-availability of the expressed protein, synthetic peptides were designed against epitope variants, and immunological studies were carried out. The antibody profile in naturally infected individuals from a malaria endemic region of India was evaluated. The analysis of naturally acquired serum IgG responses by ELISA to the epitope variants found in Indian isolates, demonstrated a high prevalence of serum IgG response against peptides studied. Peptide E1c showed the highest response among the 5 peptides studied, whereas E1d and E1e show significantly lower antibody response. We also found antibody cross reactivity against different epitope variants.

Future Perspectives:

1) Expression of the full-length protein from variant field isolates would aid in characterizing the immunological profile of this important vaccine candidate more distinctly.
2) Animal immunization studies need to be done to confirm the predictions of the results obtained from synthetic peptides.
3) All the structural predictions of variations need to be validated by more comprehensive immunological studies.
4) The polymorphism obtained in *P.vivax* isolates needs to be studied in detail, and the information could be used to elucidate the mechanisms of severe malaria cases caused by *P.vivax*.

REFERENCES

Abagyan, R.A., Batalov, S., (1997), "Do aligned sequences share the same fold?" J. Mol Biol. , Vol. 273, No. 1, 355 – 368.

Alonso, P. L., Lopez, M.C., Bordmann, G., Smith, T.A., Aponte, J. J., Weiss, N.A., Urassa, H., Armstrong-Schellenberg, J. R., Kitua, A.Y., Masania, J., Thomas, M.C., Oettli, A., Hurt, N., Hayes, R., Kilama, W. L., Tanner, M., (1998) "Immune responses to *Plasmodium falciparum* antigens during a malaria vaccine trial in Tanzanian children." Parasite Immunol., Vol. 20, No. 2, 63 – 71.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., (1990), " Basic Local Alignment Search Tool.", J. Mol. Biol., Vol. 215, 403 – 410.

Amante, F. H., Crewther, P.E., Anders, R.F., Good, M.F., (1997) "A cryptic T cell epitope on the apical membrane antigen 1 of Plasmodium chabaudi adami can prime for an anamnestic antibody response: implications for malaria vaccine design." J. Immunology, Vol. 159, No. 11, 5535 - 5544.

Anders, R. F., (1991) "Antigenic diversity in *Plasmodium falciparum."* Acta Leidensia, Vol. 60, No. 1, 57 - 67.

Anders, R. F., Crewther, P. E., Edwards, S., Margrets, M., Mathew, M. L. S. M., Pollock, B., Pye, D., (1998) "Immunization with recombinant AMA-1 protects mice against infection with *Plasmodium chabaudi* ", Vaccine, Vol. 16., No 2/3, 240 – 247.

Anders, R.F and Saul, A. J., (1994), "Candidate antigens for an asexual blood stage vaccine against *falciparum* malaria." In molecular Immunological considerations in malaria vaccine development, M.F.Good and A.J. Sauls eds., CRC Press, Boca Raton, 170.

Arévalo-Herrera, M., Herrera, S., (2001) "Review - *Plasmodium vivax* malaria vaccine development." , Mol. Immunol., Vol. 38, No.6 , 443 - 455.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., (1999), " Short Protocols in Molecular Biology" 4th edition, Published by John Wiley & Sons, Inc., NY, USA.

Baca, A. M., Hol, W. G., (2000), "Overcoming codon bias: a method for high-level overexpression of Plasmodium and other AT-rich parasite genes in Escherichia coli.", Int. J. Parasitol. Vol. 30, No. 2, 113 - 118.

Bai, T., Becker, M., Gupta, A., Strike, P., Murphy, V.J., Anders, R.F., Batchelor, A.H., (2005), "Structure of AMA1 from Plasmodium falciparum reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket.", PNAS, Vol. 102, No. 36, 12736 – 12741.

Barton, G.J., (1993) , " ALSCRIPT: A tool to format multiple sequence alignments." Protein Engg., Vol. 6, No. 1, 37 – 40.

Biswas, S., Saxena, Q. B., Roy, A., (1990), "The natural occurrence of circulating antibodies in population of endemic malarious areas." Indian J. Malariol., Vol. 27, No.3, 139 - 148.

Carlton, J., (2003), " The *Plasmodium vivax* genome sequencing project." Trends Parasitol., Vol. 19, No. 5, 227 – 231.

Casey, J.L., Coley, A.M., Anders, R.F., Murphy, V.J., Humberstone, K.S., Thomas, A.W., Foley, M., (2004) " Antibodies to Malaria Peptide Mimics Inhibit Plasmodium falciparum Invasion of Erythrocytes." Infect. Immun., Vol. 72, No. 2, 1126 – 1134.

Cheng, Q., Saul, A., (1994) "Sequence analysis of the apical membrane antigen I (AMA-1) of *Plasmodium vivax*." Mol. Biochem. Parasitol., Vol. 65, 183 - 187.

Cheng, Q., Stowers, A., Huang, T. Y., Rzepczyk, C., Saul, A., (1993) "Polymorphism in *Plasmodium vivax* MSA1 gene - the result of intragenic recombinations.", Parasitology, Vol. 106**,** 335 - 345.

Chung , J., Chun , E., Chun , J., Kho W., (2003) "Analysis of the Plasmodium vivax apical membrane antigen-1 gene from re-emerging Korean isolates" Parasitol. Res., Vol. 90, 325 – 329.

Cohen, S., McGregror, I.A., Carrington, S., (1961), " Gamma-globulin and acquired immunity to human malaria.", Nature, Vol. 192, 733 – 737.

Coley, A. M., Parisi, K., Masciantonio, R., Hoeck, J., Casey, J. L., Murphy, V. J., Harris, K. S., Batchelor, A. H., Anders, R. F., Foley, M. (2006), "The Most Polymorphic Residue on Plasmodium falciparum Apical Membrane Antigen 1 Determines Binding of an Invasion-Inhibitory Antibody." Infect. Immun., Vol. 74, 2628 - 2636.

Collins, W.E., Pye, D., Crewther, P. E., Vandenberg, K.L., Galland, G.G., Sulzer, A.J., Kemp, D.J., Edwards, S.J., Coppel, R.L., Sullivan, J.S., Morris, C.L., Anders, R.F., (1994)," Protective Immunity Induced in squirrel monkeys with recombinant Apical Membrane Antigen –1 of *Plasmodium fragile*." , Am. J. Trop. Med. Hyg., Vol. 51, No. 6, 711 – 719.

Conway, D. J., Fanello, C., Lloyd, J.M., Al-Joubori, B.M., Baloch, A. H., Somanath, S.D., Roper, C., Oduola, A.M., Mulder, B., Povoa, M. M ., Singh, B., Thomas, A.W., (2000), "Origin of *Plasmodium falciparum* malaria is traced by mitochondrial DNA." Mol. Biochem. Parasitol., Vol. 111, No. 1, 163 - 171.

Corte´s, A., Mellombo, M., Masciantonio, R., Murphy, V. J., . Reeder, J. C., and Anders, R. F., (2005) "Allele Specificity of Naturally Acquired Antibody Responses against *Plasmodium falciparum* Apical Membrane Antigen 1.", Infect. Immun., Vol. 73, No. 1, 422 – 430.

Cortes, A., Mellombo, M., Mueller, I., Benet, A., Reeder, J.C., Anders, R.F., (2003), "Geographical structure of diversity and differences between symptomatic and asymptomatic infections for *Plasmodium falciparum* vaccine candidate AMA1." , Infect. Immun. Vol. 71, No. 3, 1416 - 1426.

Crewther, P. E., Matthew, M.L.S., Flegg, R.H., Anders, R.F., (1996), " Protective Immune Responses to Apical Membrane Antigen – 1 of Plasmodium chabaudi involve recognition of Strain- specific Epitopes." Infect. Immun., Vol. 64, No. 8., 3310 – 3317.

Das, A., Holloway, B., Collins, W.E., Sharma, V.P., Ghosh, S.K., Sinha, S., Hasnain, S.E., Talwar, G.P., Lal, A.A., (1995), " Species specific 18S rRNA gene amplification for the detection of P.falciparum and *P.vivax* malaria parasites." Mol. Cell. Probes, Vol. 9 No. 3, 161 – 165.

