FUNCTIONAL CHARACTERIZATION OF *PLASMODIUM FALCIPARUM* PF10_0351 PROTEIN

A dissertation submitted to the Board of Graduate Studies, University of Ghana, Legon, Ghana

In partial fulfillment of the requirements for the award of the Master of Philosophy degree in Molecular Cell Biology of Infectious Diseases

By

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BSc. (Hons.)

JULY 2017

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DECLARATION

I, Charles-Chess Nana Appiah Essel, graduate student of the Department of Biochemistry, Cell and Molecular Biology and West African Centre for Cell Biology of Infectious Pathogens, do hereby declare that the research work presented in this thesis was conducted by me under the supervision of Prof. Gordon A. Awandare (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, and West African Centre for Cell Biology of Infectious Pathogens) and Dr Yaw Aniweh (West African Centre for Cell Biology of Infectious Pathogens).

All references have been cited accordingly.

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Dr. Yaw Aniweh (Co-supervisor)

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ACKNOWLEDGEMENT

I am grateful to Prof. Gordon A. Awandare and Dr. Yaw Aniweh for their supervisory roles in my biomedical research, career and social networking.

Also, the faculty and staff of the Department of Biochemistry, Cell and Molecular Biology (BCMB) and the Noguchi Memorial Institute for Medical Research (NMIMR), particularly Dr. Emmanuel Amlabu and Dr. Samuel Duodu, have been helpful. Members of the Awandare Research Group – Henrietta, Temitope, Baaba, Prince, Laty, Felix, Oheneba, and Francis – are all worth mentioning. Prof. Kirk Deitsch and Dr. Bismarck Dinko are two extraordinary gentlemen who always showed interest in my education and research. The past two years have been replete with the acquisition of knowledge in modern science.

My family is a constant reminder that education rekindles the mind's flame for greatness. My gratitude to Pastor Samuel Ninson and Betse Ninson transcends the radiance of sunflowers in August. I thank Selorm Kofi Dake for his brotherliness throughout the years.

Again, I appreciate the assistance from all the Erythrocyte Invasion Mechanism (EIM) project staff of LEKMA hospital, the Kintampo Health Research Centre, and the Navrongo Health Research Centre for samples used for the immunogenicity study.

I did receive enormous support from the WACCBIP leadership and secretariat throughout my studentship. This research work and graduate school tuition fees were funded by the WACCBIP-World Bank ACE Masters fellowship (ACE02-WACCBIP: Awandare).

DEDICATION

To science, society and God!

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LIST OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
ADCI	Antibody-dependent cell-mediated inhibition
AMA-1	Apical membrane antigen-1
ARDS	Acute respiratory distress syndrome
BF	Bright field
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSG	Basigin
CDC	Centre for Disease Control and Prevention
CDPK5	Calcium-dependent protein kinase 5
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester
CM	Cerebral malaria
CPM	Complete parasite medium
CR1	Complement receptor 1
CSP	Circumsporozoite protein
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl sulfoxide
DBL	Duffy binding ligand
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding ligand
EGF	Epidermal growth factor

EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GDP	Gross Domestic Product
GHS	Ghana Health Service
GPI	Glycosylphosphatidylinositol
GSK	Glasgow-Smith-Kline
GTS	Global Technical Strategy
HAP	Histo aspartic protease
HDP	*Plasmodium* haem degradation protein
HSPG	Heparin-sulfate proteoglycans
ICAM-1	Intercellular adhesion molecule 1
IDC	Intraerythrocytic development cycle
IFA	Indirect immunofluorescence assay
IgG	Immunoglobulin G
IMC	Inner membrane complex
IPTp	Intermittent preventive treatment of malaria in pregnancy
iRBCs	Infected red blood cells
IRS	Inside Residual Spraying
i-TASSER	Iterative Threading ASSEmbly Refinement
ITNs	Insecticide treated nets
LLITNs	Long lasting insecticide treated nets
MSP-1	Merozoite surface protein 1

MtDNA	Mitochondria DNA
MTIP	Myosin A tail-interacting protein
MudPIT	Multidimensional protein identification technology
NCBI	National Center for Biotechnology Information
NHS	Normal human sera
NMCP	National Malaria Control Programme
PBS	Phosphate buffer saline
PE	Phycoerythrin
PKG	Protein kinase G
PlasmoDB	*Plasmodium* database
RAP-1	Rhoptry associated proteins
RDTs	Rapid diagnostic tests
PfRh	*P. falciparum* reticulocyte-like binding homologue
PUM-HD	Humilio homology domains
PVM	Parasitophorous vacuolar membrane
PWM	Parasite wash medium
RBC	Red blood cell
RCSB	Research Collaboratory for Structural Bioinformatics
SA	Sialic acid
sCR1	Soluble complement receptor 1
SEM	Standard error of the mean
SERA	Serine repeat antigen
SMA	Severe malarial anaemia

SMART	Simple Modular Architecture Research Tool
SMC	Seasonal malaria chemoprevention
SP	Sulfadoxine-pyrimethamine
SUB-1	Subtilisin-like serine protease 1
TRAP	Thrombospondin related anonymous protein
UNICEF	United Nations Children Fund
WACCBIP	West African Centre for Cell Biology of Infectious Pathogens
WHO	World Health Organization

ABSTRACT

Inadequate understanding of the biology of *Plasmodium falciparum* and the aetiology of malaria is a hindrance to the development of effective drugs and vaccines against the backdrop of resistance to *Anopheles* insecticides and antimalarial drugs, including artemisinin-based combination therapies (ACTs). About 60% of proteins of the *P. falciparum* genome has not been characterized. Since clinical symptoms of malaria are manifested at the blood stage, parasite proteins involved in erythrocyte invasion are of major research interest in vaccine development. This study aimed to functionally characterize a probable *P. falciparum* PF10_0351 protein, and establish its role during invasion. To achieve this, bioinformatic and immunoinformatic analyses were used to map out three highly antigenic epitopes for synthesis and antibody production. ELISA was performed to test whether the peptides will react with natural human plasma from malaria-endemic areas in Ghana with varying transmission intensities (Kintampo>Navrongo>Accra). Invasion inhibition assays were also performed to assess the ability of the peptide-specific antibodies to block invasion, using flow cytometry. Immunolocalization experiments were also performed in schizont, merozoite and gametocyte stages by immunofluorescence assay (IFA) and fluorescent microscopy. The peptides, particularly PF10_0351-2 and -3, could be recognized by naturally-induced antibodies in human plasma. However, there was no significant association between plasma antibody levels and age or parasite density by Spearman's correlation. Peptide-specific antibodies inhibited parasite invasion of erythrocytes in a dose-dependent manner with significant differences in their invasion inhibitory effects (Tukey's range test, $p<0.0001$). PF10_0351 appears to co-localize with Pfs48/45 on the surface of gametocyte

stages, and also partially co-localize with MSP-1 on the surface of schizonts. Taken together, these findings suggest that the PF10_0351 protein may be localized to the surface of schizont, merozoite and gametocyte, and that it may be considered for further studies to explore its role in invasion.

CHAPTER ONE

1.0 INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium* protozoan parasites. A two-thirds of the global community is estimated to be at risk of malaria (Cibulskis *et al.*, 2016). About 212 million new malaria cases and 429,000 deaths were recorded globally in 2015 with 92% of the deaths estimated to have occurred in the African region (World Health Organization, 2016). The vast majority of deaths (99%) are due to *P. falciparum* malaria (World Health Organization, 2016) which remains the most prevalent of all the five known *Plasmodium* species (*P. falciparum, knowlesi, malariae, ovale* and *vivax*) which naturally infect humans (Cox-Singh *et al.*, 2008; Hay *et al.*, 2004; Hume *et al.*, 2003; Martinsen *et al.*, 2008; Rasti *et al.*, 2004; White, 2008).

In spite of the estimated 70% reduction in the number of people infected with malaria parasites in sub-Saharan Africa occurring from 2001 to 2015 (Cibulskis *et al.*, 2016), infection rates are higher in children aged 2–10 years and pregnant women (Geels *et al.*, 2011; World Health Organization, 2016). The emergence of drug-resistant *Plasmodium falciparum* against existing anti-malarial drugs has further necessitated the search for newer therapeutic and efficacious vaccine targets.