De la Cruz, V.F., Maloy, W.L., Miller, L.H., Lal, A.A., Good, M.F., McCutchan, T.F., (1988) "Lack of cross reactivity between variant T-cell determinants from the malaria circumsporozoite protein." J. Immunol, Vol. 141, 2456 – 2460.

Deans, J. A., Thomas, A. W., Alderson, T., and Cohen, S., (1984), "Biosynthesis of a putative protective *Plasmodium knowlesi* merozoite antigen." Mol. Biochem. Parasitol, Vol. 11, 189 - 204.

Deans, J. A., Alderson, T., Thomas, A.W., Mitchell, G.H., Lennox, E. S., Cohen, S., (1982) "Rat monoclonal antibodies which inhibit the invitro multiplication of P*lasmodium knowlesi*", Clin.Exp.Immunology Vol. 49, 297 - 309.

Deans, J.A, Knight, A. M., Jean, W. C., Waters, A. P., Cohen, S. and Mitchell, G. H., (1988) "Vaccination trials in rhesus monkeys with minor invariant *Plasmodium knowlesi", Parasite* Immunology, Vol. 10, 535 – 552.

Donahue, C. G., Carruthers, V. B., Gilk, S. D., and Ward, G. E., (2000), "The *Toxoplasma* homolog of *Plasmodium* apical membrane antigen-1 (AMA-1) is a microneme protein secreted in response to elevated intracellular calcium levels" Mol. Biochem Parasitol., Vol. 111, No. 1, 15 - 30 .

Doolan, D.L., and Hoffman, S.L., (1997), "Multigene vaccination against malaria .A Multi stage ,multi immune response approach." Parasitology Today, Vol 13 , No 5, 171 - 177.

Dutta, S., Haynes, J. D., Barbosa, A., Ware, L. A., Snavely, J. D., Moch, J. K., Thomas, A. W., Lanar, D. E. (2005). "Mode of Action of Invasion-Inhibitory Antibodies Directed against Apical Membrane Antigen 1 of *Plasmodium falciparum".* Infect. Immun.,Vol. 73, 2116 - 2122.

Dutta, S., Haynes, J.D., Moch, J.K., Barbosa, A., Lanar, D.E., (2003), "Invasion-inhibitory antibodies inhibit proteolytic processing of apical membrane antigen 1 of *Plasmodium falciparum* merozoites.",.PNAS, Vol 100, No. 21, 12295 – 12300.

Dutta, S., Lalitha, P.V., Ware, L.A., Barbosa, A., Moch, K.J., Vassell, M.A., Fileta, B. B., Kitov, S., Kolodny, N., Heppner, G.D., Haynes, D.J., Lanar, D.E., (2002) Purification , Characterization, and Immunogenecity of the Refolded Ectodomain of the *Plasmodium falciparum A*pical Membrane Antigen - 1 expressed in *Escherichia coli*. Infect. Immun. Vol 70, No 6, 3101 – 3110.

Dutta, S., Malhothra, P., and Chauhan, V.S., (1995), "Sequence analysis of the apical membrane antigen-1 of *Plasmodium cynomolgi bastianelli.*" Mol.Biochem Parasitol., Vol. 73, 267 - 270.

Eisen, D. P., Marshall, V. M., Jacobe, H. B., Coppel, R. L., (1999) "A *Plasmodium falciparum* apical membrane antigen-1 (AMA-1) gene apparently generated by intragenic recombination ." Mol. Biochem. Parasitol., Vol. 100, 243 – 246.

Escalante, A.A., Grebert, H. M., Chaiyaroj, S.C., Magris, M., Biswas, S., Nahlen, B.L., Lal, A.A., (2001), "Polymorphism in the gene encoding the apical membrane antigen –1 (AMA-1) of *Plasmodium falciparum*. X Asembo Bay Cohort Project." , Mol. Biol. Parasitol., Vol. 113, 279 – 287.

Figtree, M., Pasay, C.J., Slade, R., Cheng, Q., Cloonan, N., Walker, J., Saul, A., (2000) "*Plasmodium vivax* synonymous substitution, frequencies, evolution and population structure deduced from diversity in AMA-1 and MSP 1 genes." Mol. Biochem. Parasitol., Vol. 108, 53 - 66.

Fraser, T.S., Kappe, S.H.I., Narum, D.L., VanBuskirk, K.M., Adams, J.H., (2001) "Erythrocyte-binding activity of Plasmodium yoelli apical membrane antigen – 1 expressed on the surface of transfected COS-7 cells." Mol. Biochem. Parasitol., Vol 117, 49 - 59.

Fu, Y., Shearing, L.N., Haynes, S., Crewther, P.E., Tilley, L., Anders, R.F., Foley, M., (!997), " Isolation from Phage display libraries of single chain variable fragment antibodies that recognize conformational epitopes in the malaria vaccine candidate, Apical Membrane Antigen – 1.", J. Biol. Chem., Vol. 272, No. 41, 25678 – 25684.

Giersing, B., Miura, K., Shimp, R., Wang, J., Zhou, H., Orcutt, A., Stowers, A., Saul, A., Miller, L.H., Long, C., Singh, S., (2005), " Post translational modification of Recombinant Plasmodium falciparum Apical Membrane Antigen 1 : Impact on

Functional Immune Response to a Malaria Vaccine Candidate", Infect. Immun., Vol. 73, No. 7, 3963 – 3970.

Han, E. T., Park, J. H., Shin E. H., Choi, M. H., Oh M.D., Chai J. Y., (2002) "Apical membrane antigen-1 (AMA-1) gene sequences of re-emerging *Plasmodium vivax* in South Korea". The Korean Journal of Parasitology, Vol. 40, No. 3, 157 – 162.

Harlow, E., Lane, D., (1988), " Antibodies – A laboratory manual, Cold Spring Harbour Laboratory Press, NY, USA.

Harris, K.S., Casey, J.L., Coley, A.M., Masciantonio, R., Sabo, J.K., Keizer, D.W., Lee, E.F., McMahon, A., Norton, R.S., Anders, R. F., Foley, F., (2005) "Binding Hot Spot for Invasion Inhibitory Molecules on *Plasmodium falciparum* Apical Membrane Antigen – 1." Infect. Immun., Vol 73, No. 10, 6981 – 6989.

Healer, J., Triglia, T., Hodder, A. N., Gemmil, A .W., Cowman, A. F., (2005) "Functional Analysis of *Plasmodium falciparum* Apical Membrane Antigen 1 utilizing Interspecies domains." Infect. Immun., Vol 73, No.4, 2444 - 2451.

Hehl, A.B., Lekuter, C., Grig, M. E., Bradley, P.J., Dubremetz, J.F., Ortegabania, E., and Boothroyd, J.C., (2000) "*Toxoplasma gondii* homologue of *plasmodium* apical membrane antigen 1 is involved in invasion of host cells." Infect. Immun. Vol. 68, 7078 - 7086.

Hockmeyer, W. T. and Ballou, W. R. (1988). "Sporozoite immunity and vaccine development." Prog. Allergy, Vol. 41, 1 - 14.

Hodder, A.N., Crewther, P.E., Ander, R.F., (2001) "Specificity of the protective antibody response to Apical Membrane Antigen –1." Infect. Immun., Vol. 69, No. 5, 3286 – 3294.

Hodder, A.N., Crewther, P.E., Mathew, M. L. S., Reid, G. E., Moritz, R. L., Simpson, R. J., Anders, R.F., (1996), "The Disulphide Bond Structure of *Plasmodium* Apical Membrane Antigen –1." J. Biol. Chem. Vol. 271, No.46, 29446 - 29452.

Holder, A. A. (1999), "Malaria Vaccines." PNAS, Vol. 96, 1167-1169.

Howell, S. A., Martinez, C.W., Kocken, C. H. M., Thomas, A. W., Blackman, M. J., (2001), "Proteolytic Processing and Primary Structure of *P. falciparum* Apical Membrane Antigen – 1." J. Biol. Chem. Vol. 276, No. 33, 31311 - 31320.

Hughes, M. K., and Hughes, A. L., (1995), "Natural selection on *Plasmodium* surface proteins.", Mol. Biochem. Parasitol. , Vol. 71, 99 – 113.

Imwong, M., Pukrittakayamee, S., Looareesuwan, S., Pasvol, G., Poirreiz, J., White, N. J., Snounou G., (2001), "Association of Genetic Mutations in *Plasmodium vivax dhfr* with Resistance to Sulfadoxine-Pyrimethamine: Geographical and Clinical Correlates", Antimicrobial Agents and Chemotherapy, Vol. 45, No. 11, 3122 – 3127.

Joshi, S.K., Bharadwaj, A., Chatterjee, S., Chauvan, V.S., (2000), " Analysis of Immune Responses against T- and B-cell epitopes from Plasmodium falciparum Liver-stage Antigen 1 in rhodent Malaria Models and Malaria Exposed Human subjects in India." Infect. Immun., Vol. 68, No. 1., 141 – 150.

Kabilan, L., Sharma, V.P., Kaur, P., Ghosh, S.K., Yadav, R.S., Chauhan, V.S., (1994), " Cellular and Humoral Immune Responses to Well defined Blood stage antigens (Major Merozoite Surface Antigen) of Plasmodium falciparum in Adults from an Indian zone where malaria is endemic." Infect. Immun., Vol. 62, No.2., 685 – 691.

Kappi, S.H.I and Adams, J.H.,(1996),"Sequence analysis of apical membrane antigen-1 genes (AMA1) *Plasmodium yoelli yoelli* and *Plasmodium berghei*" Mol.Biochem.Parasitol, Vol. 78, 279 - 283.

Kato, K., Mayer, D.C., Singh, S., Reid, M., Miller, L.H., (2005), "Domain III of Plasmodium falciparum apical membrane antigen 1 binds to the erythrocyte membrane protein Kx", PNAS, Vol. 102, No. 15, 5552 – 5557.

Kemp, D. J., Coppel, R. L., Stahl, H. D., Bianco, A. E., Corcoran, L. M., McIntyre, P., Langford, C .J., Favaloro, J. M., Crewther, P. E., Brown, G. V., *et .al*. (1986). The Wellcome Trust lecture. Genes for antigens of *Plasmodium falciparum*. Parasitology 92: Suppl., S83 - S108.

Kennedy, M. C., Jin Wang., Yanling Zhang., Miles, A. P., Farideh chitsaz., Saul, A., Cong, C. A., Miller, L.H., and Stowers, A. W., (2002) "In-vitro studies with Recombinent *Plasmodium falciparum* apical membrane antigen-1 (AMA1). Production and activity of an AMA1 vaccine and generation of a multiallelic response." Infect. Immun., Vol 70, No 12, 6948 - 6960.

Kerr, P. J., Ranford-Cartwright, L. C., Walliker, D., (1994), "Proof of intragenic recombination in *Plasmodium falciparum*." Mol. Biochem. Parasitol., Vol. 66, No. 2, 241 - 248.

Knowles, R., White, R. S., and Das Gupta, B. M., (1930), "Studies in the parasitology of malaria." Indian Medical Research Memoires, Vol. 18, 1 - 436.

Kochar, D.K., Saxena, V., Singh, N., Kochar, S.K., Vijaykumar, S., Das, A., (2005), "Plasmodium vivax malaria", Emerging Infectious Diseases, Vol. 11, No. 1, 132 – 134.

Kocken, C. H., Narum, D. L., Massougbodji, A., Ayiri, B., Dubbeld, M.A., Vander wel, A., Conway, D.H., Sanni, A., and Thomas, A.W., (2000), "Molecular characterization of *Plasmodium reichnowi* apical membrane antigen 1(AMA1), Comparison with *P.falciparum* AMA-1, and antibody mediated inhibition of red cell invasion" Mol. Biochem Parasitol, Vol. 109, 143 - 150.

Kocken, C. H., Dubbeld, M. A., Van Der Wel, A., Pronk, J . T., Waters, A. P.,Langermans,J.A., Thomas,A.W. (1999), "High-level expression of Plasmodium vivax apical membrane antigen 1 (AMA-1) in Pichia pastoris: strong immunogenicity in Macaca mulatta immunized with P. vivax AMA-1 and adjuvant SBAS2", Infect. Immun., Vol. 67, No. 1, 43 - 49

Kocken, C. H. M., van der Wel, A. M., Dubbeld, M. A., Narum, D. L., van de Rijke F. M., Gemert, G.J., Linde, X., Bannister, L. H., Janse, C., Waters, A.P., and Thomas, A.W., (1998), "Precise Timing of Expression of a *Plasmodium falciparum*-derived Transgene in *Plasmodium berghei* Is a Critical Determinant of Subsequent Subcellular Localization." J. Biol. Chem. Vol. 273, 15119 – 15124.

Lal, A.A., Hughes, M.A., Oliveira, D.A., Nelson, C., Bloland, P.B., Oloo, A.J., Hawley, W.E., Hightower, A.W., Nahlen, B.L., and Udhayakumar, V., (1996), " Identification of T-cell determinants in Natural Immune Responses to the Plasmodium falciparum Apical Membrane Antigen (AMA – 1) in an adult population exposed to malaria." , Infect. Immun., Vol. 64, No. 3, 1054 – 1059.

Lalitha, P. V., Ware, L.A., Barbosa, A., Dutta, S., Moch, J.K., Haynes, J.D., Fileta, B.B., White, C.E., Lanar, D.E., (2004) " Production of the Subdomains of the *Plasmodium falciparum* Apical Membrane Antigen 1 Ectodomain and Analysis of the Immune Response", Infect. Immun., Vol. 72, No. 8, 4464 – 4470.

Leclerc, M. C., Menegon, M., Cligny, A., Noyer, J. L., Mammadov, S., Aliyev, N., Gasimov, E., Majori, G., Severini, C., (2004) "Genetic diversity of Plasmodium vivax isolates from Azerbaijan.", Malar J. , Vol. 9, No. 3, 40.

Maestre, A., Sunil, S., Ahmad, G., Mohammed, A., Echeverri, M., Corredo, M., Blair, S., (2004) "Inter-allelic recombination in the Plasmodium vivax merozoite surface protein 1 gene among Indian and Colombian isolates." Malar J., Vol. 5, No. 3, 4.

Malaria; Obstacles and Opportunities Eds; Oaks, S. C., Mitchell, V.S., Pearson, G. W., Charles, C.J., Carpenter, (1991) National Academy Press, Washington D.C., USA.

Marshall, V. M., Zhang, L. X., Anders, R. F., Coppel, R. L., (1996) "Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum.*", Mol. Biochem. Parasitol., Vol. 77, No. 1, 109-113.

Marshall, V.M., Peterson, M.G., Lew, A.M., and Kemp, D. J., (1989), "Structure of the apical membrane antigen-1 (AMA-1) of the *Plasmodium chabaudi*" Mol. Biochem. Parasitol., Vol. 37, 281 - 284.

Marshall, V.M., Xing zhang, L., Anders, R. F.,.Coppel, R.L. (1996), "Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum."* Mol. Biochem. Parasitol., Vol. 77, 109 – 113.

Miller, L. H., Hoffman, S.L., (1998) "Research toward vaccine against malaria." Nature Med., Vol. 4, 520 - 524.

Miller, L.H., Howard, R.J., Carter, R., Good, M.F., Nussenzwerg. V., Nussenzwerg. R.S., (1986), " Research towards malaria vaccines." Science, Vol. 234, 1349.

Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R., and Bannister, L. H., (2004) "Apical Membrane Antigen 1, a Major Malaria Vaccine Candidate, Mediates the Close Attachment of Invasive Merozoites to Host Red Blood Cells." Infect. Immun. Vol. 72, No. 1, 154 – 158.

Multiantigen, Multistage, Vaccine Candidate for *Plasmodium falciparum* Malaria." Infect. Immun., Vol. 96, No. 9, 2201 – 2207.

Nair, M., Hands, M. G., Coley, A. M., Modder, A. N., Foley, M., Anders, R. F., and. Norton, R.S., (2002), "Structure of Domain – III of the blood stage malaria vaccine

canditate *P.falciparum* Apical Membrane Antigen –1(AMA-1)" J. Mol. Biol., Vol. 322, 741 - 753.