The life cycle of *P. falciparum* comprises a sexual phase in the mosquito vector and an asexual phase in the human host. An essential step in the asexual blood stage of the life cycle of *Plasmodium* parasites is the invasion of host erythrocytes by merozoites which release proteins from their apical secretory organelles to mediate a complex set of

interactions between different parasite ligands and erythrocyte receptors (Cowman and Crabb, 2006; Iyer *et al.*, 2007; Preiser *et al.*, 2000). The intraerythrocytic development cycle (IDC) exhibits a highly regulated and progressive cascade of gene expression which are critical for the functional processes and survival of the parasite throughout the specific stage of development in the erythrocyte (Bozdech *et al.*, 2003; Ganesan *et al.*, 2008; Le Roch *et al.*, 2004). This has been studied as one of the critical points in the parasite's development, hence a possible area to study for development of potential blood-stage vaccine candidates (Cowman and Crabb, 2006). Since the clinical manifestation of malaria mainly occurs at the erythrocytic stage of the parasite (Bledsoe, 2005; Chen *et al.*, 2000; Lindner *et al.*, 2012; Miller *et al.*, 2002), it is necessary to study the characteristics and functions of antigens predicted to play a role in erythrocyte invasion.

The current genomic information on the *Plasmodium falciparum* (3D7) genome sequence reveals some 5400 genes which encode fewer enzymes and transporters but a large proportion of genes are devoted to immune evasion and host-parasite interactions (Gardner *et al.*, 2002). While 60% of these proteins have no assigned functions (Gardner *et al.*, 2002), the vast majority of the *P. falciparum* proteins have not been characterized experimentally (LaCount *et al.*, 2005). The inadequate experimental data on the function, structure, and characteristics of the *Plasmodium* genome are major setbacks in the quest for rapid development of vaccine candidates.

PF10_0351 (PlasmoBD Gene ID: PF3D7_1035900) is one of the hypothetical proteins predicted to play a role in erythrocyte invasion (Cowman *et al.*, 2012). The PF10_0351

gene was selected because it is conserved across *Plasmodium* species, transcriptionally expressed during late schizogony and possesses a signal sequence in the protein.

1.1 *Rationale*

Inadequate understanding of the biology of *Plasmodium falciparum* and aetiology of malaria is a hindrance to the development of effective drugs and vaccines against the backdrop of resistance to insecticides and antimalarial drugs including ACTs. Many antimalarial drugs are losing their efficacy owing to the emergence of drug-resistant *P. falciparum* parasites (Dondorp *et al.*, 2009; Rieckmann *et al.*, 1978). Since clinical symptoms of malaria are manifested at the erythrocytic stage of the parasite's life cycle, parasite proteins involved in erythrocyte invasion are of major interest for vaccine development.

In order to establish the importance of uncharacterized proteins in erythrocyte invasion, experimental data that validate novel genes and their functional roles would be required. Using bioinformatics and already published data on the parasite genome, transcriptome, and proteome, the PlasmoDB database was screened for probable proteins that may play a role in invasion. The selection criteria for this screening included:

1. conservation of the protein across *Plasmodium* species
2. expression of the protein during late schizogony of asexual blood stage
3. the presence of a signal peptide/anchor sequence, and
4. the presence of a GPI anchorage or transmembrane domain

Based on the above criteria and other experimental approaches, the study aimed to characterize portions of *P. falciparum* PF10_0351 (PF3D7_1035900) protein. It has been previously suggested that PF10_0351 may be an "MSP3-like" peripheral surface protein with no known functions (Cowman and Crabb, 2006). Additionally, preliminary data in PlasmoDB (http://www.plasmodb.org) suggest the protein consists of highly polymorphic amino acid sequences with the interspersed central repetitive region.

1.2 *Hypothesis*

This study hypothesized that the *P. falciparum* PF10_0351 (PF3D7_1035900) protein plays a role in the invasion of erythrocytes.

1.3 *Aim*

The aim of this study was to functionally characterize portions of the *P. falciparum* PF10_0351 protein and establish their role during erythrocyte invasion.

1.3.1 *Specific objectives*

1. To determine the functional interactions of the PF10_0351 protein
2. To determine the stage-specific expression and localization of PF10_0351 protein across the sexual and asexual stages
3. To test the essentiality of the PF10_0351 protein for merozoite invasion

1.4 *Significance of the study*

1. This study will provide additional insight into the biology of the *P. falciparum* parasite in terms of function, molecular structure and sequence of the Pf10_0351 protein.

2. It will also help to obtain information about the relationship of the Pf10_0351 protein homology to other similar known or predicted proteins.
3. This study may help identify a potential antigen target and contribute to studies which assess multi-antigen, blood-stage vaccines for protection against malaria.

CHAPTER TWO

2.0 **LITERATURE REVIEW**

2.1 *Global view of malaria*

Malaria remains a public health problem worldwide. There has, however, been tremendous progress made in combating the disease due, in part, to the implementation of multiple control interventions (Cibulskis *et al.*, 2016) as well as general economic development and urbanization, leading to improved housing and nutrition (World Health Organization, 2016). For instance, the disease has not been re-established in any of the countries that eliminated malaria between 2000 and 2015 as reported by the WHO Global Technical Strategy (World Health Organization, 2016). The global case incidence and mortality rate between 2000 and 2015 decreased by 41% and 62% respectively. The number of malaria-endemic countries and territories in 2000 (108) decreased by 17 as of 2015 (Figure 2.1) (World Health Organization, 2016). These gains made in the reduction of malaria mortality, observed between 2000 and 2015, have resulted in an increase in life expectancy by 0.26 years. The decline of world poverty may be correlated with the decline of malaria prevalence (https://ourworldindata.org/malaria/). There has been an increase from 37% in 2010 to 57% in 2015 among persons who use insecticide-treated mosquito net (ITN), or are protected by indoor residual spraying (IRS) in the proportion of the population at risk in sub-Saharan Africa (World Health Organization, 2016).

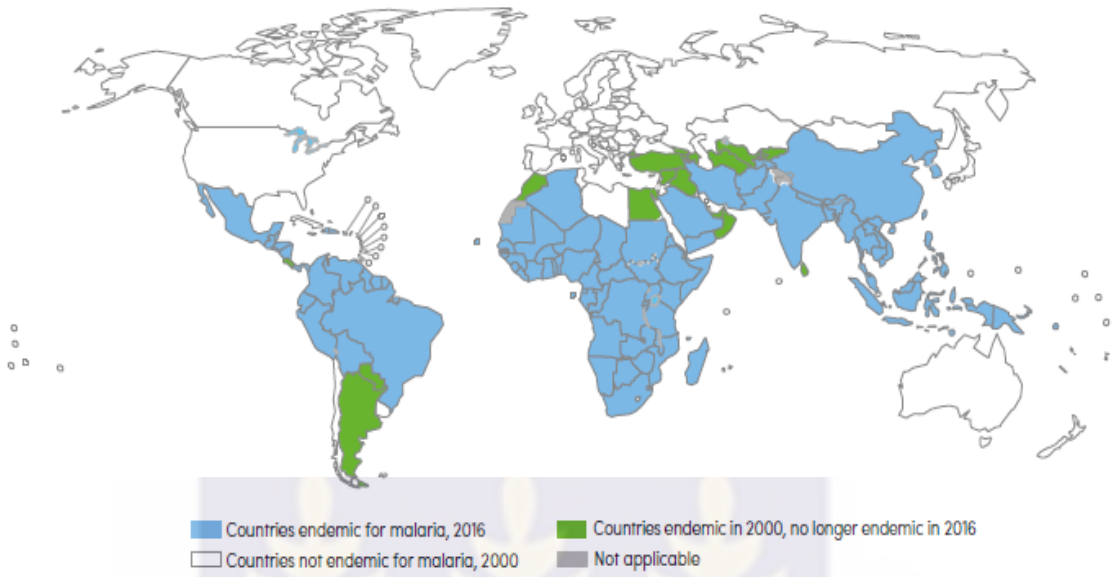


Figure 2.1: Global distribution of malaria transmission

The colour-coded world map depicts the endemicity of malaria from 2000 to the beginning of 2016. Blue: countries endemic for malaria, 2016; white: countries not endemic for malaria, 2000; green: countries endemic in 2000, no longer endemic in 2016; grey: not applicable to malaria zones. In general, malaria endemicity in 108 countries and territories in 2000 declined to 91 countries at the beginning of 2016. (Source: World Health Organization, 2016)

In spite of the global progress made in reducing the burden of the disease, malaria is still heavily endemic in the poorest parts of the world. Treatment cost and loss of workforce resulting from malaria in sub-Saharan Africa essentially reduce economic growth (Gallup and Sachs, 2001). In 2015, most malaria cases recorded were 90% in WHO African, 7% in South-East Asia, and 2% in Eastern Mediterranean Regions. Some 70% of the global malaria mortality (429,000) occurred in children under five years (WHO World Malaria Report, 2016). Whereas 99% of all deaths were estimated to have been caused by *P. falciparum*, some 86% of the deaths occurring outside Africa were caused by *P. vivax* (World Health Organization, 2016). Children under five years, pregnant women, and non-

immune adults or travellers are major risk groups of malaria. The tools – drugs and vaccines – for preventing and treating malaria face some challenges. Drugs may not easily be available and vaccines are also not available everywhere. WHO World Malaria Report usually gathers data on malaria endemicity from country-specific national malaria control programmes (NMCP).