Narum, D. L., Ogum, S, A., Thomas, A. W., Hodder, A. A., (2000), "Immunization with parasite derived apical membrane antigen 1 or passive immunization with a specific monoclonal antibody protects BACB/C mice against lethal *Plasmodium yoelli yoelli* 1/m blood stage infection." Infect. Immun., Vol. 68, 2899 – 2906.

Narum, D. L., Thomas, A. W., (1994), "Differential localization of full length and processed forms of PF83/AMA-1 an apical membrane antigen of *P.falciparum* merozoites." Mol. Biochem. Parasitol., Vol. 67, 59 - 68.

Narum, D. L., Welling, G. W., Thomas, A. W., (1993) "Ion – exchange- immunoaffinity purification of a recombinant baculovirus *Plasmodium falciparum* apical membrane antigen, PF83/AMA-1." J. Chromat., Vol. 657, 357 – 363.

Narum, D.L., Ogun, S.A., Thomas, A.W., Holder, A.A., (2000) "Immunization with parasite-derived apical membrane antigen 1 or passive immunization with a specific monoclonal antibody protects BALB/c mice against lethal Plasmodium yoelii yoelii YM blood-stage infection.", Infect. Immun., Vol 68, No. 5, 2899 – 2906.

NMEP, *Malaria and its Control in India* (1995), Directorate General of Health Services, Directorate of National Malaria Eradication Programme, Ministry of Health and Family Welfare, Government of India, New Delhi.

Novy, R., Drott, D., Yaeger, K., Mierendorf, R., — Novagen, Inc., (2001), "Overcoming the codon bias of E. coli for enhanced protein expression", InNovations, News letter of Novagen, Inc . Advanced Products and Protocols For Molecular biology Research, NUMBER 12.

Oliveria, D. A., Udhayakumar, V., Bloland, P., Shi, Y. P., Nahlen, B. L., Oloo, A. J., Hawley, W. E., Lal, A. A., (1996) "Genetic conservation of the *Plasmodium falciparum* apical membrane antigen-1 (AMA-1)". Mol. Biochem. Parasitol., Vol. 76, (No. 1-2), 333-336.

Olivier, S., Franetich, J.F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C H., Thomas, A.W., Gemert, G. Jan van., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., and Mazier, D., (2004), "A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by *Plasmodium falciparum* Sporozoites" J. Biol. Chem., Vol. 279, No. 10, 9490 – 9496.

Pan, S.H., Malcolm, B.A., (2000), "Reduced background expression and improved plasmid stability with pET vectors in BL21 (DE3).", Biotechniques, Vol. 29, No. 6, 1234 – 1238.

Pan, W., Huang, D., Zhang, Q., Qu, L., Zhang, D., Zhang, X., Xue, X., and Qian, F., (2004), "Fusion of Two Malaria Vaccine Candidate Antigens Enhances Product Yield, Immunogenicity, and Antibody-Mediated Inhibition of Parasite Growth *In Vitro*." J. Immunol. , Vol. 172, No. 10 , 6167 - 6174.

Peterson, M.G., Marshall, V. M., Symthe, A. J., Crewther, P.E., Lew A., Silva, A., Anders, R. F and Kemp, D. J., (1989), "Integral membrane protein located in apical complex of *Plasmodium falciparum.*" Mol. Cell. Biol Vol. 9, No. 7, 3151 - 54.

Peterson, M.G., Nguyen-Dinh, P., Marshall, V.M., Elliot, J.F.E., Collins, W.E., Anders, R.F., Kemp, D.J., (1990), "Apical membrane antigen of *Plasmodium fragile*." Mol. Biochem. Parasitol., Vol. 39**,** 279 – 284.

Pizarro, C.J., Normand, B. V. L., Chesne-Seck, M.L., Collins, C.R., Martinez, C.W., Hackett, F., Blackman, M.J., Faber, B.W., Remarque, E.J., Kocken, C.H.M., Thomas,

A.W., Bentley, G.A., (2005), "Crystal Structure of the Malaria Vaccine Candidate Apical Membrane Antigen", *Science*, Vol. 308, No. 5720, 408 – 411.

Polley S.D., Chokejindachai W., Conway D.J., (2003) "Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen." Genetics, Vol. 165, No. 2, 555 - 561.

Preiser, P.R., Kaviratne, M., Khan, S., Bannister, L., Jara, W., (2000) "The apical organelles of malaria merozoites: host cell selection, invasion, host immunity and immune evasion." Microbes. Infect., Vol. 2, 1461 - 1477.

Qari, S.H., Shi, Y. P., Goldman, I.F., Nahlen, B.L., Tibayrenc, M., Lal, A. A., (1998) "Predicted and observed alleles of *Plasmodium falciparum* merozoite surface protein-1 (MSP-1),a potential malaria vaccine antigen**.**" Mol. Biochem. Parasitol., Vol. 92**,** 241 – 252.

Rich, S. M., Ferreira, M. U., Ayala, F. J., (2000), "The origin of antigenic diversity in *Plasmodium falciparum*." Parasitol. Today, Vol. 16, No. 9, 390 - 395.

Richie, T. L., Saul, A., (2002) "Progress and challenges for malaria vaccines**.**" Nature , Vol. 415 **,** 694 - 701.

Rodrigues, M. H. C., Rodrigues, K. M., Oliveira, T. R., Cômodo, A. N., Rodrigues, M. M. ,. Kocken, C. H. M., Thomas A. W., and Soares, I. S., (2005), "Antibody response of naturally infected individuals to recombinant Plasmodium vivax apical membrane antigen-1.", International Journal for Parasitology , Vol. 35, No. 2, 185 – 192.

Sambrook, J., Fritch, E.F., Maniatis, T., (1989), " Molecular cloning: A laboratory manual. 2nd Ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, NY, USA.

Saul, A., Lawrence, G., Allworth, A., Elliot, S., Anderson, K., Rzepczyk, C., Martin, L. B., Taylor, D., . Eisen, D. P., Irving, D. O., Pye, D., Crewther, P. E., Hodder, A. N., Murphy, V. J., Anders, R. F., (2005) "A human phase I vaccine clinical trial of the *Plasmodium falciparum* malaria vaccine candidate apical membrane antigen 1 in Montanide ISA720 adjuvant." Vaccine, Vol. 23, 3076 – 3083.

Sekar, V., (1987), " A Rapid Screening Procedure for the identification of Recombinant Bacterial Clones." Biotech., Vol. 5, No.1, 11 – 13.

Silvie, O., Franetich, J. F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, Eric., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., Gemert, G. J. V., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., Mazier, D., (2004), "A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by *Plasmodium falciparum* Sporozoites.", J. Biol. Chem. Vol. 279, No. 10, 9490 – 9496.

Sina, B., (2002), " Focus on Plasmodium vivax." Trends. Parasitol., Vol. 18, No. 7, 287 – 289.

Smythe, J. A.; Coppel, R. L.; Brown, G. V.; Ramasamy, R.; Kemp, D. J.; Anders, R. F. (1988), "Identification of two integral membrane proteins of *Plasmodium falciparum*", PNAS, Vol**.** 85, No. 14, 5195 – 5199.

Thomas, A. W., Deans, J. A., Mitchell, G. H., Alderson, T., and Cohen, S., (1984) "The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes." Mol. Biochem. Parasitol., Vol. 13, 187 – 199.

Thomas, A. W., Trape, J. F., Rogier, C., Goncalves, A ., Rosario, V. E., Narum, D. L., (1994) "High prevalence of natural antibodies against *P.falciparum 83* kilo Dalton Apical Membrane Antigen pf83/AMA1." Am . J. Trop. Med. Hyg, Vol. 51, 730 - 740.

Thomas, A. W.,.Waters, A. P., Debra Carr., (1990) "Analysis of variation in PF83,an erythro cytic merozoite vaccine candidate antigen of *Plasmodium falciparum"* Mol. Biochem. Parasitol., Vol. 42, 283 - 288.

Thompson, J.D., Higgins, D.G., Gibson, T.J., (1994), " CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties, and weight matrix choice." Nucleic Acid Res., Vol. 22, 4673 – 4680.

Tine, J. A., Lanar, D. E., Smith, D.M., Wellde, B.T., Schultheiss, P., Ware, L.A., Kauffman, E.B., Wirtz, R.A., De Taisne, C., Hui, G.S., Chang, S.P., Church, P., Hollingdale,. R., Kaslow, D.C., Hoffman, S., Guito, K.P., Ballou, W.R., Sadoff, J.C., Paoletti, E., 996), "NYVAC-Pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria.", Infect Immun., Vol. 64, No. 9, 3833 – 3844.

Triglia, T., Healer, J., Caruana, S.R., Hodder, A.N., Anders, R.F., Crabb, B.S., Cowman, A.F., (2000) Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. Mol. Microbiol., Vol. 38, No. 4, 706 - 718.

Udhayakumar, V., Kariuki, S., Kolczack, M., Girma, M., Roberts, J. M., Oloo, A.J., Nahlen, B.L., Lal, A.A., (2001), " Longitudinal Study of natural Immune Responses to the Plasmodium falciparum Apical Membrane Antigen (AMA-1) in a holoendemic region of Malaria in Western Keny: Asembo Bay Cohort Project VIII." , Am. J. Trop. Med. Hyg., Vol. 65, No. 2, 100 – 107.

Urquiza, M., Suarez, J. E., Cardenas, C., Lopez, R., Puentes, A., Chavez, F., Calvo, J. C., Patarroyo, M. E., (2001), "Plasmodium falciparum AMA-1 erythrocyte binding peptides implicate AMA-1 as erythrocyte binding protein." , Vaccine, Vol. 19, 508 – 513.

Verra., and Hughes., (2000), "Evidence for ancient balanced polymorphism at the Apical Membrane Antigen – 1 (AMA-1) locus of *P.falciparum.*" Mol. Biochem.Parasitol., Vol. 105, 149 -153.

Waters, A.P., Higgins, D.G., McCutchan, T.F., (1991), " *Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human host." PNAS, Vol. 88, 3140 –3144.

Waters, A.P., Thomas, A.W., Deans, J.A., Mitchell, G.H., Hudson, D.E., Miller, L.H., McCutchan, T.F., Cohen, S., (1990), "A merozoite receptor protein from *P.knowlesi* is highly conserved and distributed throughout *Plasmodium*." J. Biol. Chem., Vol. 265, 17974 – 17979.

W.H.O. Expert Committee on Malaria: twentieth report, Geneva, WHO, (2000), W.H.O. Technical Report Series.

Wilairatana, P., Looareesuwan, S. & Gilles, HM. (1999) "Pathogenesis, pathology, clinical manifestations and differential diagnosis." In: Protozoal Diseases. Gilles HM (ed.). Arnold Publications, London, UK.

Xu, H., Hodder, A. N., Yan, H., Crewther, P.E., Anders, R.F., Good, M.F., (2000), "CD4+ T cells acting independently of antibody contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization." J. Immunol. , Vol. 165, No. 1, 389 - 396.

Web sources:

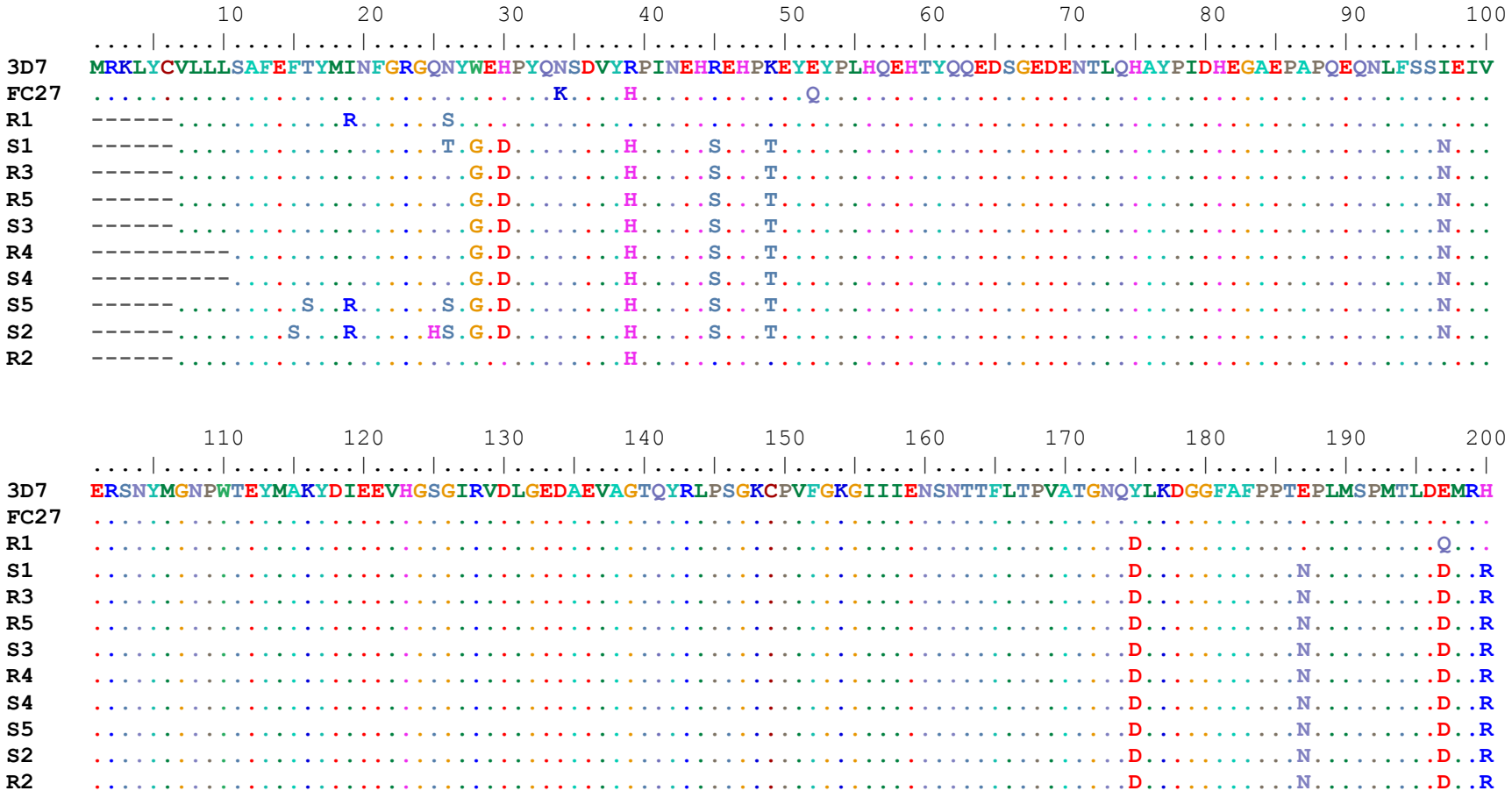
http://www.malariasite.com

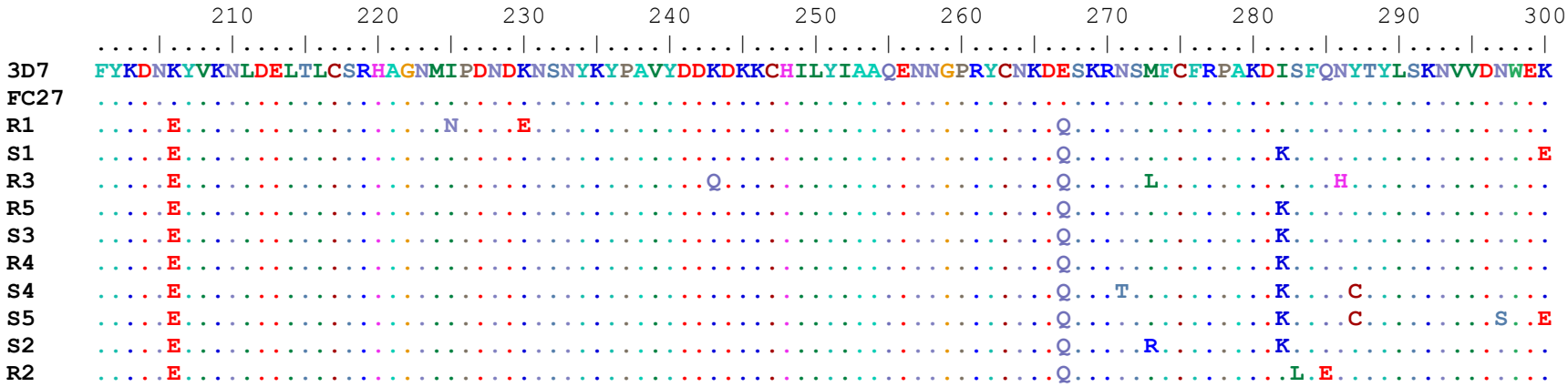
http://www.stratagene.com/newsletter/pdf/Easier Protein Expression in *E. coli.*

http://www.cdc.gov/malaria/biology/lifecycle.htm

Appendix 1

Alignment of AMA-1 sequences of *P. falciparum* from 10 Indian Isolates at protein level using Bioedit software