To accelerate the efforts to combating malaria, the WHO has developed the Global Technical Strategy for the elimination of malaria between 2016 and 2030, and this strategy recognizes innovation and research as key indicators of tracking the progress of this target.

2.1.1 *Malaria situation in Ghana*

The malaria situation in Ghana follows the global trend of reduction in cases and mortality. Ghana accounted for about 6% of the estimated global malaria cases and 3% of the total malaria deaths in 2015 (World Health Organization, 2016). According to the World Health Organization (2016), the country continued to distribute ITNs/LLINs through mass campaigns to all age groups free of charge. In addition, the country's National Malaria Control Programme (NMCP) also implemented indoor residual spraying (IRS) as well as the use of IPTp to prevent malaria during pregnancy. These policy adoptions, however, did not include the use of DDT for IRS nor seasonal malaria chemoprevention (SMC or IPTc). The proportion of Ghanaians who slept under ITN or were protected with IRS is more than 60% of the population (World Health Organization, 2016). It is estimated that the annual economic burden of malaria is 1-2% of Ghana's gross domestic product (GDP).

2.2 *Malaria control strategies*

The current efforts aimed at controlling and eliminating malaria combine antimalarial drugs, bed nets, indoor residual spraying and a long-term consideration for the development of a vaccine to overcome the chronic and widespread nature of the disease. These strategies also include improvement of infrastructure and disease transmission monitoring.

2.2.1 *Vector control*

Insect control and management programmes involve the use of strategies to eliminate the *Anopheles* mosquito by targeting its feeding and resting dynamics in different transmission areas. Some vector management strategies target the environment of the mosquito by eliminating its breeding grounds. Others target the agent biologically by using niche competitors, insect pathogens, or plasmodicidal symbiotic organisms. The chemical management strategy involves the use of new effective insect repellents.

In areas of low-moderate transmission, the combined use of indoor residual spraying (IRS) with pyrethroid insecticides in households and long-lasting insecticide-impregnated nets (LLINs) has been effective in controlling the disease in areas where pyrethroid-susceptible indoor-feeding vectors predominate (World Health Organization, 2016).

In high transmission and vector-resistant areas where there is resistance to pyrethroid and related chemicals (Ranson *et al.*, 2009), or where mosquitoes have a characteristic outdoor-feeding cycle, vector replacement strategies using mosquitoes which are resistant to

Plasmodium or those which are killed upon infection with *Plasmodium spp.* (Terenius *et al.*, 2008) have been deployed on an experimental basis.

2.2.2 *Antimalarial therapy*

Many antimalarial drugs have been developed over the years. Some common antimalarial drugs in use include atovaquone, sulfadoxine-pyrimethamine, mefloquine and the artemisinin-based combination therapies (ACTs) such as Artemether-Lumefantrine.

From the discovery of quinine in the seventeenth century to the development of chloroquine in the twentieth century as chemotherapy for the treatment of malaria (Carter and Mendis, 2002; Meshnick, 2002), resistance to these drugs and undesirable side effects have necessitated the development of newer and more effective therapeutic agents such as the ACTs (Achan *et al.*, 2011; Cross *et al.*, 2011; Stead *et al.*, 2001). The WHO, charities such as Bill and Belinda Gates Foundation, and local governments have collectively invested resources in preventing resistance to the ACTs.

Unfortunately, the recent emergence of artemisinin resistance in Cambodia (Dondorp *et al.*, 2009) and subsequently in Myanmar (Phyo *et al.*, 2012) have altogether prompted fears of the spread of artemisinin resistance to other parts of the world.

The intermittent preventive treatment of malaria in pregnancy (IPTp) and infants with sulfadoxine-pyrimethamine (SP) as well as the seasonal malaria chemotherapy (SMC) protect against clinical malaria, reducing anaemia and low-birth rates (Bhatt *et al.*, 2015; Cibulskis *et al.*, 2011).

2.2.3 *Vaccine development*

The widespread nature of malaria and recurrent resistance against frontline chemotherapeutic compounds and insecticides threaten the fight against malaria (Ganesan *et al.*, 2008) but have also renewed the urgent need for newer and improved strategies to eradicating the disease. Antimalarial vaccine development promises to be an effective eradication method to complement the current WHO preventive, diagnostics and treatment strategies (World Health Organization, 2016). These vaccines represent a beneficial boost to the immune defence. This is true for the fact that natural immunity against severe and clinical *Plasmodium* malaria is acquired over time with increasing exposure to malaria-endemic areas (Day and Marsh, 1991). Age and the immune status of the individual also influence the progression of malaria, particularly in endemic regions of the world.

In spite of the over six decades of global efforts by many laboratories to develop an effective vaccine (Hill, 2011), there are still limited insights into the best antigen components which will elicit protection as well as the vaccine development technologies available for use. In the 1960s, immunization studies of mice with irradiated sporozoites led malaria vaccine development (Nussenzweig *et al.*, 1967). This was followed by further studies to analyze the mechanisms of immunity in the irradiated mice (Doolan and Hoffman, 1997). Today, the WHO Rainbow tables for malaria vaccines entail several clinical trial vaccines designed to target the pre-erythrocytic (liver) stage, the asexual blood stage, and gametocytic stages (transmission blocking vaccine) (WHO. 20/03/2016. Malaria Vaccine Rainbow Tables. http://www.who.int/vaccine_research/links/Rainbow/en/index.html). Although the

parasite exhibits several immune evasive mechanisms, exposure of *Plasmodium* antigens to the host humoral immune surveillance during the asexual blood stage makes vaccines targeting this stage conceptually attractive.

Malaria vaccine clinical trial efforts have considered single or a combination of antigens, delivery systems and adjuvants. At present, the leading first-generation malaria vaccine candidate is the RTS, S/AS01, a collaborative effort from GSK Biologicals and Walter Reed Army Institute of Research, USA (Ballou and Cahill, 2007). It is a sub-unit vaccine comprising the central repeat (R) of circumsporozoite protein (CSP) fused to the T-cell epitope C-terminal region (T) and hepatitis B surface antigen (S) formulated with an AS01 adjuvant containing liposomes (Hill, 2011). An association between anti-PfCSP antibody levels and CD4+ T cell activation and protection against malaria has been shown, albeit there is no established correlate of protection (White *et al.*, 2014; White *et al.*, 2013). The RTS,S/AS01 vaccine is thought to induce a specific anti-PfCSP immune response that prevents initiation of patent blood-stage infection by killing parasites at the pre-erythrocytic stage. Gosling and von Seidlein (2016) have reported that, in a Phase III trial of RTS,S/AS01 involving 11 study sites in 7 sub-Saharan Africa countries, vaccine efficacy among 5-17 month-old children was found to reduce drastically from 45.1% to 28.3% over 48 months but improved to about 36.3% upon booster dose at 18 months later.

2.3 *Pathogenesis of malaria*

Parasite and human host factors modulate the pathogenesis of malaria, ensuring the survival of the parasite in both niches – vector and human host (Acharya *et al.*, 2017). The clinical symptoms of malaria are manifested during the intraerythrocytic stage of the

parasite (Malaguarnera and Musumeci, 2002). During this stage, the parasite releases toxic metabolic by-products such as hemozoin which accumulate and prompt macrophages to release cytokines when schizonts rupture to release merozoites to infect new RBCs. This produces acute inflammatory responses (Heddini, 2002). This intra-erythrocytic cycle seems synchronized to host circadian rhythm, influencing an hourly sequential rupture of schizonts (Hawking *et al.*, 1968). The clinical manifestations may range from mild to severe, and in some cases, death, but generally, these conditions could be complicated or uncomplicated.