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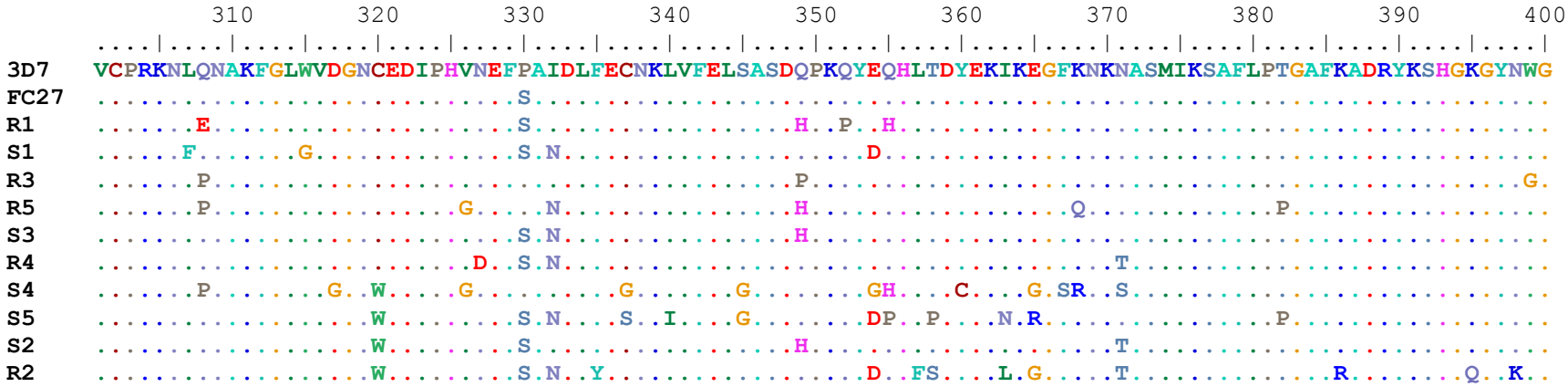
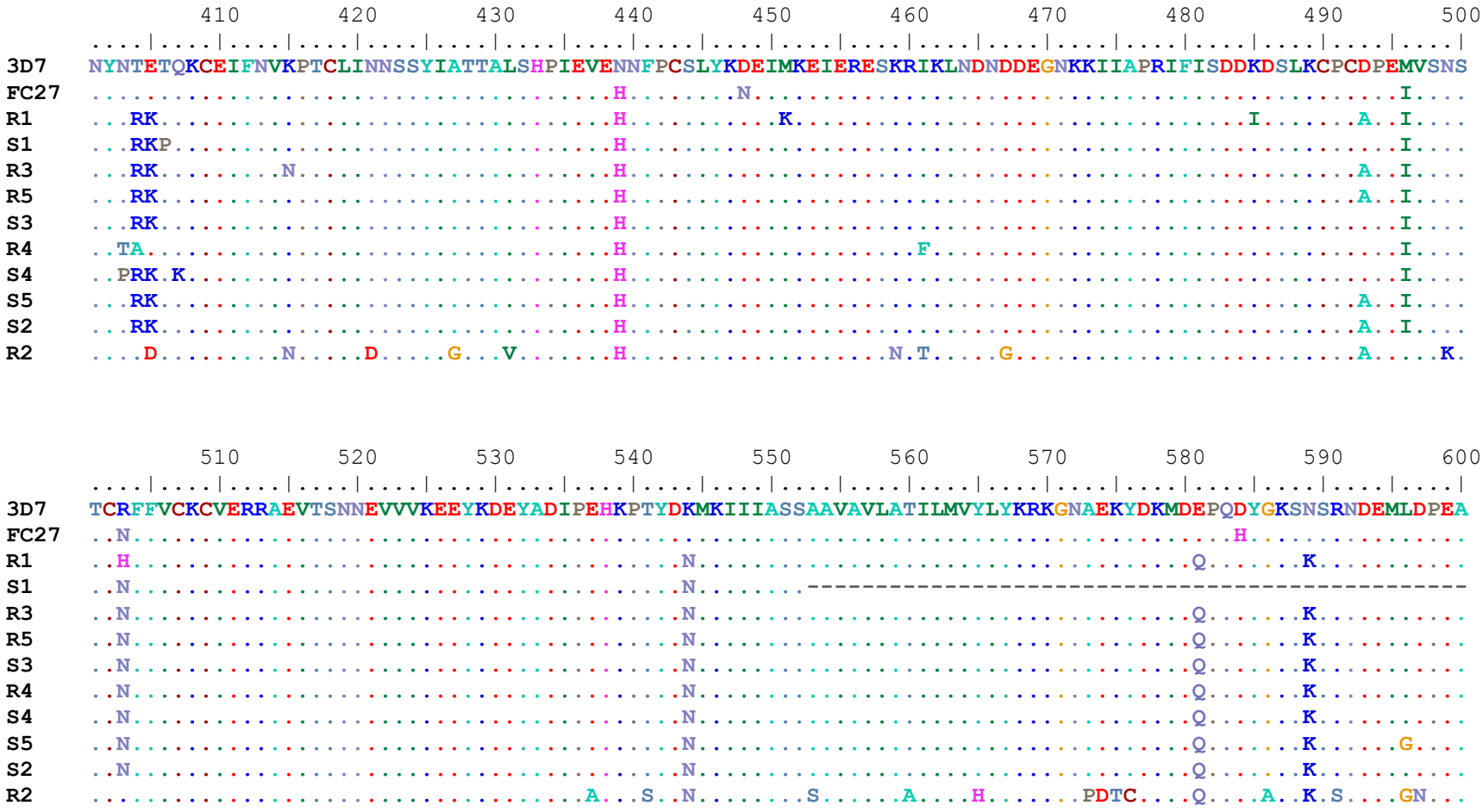
FC27
R1
S1
R3
R5
S3
R4
S4
S5
S2
R2