Uncomplicated malaria may present features such as fever, chills, sweats, headaches, nausea, body aches and general malaise (Lindner *et al.*, 2012), which may be confused with similar presentations by common cold or influenza unless malaria diagnostic testing confirms otherwise (Lillie *et al.*, 2012). Complicated malaria, such as severe malarial anaemia (SMA) and cerebral malaria (CM), is associated with hypoglycaemia, haemoglobinuria, acute respiratory distress syndrome (ARDS), renal failure, and coma (Heddini, 2002; Rasti *et al.*, 2006; Wells *et al.*, 2009). SMA may result from immune-mediated lysis, RBC rupture and dyserythropoiesis. The cytoadherence of parasite-infected erythrocytes may also occlude blood vessels, and restrict blood flow to parts of the brain and other organs (Marsh and Snow, 1997), causing cerebral malaria.

The *Plasmodium* parasite employs several mechanisms such as antigenic variation to avoid immune recognition by the host. For instance, it expresses variant surface antigens, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), which are exported to the

surface of erythrocytes to mediate the binding of infected red blood cells (iRBCs) to vascular receptors, thereby avoiding recognition and clearance by the spleen (Fairhurst *et al.*, 2005; Kraemer and Smith, 2006). PfEMP-1 is encoded by the var gene superfamily which consists of about 60 var genes which switch to express one gene at a time (Roberts *et al.*, 1992; Smith *et al.*, 1995). It is capable of binding to many endothelial receptors, such as CD36, which are associated with uncomplicated malaria and intercellular adhesion molecule (ICAM)-1 implicated in severe and cerebral malaria (Newbold *et al.*, 1997; Ochola *et al.*, 2011; Silamut *et al.*, 1999). Severe manifestations of malaria may also be caused by parasite sequestration (Grau and Craig, 2012), a phenomenon which enables infected mature RBCs to cytoadhere to human cells within the microvascular circulatory system (Autino *et al.*, 2012; Udeinya *et al.*, 1981), as well as the parasite binding to receptors such as endothelial protein C receptor (EPCR) (Turner *et al.*, 2013). The brain's vulnerability to malaria-infected erythrocytes (IE) may be due to the low cerebral constitutive expression of EPCR and thrombomodulin (TM) (Moxon *et al.*, 2013). Other mechanisms of immune evasion are rosetting and platelet-mediated clumping. The former occurs when infected erythrocytes adhere to uninfected erythrocytes (Rowe *et al.*, 1995), while the latter involves the binding of infected erythrocytes to platelets (Pain *et al.*, 2001).

2.4 *Lifecycle of Plasmodium*

Plasmodium is a protozoon of the apicomplexan parasites (Cavalier-Smith, 1993). The *Plasmodium* parasite has a complex life cycle which involves a sexual phase in the female *Anopheles* mosquito vector and an asexual phase in the human host. An infective female *Anopheles* mosquito transfers the parasite to the human host by bites during a blood meal.

Anopheles mosquitoes normally obtain their energy from feeding on nectars but the female requires blood for the development and production of eggs (http://www.cdc.gov/malaria/).

During the blood meal, the mosquito injects a mixture of saliva and sporozoites as well as an anti-coagulation factor Xa in its salivary glands into the skin of the human host (Stark and James, 1996). The sporozoites penetrate into the microvasculature by traversing various layers of the dermis through gliding motility until they pass through the blood to reach the liver (Amino *et al.*, 2005). Sometimes, they drain the lymph nodes before reaching the liver. Here, they adhere to the endothelium and bind to highly sulphated heparin sulphate proteoglycans (Coppi *et al.*, 2007) which are presented by hepatocytes through the fenestrae. The sporozoites switch to an invasive phenotype upon the cleavage of the parasite's CSP from the activation of calcium-dependent protein kinase 6 in a signalling cascade (Coppi *et al.*, 2007). They pass through sinusoidal cells of the capillaries and several layers of hepatocytes before selectively and actively invading one of the hepatocytes (Mota and Rodriguez, 2002). The sporozoite subsequently forms a parasitophorous vacuolar membrane (PVM) around itself and undergoes several rounds of replication to form hepatic schizonts containing merozoites (Graewe *et al.*, 2012; Lindner *et al.*, 2012). The PVM eventually disrupts to release mature merozoites which evade the liver Kupffer cells (sessile macrophages) and enter the bloodstream to infect erythrocytes (Graewe *et al.*, 2011).

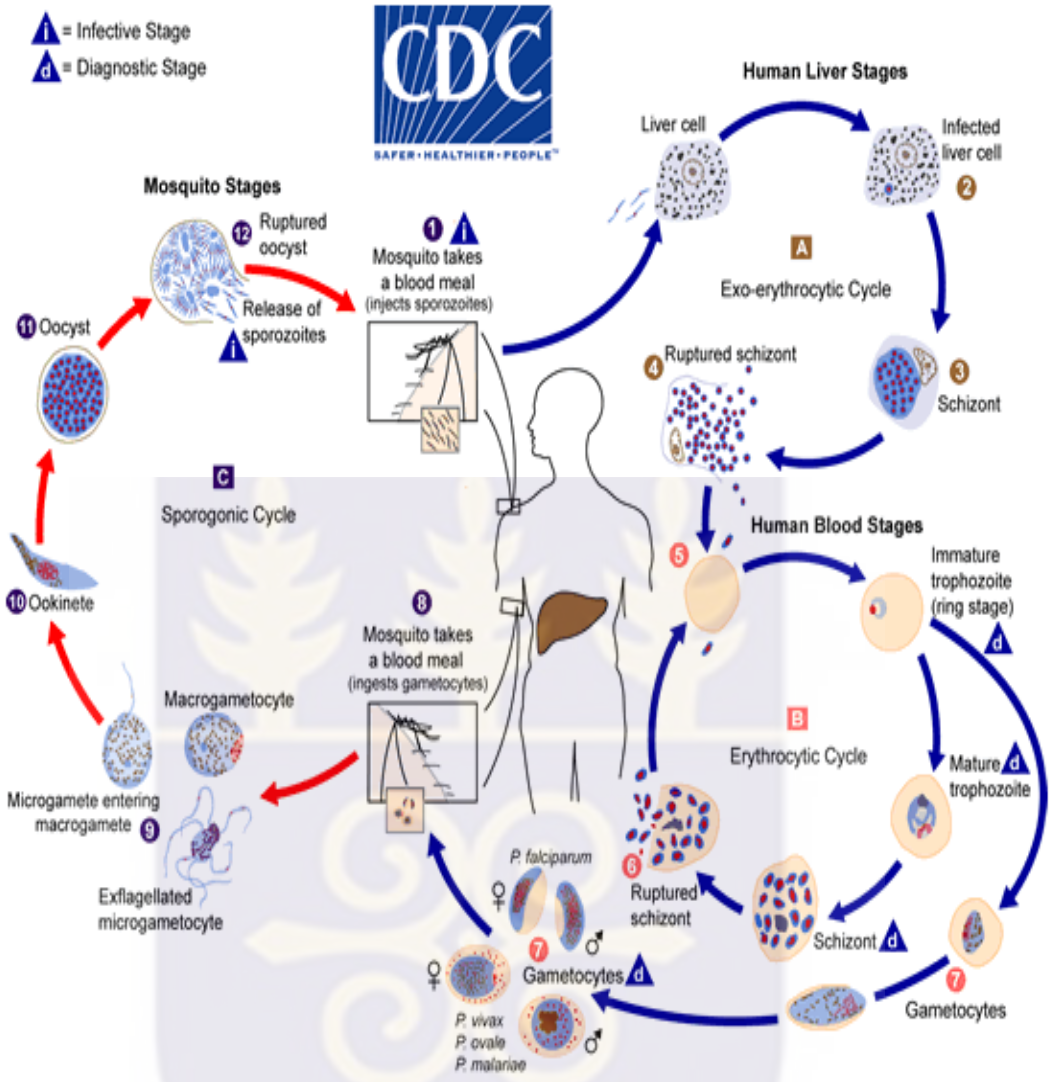


Figure 2.2: **The *Plasmodium* life cycle**

An infective female *Anopheles* mosquito takes a blood meal from a human host during which it injects sporozoites into the skin. Sporozoites rapidly migrate through the bloodstream to the liver where they differentiate into liver merozoites. Merozoites exit the hepatocytes to re-enter the bloodstream where they grow and develop through ring, trophozoite and schizont stages in the erythrocytes. Mature schizonts burst to release merozoites, some of which re-infect circulating erythrocytes while others differentiate into gametocytes to begin the sexual phase of the life cycle. Gametocytes ingested by *Anopheles* mosquito during a blood meal are able to produce male and female gametes which fuse and develop into ookinetes which traverse the midgut to form oocysts and finally sporozoites that migrate to the salivary glands to begin the cycle once more. (Adapted from http:/www.cdc.gov/malaria/about/biology/ Accessed: 03/06/17)

The mechanism of egress of liver stage parasites is unknown but it is believed that cysteine proteases of the serine repeat antigen (SERA) family are involved in the breakdown of PVM, degradation of mitochondria, cell detachment and merosome formation around the parasite (Gerald *et al.*, 2011; Sturm *et al.*, 2006). The merosomes squeeze their ways between two adjacent cells of the fenestrated endothelium of the liver sinusoid into the bloodstream (Wirth and Pradel, 2012), and finally to the pulmonary capillary system where the parasites are released to mark the beginning of the erythrocytic stage of the life cycle (Baer *et al.*, 2007; Gilson and Crabb, 2009; Mazier *et al.*, 2009). Other *Plasmodium* species such as *P. vivax* and *P. ovale* are able to produce a dormant stage hypnozoite during liver development that can result in a relapse of malaria (Cogswell *et al.*, 1983; Krotoski *et al.*, 1982). Host cell invasion processes such as attachment, entry, and formation of PVM require the release of proteins from apical secretory organelles.

The invasion of host erythrocytes by the parasite is mediated by the interactions of merozoite surface proteins and receptors on the RBCs as well as molecular motors. Once inside the erythrocyte, the merozoite forms another PVM around itself using part of the erythrocyte membrane and lipids from its secretory organelles (Ward *et al.*, 1993). It takes about 48 hours for the discrete developmental stages within the vacuolar membrane. The PVM ensures the exchange of nutrients between the parasite and the host cell. It also forms a food vacuole which is highly acidic and contains several proteases such as falcipain 2, plasmepsin II, plasmepsin IV, histo-aspartic protease (HAP) and *Plasmodium* haem degradation protein (HDP) which form a complex for the digestion of about 60-80% of erythrocytic haemoglobin (Krugliak *et al.*, 2002), thereby deriving amino acids from the

globin chain and the polymerization of haem to haemozoin (Chugh *et al.*, 2013). With available nutrients, the parasite undergoes aggressive feeding, growth, and development. It transforms from the ring forms to trophozoites, which undergo DNA replication to form schizonts. Nuclear division within the schizonts results in the segmentation of daughter cells, producing 16-32 merozoites (Bannister and Mitchell, 2003) which egress upon the breakdown of both PV and erythrocyte membranes. Subtilisin-like serine protease (SUB)-1, responsible for the necessary proteolytic processing of MSP-1, 6 and 7 precursor proteins (Koussis *et al.*, 2009), are discharged into the parasitophorous vacuolar space (Yeoh *et al.*, 2007) by exonemes prior to the release of merozoites. Essential to the process of egress is also a cascade of kinases including calcium-dependent protein kinase 5 (CDPK5) (Dvorin *et al.*, 2010), and subsequently cGMP-dependent protein kinase (PKG) (Taylor *et al.*, 2010). The primed and ready merozoites invade uninfected RBCs within 2 minutes of egress (Gilson and Crabb, 2009).

Occasionally, the mother schizont can produce sexually committed merozoites whose intracellular development in the erythrocyte would be geared toward producing male and female gametocytes (Bruce *et al.*, 1990; Silvestrini *et al.*, 2000; Smith *et al.*, 2000). *P. falciparum* gametogenesis takes over a period of 8-12 days within the human host. It is unclear what factors bring about the commitment to gametocytogenesis but Baker (2010) has suggested stress stimuli from the surrounding environment, such as host immune factors, haematological disruption, high parasite density, low nutrient levels and antimalarial drugs. The expression of protein Pfs16 within 24 hours of post-invasion in sexually committed parasites is an extremely valuable distinguishing marker between early

gametocytes and asexual trophozoites (Bruce *et al.*, 1994). Later mature stage gametocytes take on a characteristic crescent form (Sinden, 1982). Gametocytes may be arrested at G0 but the final maturation of the gametocytes into gametes is stimulated by a change in pH, a 5ºC drop in temperature and the gametocyte activating factor xanthurenic acid when a female *Anopheles* mosquito takes a blood meal (Arai *et al.*, 2001; Wirth and Pradel, 2012). The gametes subsequently fuse to form a zygote, which rapidly develops into a motile invasive ookinete that migrates through various layers of the midgut epithelium by gliding motility and reliance on adhesins released by microneme secretions. Upon reaching the basal lamina of the gut, it forms an oocyst (Vlachou *et al.*, 2004) within which sporogony occurs. Developed sporozoites are released into the haemocoel (Canning and Sinden, 1973). They migrate and actively invade the salivary glands where they remain until their injection along with saliva into human host skin, during a blood meal, to commence the lifecycle (Figure 2.2).

2.5 *The Plasmodium Merozoite*

The merozoite is the invasive form of the human *Plasmodium* parasite at the blood stage. A small ovoid cell (~1–2 μm), the merozoite is essential for erythrocyte invasion (Bannister *et al.*, 1986). It has a repertoire of organelles and a typical apicomplexan cytoskeletal organization (Morrissette and Sibley, 2002).

The merozoite has a nucleus, mitochondrion, apicoplast and apical secretory organelles such as micronemes, rhoptries, and dense granules (Bannister *et al.*, 2000; McFadden *et al.*, 1996; Roos *et al.*, 1999). Located at the posterior end, the nuclear genome comprises 14 chromosomes and 23 Mb of DNA. The mitochondrion contains 6 kb mtDNA (Gardner *et al.*, 2002). In spite of its own 35 kb circular genome, the apicoplast traces the majority of its protein expressions to the nuclear genome. A relic of rhodophytes, the apicoplast is involved in the biosynthesis of fatty acids, isoprenoids and the digestion of haem to provide metabolites essential for the parasite's survival (Striepen, 2011).

The endoplasmic reticulum (ER) surround the nucleus and plays host to newly synthesized proteins meant for secretory or cellular compartment roles. In order to be trafficked through the ER to their appropriate destinations, secretory proteins must contain signal peptides at the N-terminus (Foth *et al.*, 2003). Bioinformatics data on sequence similarity show that protein translocation machinery is conserved (Tuteja, 2007). XDEL motifs retrieve escaped resident ER proteins from the Golgi and retain them in the ER (Elmendorf and Haldar, 1993; van Dooren *et al.*, 2005). A Golgi apparatus exists with cis- and trans-compartments with limited evidence for stacks (Elmendorf and Haldar, 1993; Noe *et al.*, 2000). The

addition of glycosylphosphatidylinositol (GPI), post-translational modification, to the cleaved C terminus of would-be surface proteins allows membrane insertion.

A network of flattened vesicular inner membrane complex (IMC) underlies the thick plasma membrane of the merozoite - subpellicular microtubules subtend the IMC (Bannister *et al.*, 2000). Electron images obtained from Aikawa *et al.* (1978); Bannister *et al.* (1975) indicate the surface of the *Plasmodium* merozoite may be covered by a protein coat which seemed to be sloughed during the invasion of erythrocytes.

The merozoite makes and deploys an array of proteins (Table 2) essential for erythrocyte invasion. These proteins are either located within apical secretory organelles or found on the surface of merozoites. The latter may be glycophosphatidylinositol (GPI)-anchored proteins, integral membrane proteins or as peripherally-associated proteins which interact with membrane-bound proteins. During schizont development, merozoite proteins which are retained in the rhoptries and micronemes are deployed to the merozoite surface before, or soon after, schizont rupture and release of merozoite. The mechanisms underlying this development are not well understood. There is substantial processing of merozoite surface proteins before, during and after the invasion of erythrocytes (Beeson *et al.*, 2016).

A defining feature of apicomplexan species, the apical organelles are secreted to ensure motility and invasion of erythrocytes. The apical organelles of *Plasmodium* merozoite (Figure 2.3) are formed during late schizogony. Protein secretion in these organelles is limited to the microneme, rhoptry, and the dense granules.