310 320 330 340 350 360 370 380 390 400
3D7 VCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNASMIKSAFLPTGAFKADRYKSHGKGYNWG
FC27
R1
S1
R3
R5
S3
R4
S4
S5
S2
R2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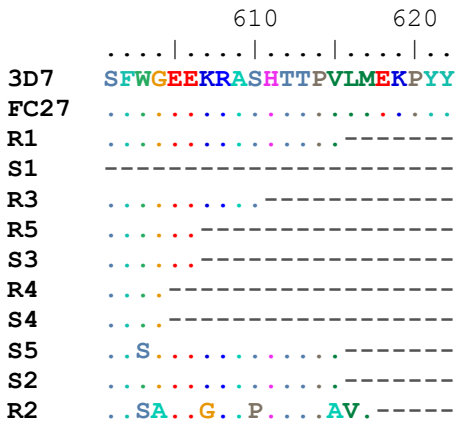
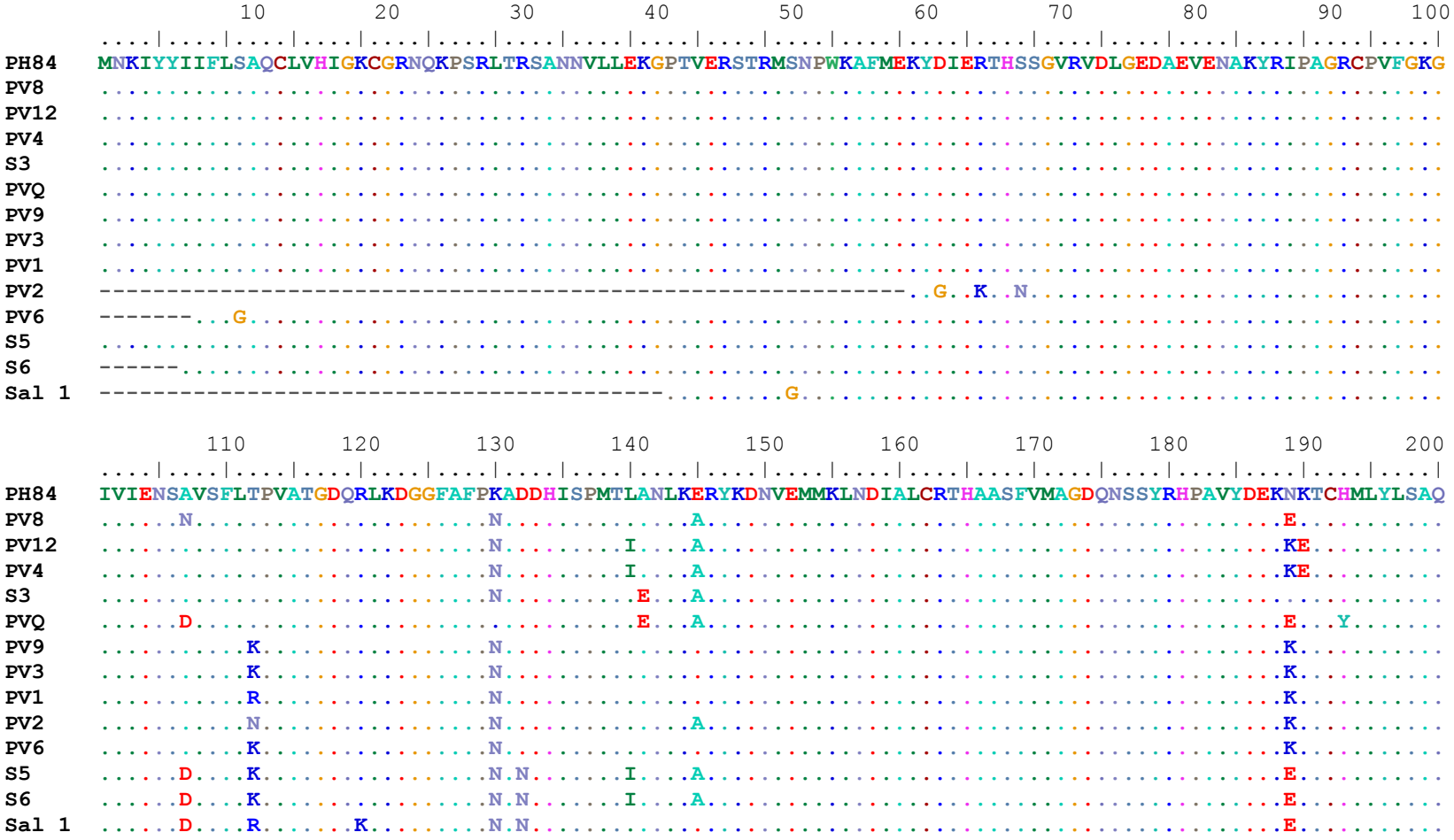
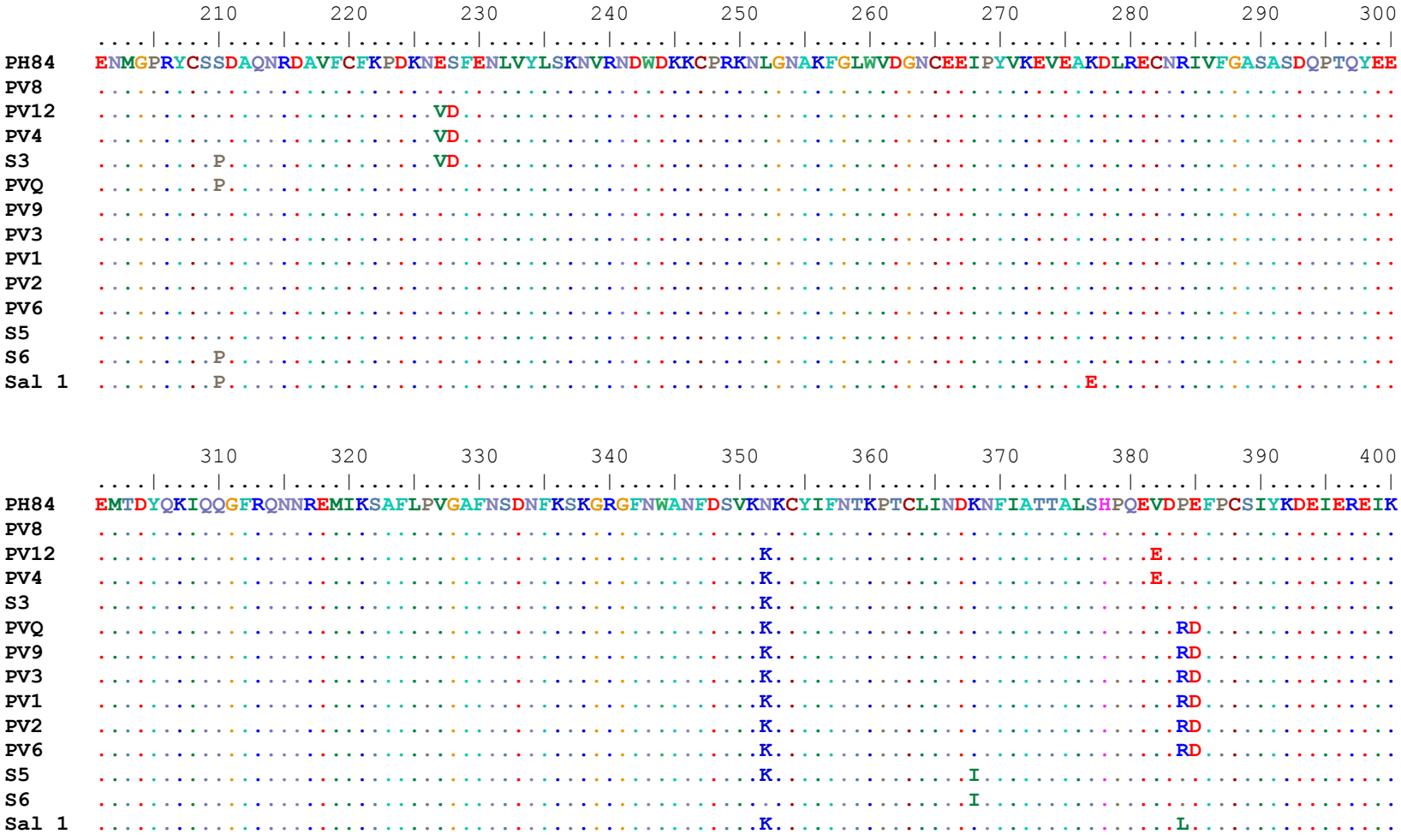
FC27
R1
S1
R3
R5
S3
R4
S4
S5
S2
R2
510 520 530 540 550 560 570 580 590 600
3D7 TCRFFVCKCVERRAEVTSNNEVVVKEEYKDEYADIPEHKPTYDKMKIIIASSAAVAVLATILMVYLYKRKGNAEKYDKMDEPQDYGKSNSRNDEMLDPEA
FC27
R1
S1
R3
R5
S3
R4
S4
S5
S2
R2


610
620
3D7 SFWGEEKRASHTTPVLMEKPYY
FC27 .....
R1 .....-------
S1 ----------
R3 .....----------
R5 .....----------
S3 .....----------
R4 .....----------
S4 .....----------
S5 ..S.....-------
S2 .....-------
R2 ..SA..G..P....AV.-----

Appendix 2

Alignment of AMA-1 sequences of *P. vivax* from 10 Indian Isolates at protein level using Bioedit software