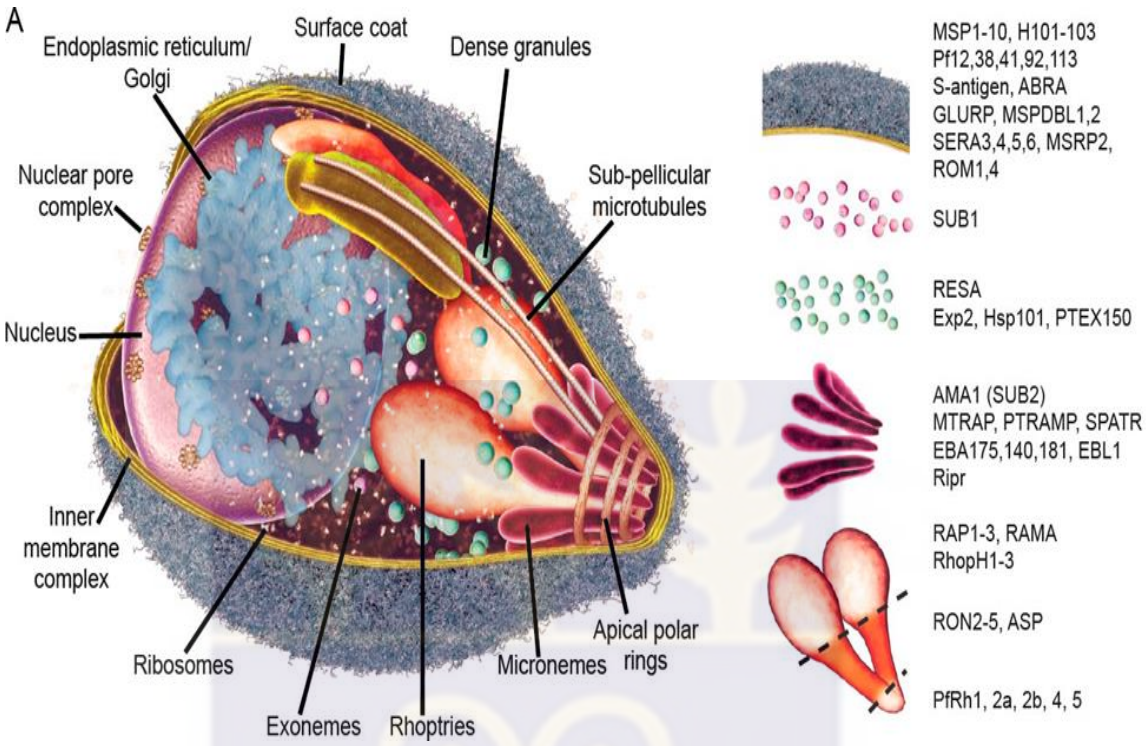


Figure 2.3: Diagram of merozoite and a repertoire of invasive proteins

The apical secretory organelles – micronemes, rhoptries and dense granules – exhibit subpopulations of organelles and sub-compartmentalization. The rhoptries are organized into three segments displaying the PfRh proteins (1, 2a, 2b, 4, and 5) to the most distal segment, followed by RON2-5 (Riglar *et al.*, 2011). The dense granules include the translocon protein which is inserted into the parasitophorous vacuole membrane as well as the ring-infected erythrocyte surface antigen (RESA) which is exported to the infected red blood cells (iRBCs). RAP1-3 and RAMA are proteins of the rhoptry bulb involved in the formation of the parasitophorous vacuole. (Source: Cowman *et al.*, 2012)

2.5 *Mechanism of erythrocyte invasion*

Invasion of host erythrocytes by merozoites is an essential event for the parasite survival and malaria pathogenesis. It is a highly regulated step which involves the release of apical organelle proteins to mediate complex and rapid sets of interactions between different parasite ligands and erythrocyte (Cowman and Crabb, 2006; Iyer *et al.*, 2007; Preiser *et al.*, 2000).

Structure and electron microscopy studies (Aikawa *et al.*, 1978; Bannister *et al.*, 1975) reveal that upon release from schizont rupture, it takes few minutes for the merozoites to invade other erythrocytes (Gilson and Crabb, 2009) via the following distinct invasion processes: initial attachment, apical reorientation, junction formation and entry into the host RBCs as depicted in Figure 2.4. Ultimately, an actin-myosin type motor drives the invasion of erythrocytes (Baum *et al.*, 2006).

Table 2: Invasion-related proteins of *Plasmodium falciparum* merozoites

Peripheral surface proteins	PlasmoDB accession number	Genetic knockout	Localization in merozoite before/during invasion	Potential function	Feature/structure
Pf113	PF3D7_1420700	N	Surface/shed	Not known	No data
MSP-9 (ABRA)	PF3D7_1228600	Y	Surface/shed	Putative protease	No data
S-antigen	PF3D7_1035200	N	Secreted into PV of schizont and released on egress	Not known; potential immunomodulatory role	Highly repetitive and diverse protein
GLURP	PF3D7_1035300	Y	Secreted into PV of schizont and released on egress	Not known	Repetitive Glutamate-rich
MSP-3	PF3D7_1035400	Y	Surface/shed	Not known; binds to MSP-1	Repetitive and Glutamate-rich
MSP-6	PF3D7_1035500	Y	Surface/shed	Not known; binds to MSP-1	Leucine zipper-like C-terminal domain
H101 (MSP-11)	PF3D7_1035600	Y	Surface/shed	Not known	MSP-3 family, leucine zipper-like C-terminal domain
H103	PF3D7_1035900	Y	Surface/shed	Not known	MSP-3 family, leucine zipper-like C-terminal domain
MSP-7	PF3D7_1335100	Y	Surface/shed	Associates with MSP-1, gene knockout in *P. berghei* shows importance in invasion of mature erythrocytes	No data
MSP-7-like (MSRP2)	PF3D7_1334800	Y	Surface/shed	Not known; may associate with MSP-1	MSP-7 family
MSPDBL-1	PF3D7_1036300	Y	Surface/shed	Binds to unknown receptor on red cell	Member of EBL family, DBL and leucine zipper-like domains
MSPDBL-2	PF3D7_1035700	Y	Surface/shed	Binds to unknown receptor on red cell	Member of EBL family, DBL and leucine zipper-like domains
SERA3	PF3D7_0207800	Y	Secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA4	PF3D7_0207700	N	Most secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA5	PF3D7_0207600	N	Secreted into PV of schizont and released on egress	Cysteine protease domain with active site serine	Cysteine protease domain
SERA6	PF3D7_0207500	N	Most secreted into PV of schizont and released on egress	Cysteine protease domain with active site cysteine	Cysteine protease domain
Pf41	PF3D7_0404900	Y	Surface/shed	Potential adhesive protein; binds Pf12 on merozoite	6-Cys domains

Grouping of peripheral surface proteins from invasion-related proteins based on their localization.

N = knockout attempt unsuccessful; Y = knockout generated; ND = knockout not attempted; PV = parasitophorous vacuole; MSP = merozoite surface protein. (Source: *Review* in Cowman *et al.*, 2012)

2.5.1 *Initial attachment*

When matured blood stage schizonts rupture, free merozoites are released to invade new erythrocytes. The merozoites must first establish selective contact with the surface of RBC by specific molecular interactions (Miller, 1977). This initial attachment is usually weak and can occur at any point of the merozoite surface (Bannister and Dluzewski, 1990).

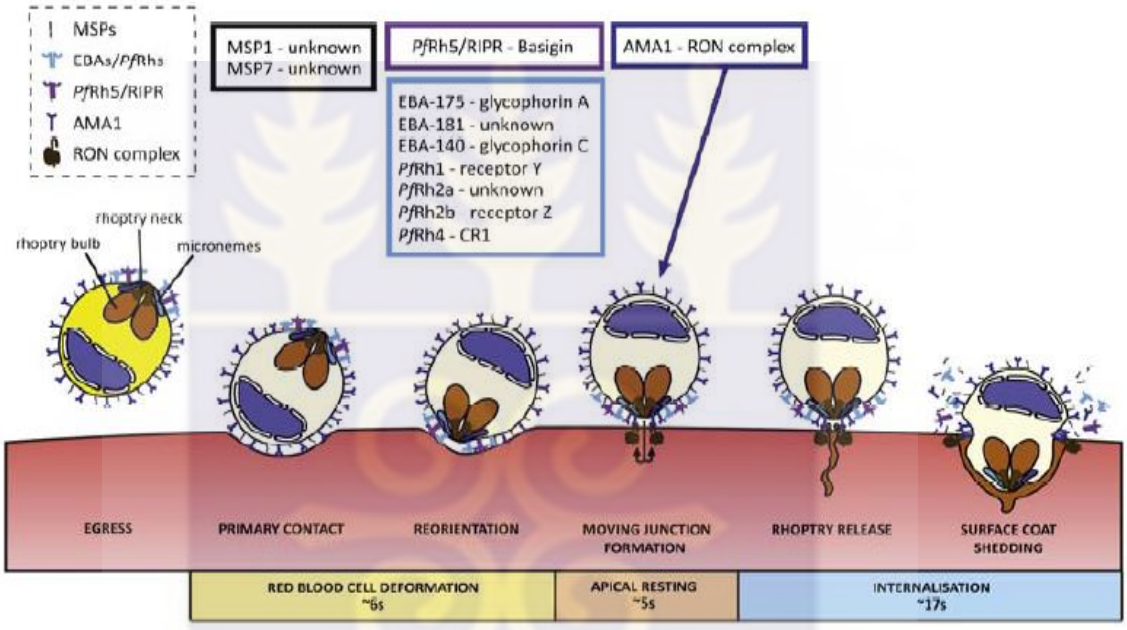


Figure 2.4. Erythrocyte invasion processes by merozoite from egress

Upon egress, calcium levels (yellow) surge owing to low potassium environment result in the release of micronemal proteins onto merozoite surfaces. The merozoite establishes a reversible contact on the erythrocyte membrane probably by GPI anchorage (black) and forms a moving junction with AMA1-RON complex after apical reorientation. Through the proteolytic activities of proteins such as ROM4, ROM1, SUB1, and SUB2, the parasite sheds its proteins into the supernatant as the actomyosin motor-mediated tight junction moves across the merozoite surface. The action of the actomyosin motor moves the parasite into the parasitophorous vacuole and membrane formed primarily by the rhoptries. The membranes seal once the tight junction reaches the posterior end of the parasite. Most of the mechanisms of erythrocyte invasion are not well-established. (Source: Harvey *et al*., 2012)

Merozoite surface proteins (MSPs) are involved in the initial attachment process. MSP-1, 2, 4, 5, 8 and 10 are attached to the merozoite surface proteins. Interestingly, MSP1, 4, 5, 8 and 10 tether to the parasite membrane by GPI-anchorage and share some common epidermal growth factor (EGF)-like domain at their C-terminus. MSP-3 and -7 as well as serine repeat antigen (SERA) indirectly associate with the merozoite surface (Cowman and Crabb, 2006). Most abundant and functionally conserved protein on the merozoite surface, precursor MSP-1 protein (~200 kDa) is cleaved into three fragments (83, 30 and 38 kDa) by PfSUB-1 (Holder *et al.*, 1987). Attached to the membrane is a GPI-anchored 42 kDa fragment, which links these three fragments by noncovalent bonds (Gerold *et al.*, 1996; McBride and Heidrich, 1987). Kauth *et al.* (2006) have reported that MSP-3, -6 and -7 associates indirectly with the merozoite surface by forming a complex with MSP-1. Separate studies by Goel *et al.* (2003) and Li *et al.* (2004) also suggest that, within this complex, soluble MSP-9 protein associates with the 42 kDa MSP-1 fragment thereby binding to Band 3 on erythrocyte surface.

The exact mechanisms driving the specificity of host cell selection for initial attachment as well as the complexes formed by MSPs are yet to be elucidated.

2.5.2 *Apical orientation and irreversible attachment*

Following attachment, the merozoite reorients its apical end such that it is in direct contact with the erythrocyte surface, making the merozoite to irreversibly attach and bind to

specific erythrocyte receptors through the erythrocyte binding-like (EBL) proteins and *P. falciparum* reticulocyte binding-like (PfRH) proteins (Cowman *et al.*, 2012).

Though the mechanism behind reorientation is not well-established, Mitchell *et al.* (2003) have demonstrated the essential role of apical membrane antigen (AMA-1) in this process by blocking *P. knowlesi* merozoites with specific mAb. Conserved across the apicomplexan species, AMA-1 is expressed in late schizonts and localized to the micronemes which secrete their contents before the invasion (Bannister and Mitchell, 2003; Healer *et al.*, 2002). AMA-1 appear to diffuse around the merozoite surface upon release from the micronemal location (Singh *et al.*, 2010). Additional experimental evidence suggests that AMA-1 may have adhesive properties. For instance, while peptides derived from AMA-1 bind to human erythrocytes (Urquiza *et al.*, 2000), the expression of the protein in COS cells resulted in aggregation of erythrocytes (Fraser *et al.*, 2001).

The apical juxtaposition of the merozoite to the erythrocyte membrane brings about tighter interactions involving ligand-receptor recognitions. EBL superfamily proteins are deployed from the micronemes to the apical end of the merozoite surface where they remain for the greater part before interacting with their binding partners to effect the process of invasion (Singh *et al.*, 2010). The erythrocyte sialic acid residues to which EBA-175, EBL-1 and EBA-140 bind are shown in Figure 2.4. EBA-175 binds to glycophorin A (Adams *et al.*, 1992; Sim *et al.*, 1994), EBL-1 binds to glycophorin B (Mayer *et al.*, 2009), EBA-140 binds to glycophorin C (Rydzak *et al.*, 2013) but the receptor for EBA-181 remains unknown (Gilberger *et al.*, 2003).

Located in the rhoptries are five functional PfRH proteins, namely PfRH1, 2 (2a and 2b), 3, 4, and 5. Rayner *et al.* (2001) have demonstrated that PfRH1 exhibits erythrocyte binding activity and it is localized at the merozoite apex. Although they have binding roles, PfRH2a and PfRH2b do not have known receptors (Duraisingh *et al.*, 2003; Dvorin *et al.*, 2010; Rayner *et al.*, 2000; Triglia *et al.*, 2001). PfRH3 is a transcribed pseudogene (Taylor *et al.*, 2001). PfRH4, which is a rhoptry neck protein, interacts with Complement receptor 1 (CR1) (Tham *et al.*, 2010) while PfRH5 has been shown to interact with Basigin on the erythrocytes (Crosnier *et al.*, 2011). An essential gene, PfRH5 has been demonstrated, for instance in *Aotus* monkeys, to be a possible vaccine candidate against blood-stage *P. falciparum* infections (Douglas *et al.*, 2015).

A study by Dolan *et al.* (1990) has shown that *P. falciparum* can switch its invasion pathways from sialic acid dependence to sialic acid independence. Additionally, Stubbs *et al.* (2005) have reported that genetic deletion of *eba-175* resulted in the upregulation of *PfRh4*, indicating a level of cooperativity among the erythrocyte binding antigens. This degree of redundancy appears to safeguard the success of erythrocyte invasion as well as provides escape from host immune surveillance.

2.5.3 *Junction formation*

In addition to parasite-host interactions at the tight junction, parasite proteins form complexes which enhance the molecular motor for the internalization of merozoites. The merozoite remains irreversibly attached at the apical end, allowing the translocation of

rhoptry neck proteins and apical membrane antigens into the erythrocyte (Harvey *et al.*, 2012).

Secreted RON2, -4 and -5 proteins translocate across and inject into the erythrocyte membranes, residing at the posterior end of the zoite. Anchored in the merozoite membrane by a transmembrane domain, AMA1 has been proposed to form a complex with RON4 which also interacts with the transmembrane portion of RON2 during moving junction interaction (Cao *et al.*, 2009; Richards *et al.*, 2010). It has been shown that AMA-1 cannot be deleted in the blood stage parasite of *P. falciparum*, suggesting an essential function of the antigen (Triglia *et al.*, 2000).

2.5.4 *Merozoite entry into erythrocyte*

The movement of the merozoite into the erythrocyte is enhanced by the conserved actin-myosin motor complex found between the plasma membrane and the inner membrane complex (IMC). The complex comprises membrane-anchored glideosome associated proteins GAP50, GAP45; myosin A tail-interacting protein (MTIP); myosin A; F-actin and aldolase (Baum *et al.*, 2008; Baum *et al.*, 2006; Green *et al.*, 2006).

The junction moves from the apical end to the extreme end to ensure that the merozoite is fully engulfed by the erythrocyte membrane. An interaction between myosin A and F-actin initiated by a merozoite binding signal drives the merozoite forward. In a model proposed by Baum *et al.* (2008), formin and profilin polymerize actin when signalled. The myosin A drives the actin and thrombospondin-related anonymous protein (TRAP) towards the

posterior of the merozoites. Thereon, the adhesive molecules are shed to allow internalization while the actin depolymerizes and monomers are recycled.

Whereas the exact link between surface adhesive molecules and motor activation remains elusive in merozoites, a study by Buscaglia *et al.* (2003) has suggested that TRAP may interact with both hepatocyte membrane and fructose-1,6-bisphosphate aldolase which may bind the F-actin to link host cell binding with the sporozoite's invasion mechanism. The *Plasmodium* merozoite equivalent of TRAP (MTRAP) has been shown in an *in vitro* experiment to exhibit erythrocyte binding capacity, suggesting a possible linkage with activation of the motor (Uchime *et al.*, 2012).