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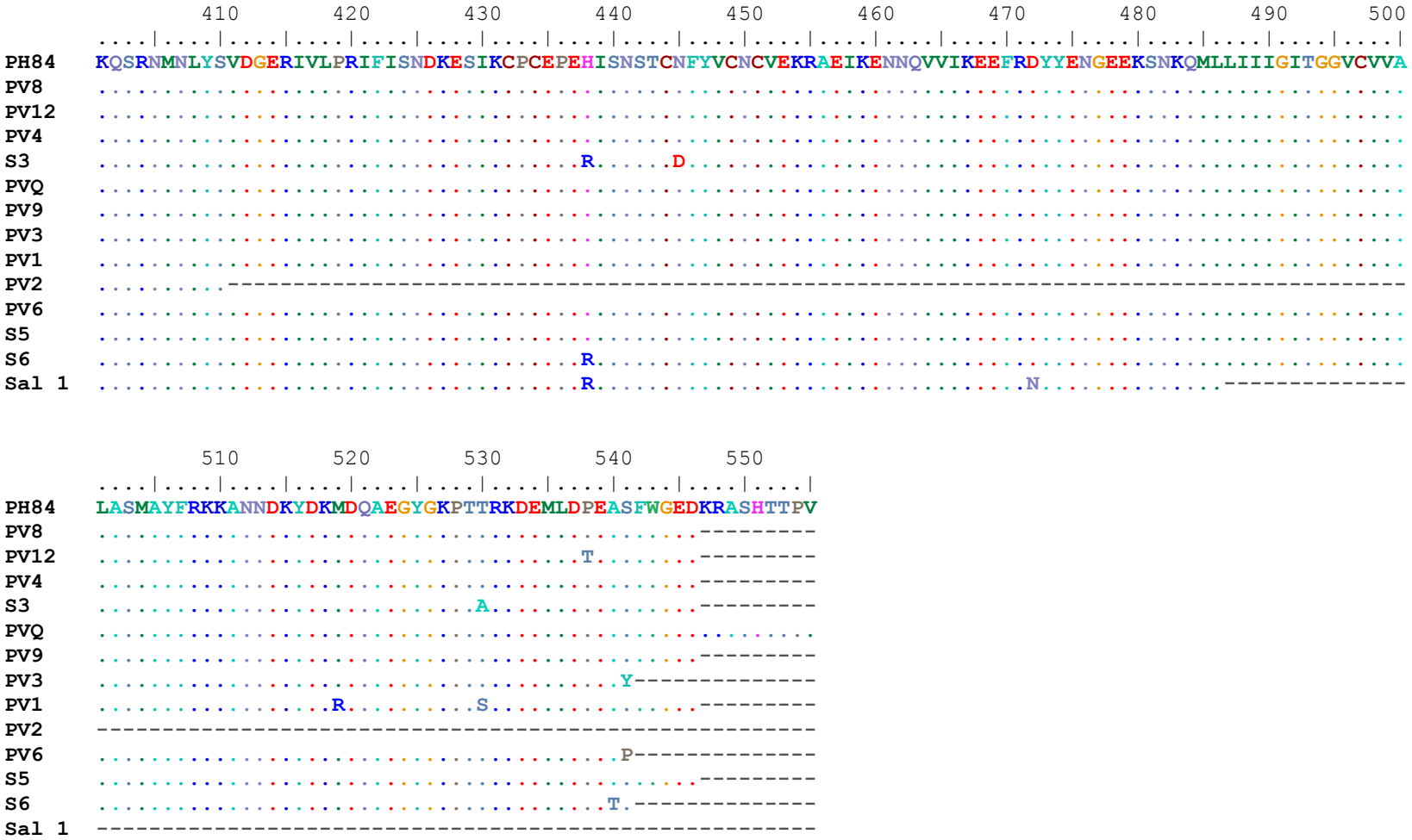
PV8
PV12 VD
PV4 VD
S3 P VD
PVQ P
PV9
PV3
PV1
PV2
PV6
S5
S6 P
Sal 1 P E
310 320 330 340 350 360 370 380 390 400
PH84 EMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKNKCYIFNTKPTCLINDKNFIATTALSHPQEVDPEFPCSIYKDEIEREIK
PV8
PV12 K E
PV4 K E
S3 K
PVQ K RD
PV9 K RD
PV3 K RD
PV1 K RD
PV2 K RD
PV6 K RD
S5 K I
S6 I
Sal 1 K L


410 420 430 440 450 460 470 480 490 500
PH84 KQSRNMNLYSVDGERIVLPRIFISNDKESIKCPCEPEHISNSTCNFYVCNCVEKRAEIKENNQVVIKEEFRDYYENGEEKSNKQMLLIIIGITGGVCVVA
PV8
PV12
PV4
S3
PVQ
PV9
PV3
PV1
PV2
PV6
S5
S6
Sal 1
510 520 530 540 550
PH84 LASMAYFRKKANNDKYDKMDQAEGYGKPTTRKDEMLDPEASFWGEDKRASHTTPV
PV8
PV12
PV4
S3
PVQ
PV9
PV3
PV1
PV2
PV6
S5
S6
Sal 1

Appendix 3

List of Publications

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1. S.Vijay Kumar, Sarita Ranjan, Vishal Saxena, **Vidya Rajesh**, S.K.Roy, Dhanpat Kochar, Akash Ranjan, Ashis Das. "*Plasmodium falciparum :* Genetic diversity of C terminal region of MSP-1 in isolates from Indian sub-continent", Experimental Parasitology, 110 (2005) 384-388.

2. **Vidya Rajesh**, Elamaran M, Vidya S, Gowrishankar M, Dhanpat Kochar, Ashis Das (2005) **"**Genetic diversity of the *P. vivax* Apical Membrane Antigen –1 (AMA-1) in isolates from India." (In Press - **Experimental Parasitology**).

Papers in Conferences/Symposiums

1. **Vidya Rajesh**, Akash Ranjan, Ashis Das, " Polymorphism studies of Apical Membrane Antigen (AMA-1) of *P. falciparum*." Poster presentation in International Symposium On Emerging Trends in Genomics And Proteomics Education and Research, January 12th and 13th , 2003 – BITS, PILANI**.**

2. **Vidya Rajesh**, Vijay S, Vishal Saxena, Samayita C, Kochar D, Akash Ranjan, Ashis Das "Polymorphism studies and structural analysis of variation in AMA-1 from field isolates in India." Oral Presentation in the International Conference on Malaria, November 4-6, 2005, New Delhi, India.

3. Shilpi Garg, Vishal Saxena, **Vidya Rajesh**, Dhanpat Kochar, Ananias Escalante, Ashis Das "Genetic Analysis of *Plasmodium vivax* isolates from severe malaria cases." Poster presentation in the National Symposium on Tribal Health, 19-20 October 2006, Jabalpur, India.

4. **Vidya Rajesh**, Ashish Runthala, Gowrishankar M, Dhanpat Kochar, Ashis Das "Evaluation of Antibody Profiles in naturally infected Individuals, to peptides based on different AMA-1 epitope variants." Poster presentation in the 33rd Indian Immunology Society Conference, 28 –31 January 2007, New Delhi, India.

Biography of Dr. Ashis K. Das

Dr. Ashis K. Das is presently working as an Associate Professor and Group Leader, at Biological Sciences Group, Birla Institute of Technology and Sciences, Pilani. He received his Ph.D. degree in 1993 from National Institute of Immunology, Jawaharlal Nehru University, New Delhi. Worked as Post Doctoral Fellow in the Department of Molecular Biology and Immunology, SHPH, Johns Hopkins University, Baltimore, USA and WHO Fellow at the Malaria Branch, Center for Disease Control, Atlanta, Georgia USA. He has been involved in teaching and research for 20 years. His research areas of interest include Molecular and Immuno – Parasitology, Molecular Diagnostics and Industrially oriented projects dealing with different aspects of vector design, cloning and expression and bioinformatics. He has published various research articles in peer reviewed international journals. He is currently working on various projects that are funded by CSIR, Dabur Research Foundation and UGC .

Biography of Ms. Vidya Rajesh

Ms. Vidya Rajesh completed her M.Sc. in Microbiology, in the year 1995 from Nagpur University, Maharashtra. She stood second in the order of merit. She worked in various capacities in several institutions like NEERI, Nagpur and Springer Verlag Group before joining M.E. Biotechnology, at BITS, Pilani. She joined BITS, Pilani as a regular faculty in the year 2001. Her research areas of interest are Molecular Immunology, Advanced Cell Biology and Microbiology. Ms. Vidya Rajesh has been pursuing her part time Ph.D. at BITS, Pilani from 2001-2006. She has presented papers in various International and National conferences/symposiums and is interested in pursuing active research in malaria.