The ultimate entry of the merozoite is executed by two groups of proteases: subtilisin-like (SUB) and rhomboid (ROM) serine proteases. PfSUB-2 initiates the dissociation of the link between the erythrocyte membrane and MSP-1, AMA-1 and PTRAMP (Green *et al.*, 2006; Harris *et al.*, 2005). Howell *et al.* (2005) have reported that PfSUB-2 cleaves majority of AMA-1, leaving a small portion for rhomboid proteolytic activity. The MSP1-42 fragment is cleaved, leaving a 19 kDa fragment which enters the PV (Blackman *et al.*, 1996) followed by rhomboid proteases which ultimately disengage the adhesive interactions of the merozoite from the erythrocyte (Riglar *et al.*, 2011). The rhomboid proteases usually cleave target proteins within the transmembrane domains (Freeman, 2008). Notably, ROM1 and -4 cleave adhesins involved in erythrocyte attachments such as EBA-175 (O'Donnell *et al.*, 2006).

2.5.5 *Formation of parasitophorous vacuole and completion of invasion*

The entry of the merozoites accompanies the discharge of lipid-rich rhoptry contents into the erythrocyte to aid the formation of the PV within which the merozoite will reside for the remainder of its intraerythrocytic stage (Bannister *et al.*, 1986). Mid-way into the invasion, rhoptry-associated proteins (RAP-1) move to the apex to assume the position of rhoptry neck proteins and ultimately gets to reside in the PVM (Riglar *et al.*, 2011). As the moving junction progresses to the distal end of the merozoite, the PVM merges to enclose the merozoite completely from the cytosolic milieu, sealing the process of erythrocyte invasion (Lingelbach and Joiner, 1998).

2.6 *Bioinformatics principles and applications to proteins*

Bioinformatics uses computational tools to organize, analyze, store and understand information such as biological sequences, biomolecular structures and evolutionary relationship between species (Diniz and Canduri, 2017; Luscombe *et al.*, 2001). The characterization of biomolecules, such as proteins, is essential to the understanding of the molecular processes underlying cellular physiology (Schmidt *et al.*, 2014). Proteomics, therefore, seems to systematize the study of proteins in space and time (Jensen, 2006) such that the research ideas can first be tested *in silico* before they are experimented through wet labs.

The interdisciplinary nature of bioinformatics has revolutionized and improved the identification, quantification and analysis of proteins, overcome the challenges of data processing and storage (Altelaar *et al.*, 2013). These significant advances have been

possible through the computing power and genome projects, such as sequencing and annotation (Diniz and Canduri, 2017). Briefly, bioinformatics methods can be applicable to amino acid sequence (peptide or protein) analysis, analysis of protein expression, protein structure prediction, homology modelling, and protein-protein docking and interaction (Lee, 2008; Nayeem *et al.*, 2006).

The alignment and comparison of protein structures is an important bioinformatics tool which enables the determination of equivalent amino acids between two or more structures, and their sequence similarity analysis using an algorithm. Another valuable tool for determining the quality of the protein structure is the Ramachandran plot which shows the stereochemical impediments in the main chain of amino acids (Diniz and Canduri, 2017).

Homology modelling helps to determine the 3-D structure of a target protein from the homologous structure of previously determined protein structure because the protein sequences of evolutionary relatedness exhibit the same folding pattern of the tertiary structure. The understanding of protein function and interaction, and the functional prediction and identification of therapeutic targets, from the determination of the 3-D structure (Vyas *et al*., 2012). SWISS-MODEL software can be used to construct models while the amino acid sequences can be compared using Basic Local Alignment Search Tool (BLAST).

Bioinformatics databases are repositories which collect, organize, archive and retrieve data for interpretation. An information web resource for understanding the relationship between

sequence, structure and function of biological macromolecules is the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Kouranov *et al.*, 2006). Computer-aided programmes for the analysis and comparison of mass spectrometry data has also improved proteomics (Altelaar *et al.*, 2013).

2.7 *Parasite transcriptome, proteome and comparative genomic data*

The huge number of uncharacterized proteins in the *P. falciparum* genome could be the source of new therapeutic or vaccine targets. Functional genomics has made it possible for the identification and selection of stage-specific expression of genes for further study. Existing bioinformatics data on the parasite's genome, transcriptome, and proteome have been used to identify novel parasite genes predicted to play a role in erythrocyte invasion. Features such as microarray expression timing or transcriptome analysis from RNAseq were used to uncover invasion-related proteins (Figure 3.1).

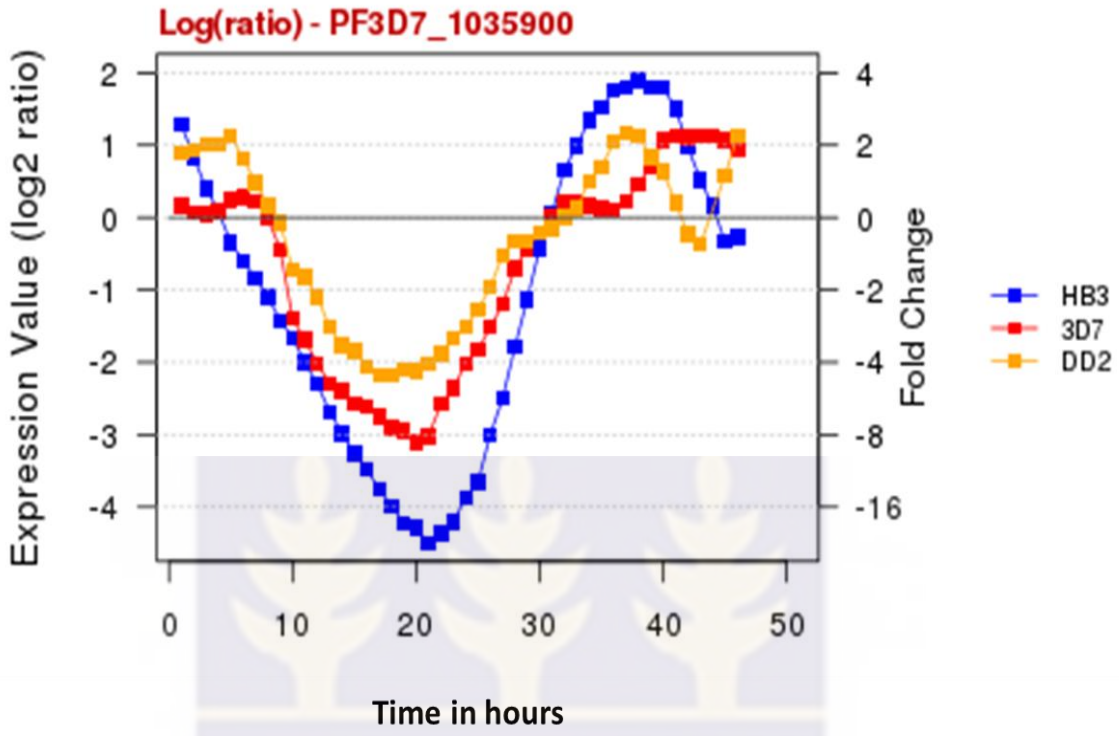


Figure 3.1: Erythrocyte expression time series

This is a panel of DNA microarray of expression across 48 individual 1-h time points from the intra-erythrocytic development cycles of *P. falciparum* 3D7, Dd2, HB3. The left vertical axis shows the expression values for 2 channel microarray experiments as log ratios while the right one shows the fold change. The horizontal axis shows the time in hours after adding a synchronized culture of parasite strains to fresh blood. Periodicity in the expression pattern of 3D7 peaks essentially from 36 to 48 h post-invasion with higher fold changes. (Source: Bozdech *et al*. PLoS Biol. 2003)

Several transcriptome data show a strong relationship between the time of expression of genes and their functions. For instance, DNA microarray study by Bozdech *et al.* (2003) observed periodicity in the expression profiles of genes during the 48-hour intraerythrocytic developmental cycle of *P. falciparum* HB3 lines. The formation of the micronemes coincides with the transcriptional expression of *ama1*, which peaks at 40 hours post-invasion. Further, evidence from an alternative transcriptome by Le Roch *et al.* (2004)

indicate that the similarity in the time of expression of *msp1*, *ama1*, *sub2* and *eba* genes in the same cluster (cluster 15) suggests functions in erythrocyte invasion.

Proteomic data available through mass spectrometry have provided reliable means to identify stage-specific proteins of the *Plasmodium* life cycle. For instance, using multidimensional protein identification technology (MudPIT), almost 2400 proteins have been identified from sporozoites, trophozoites, merozoites, and gametocytes (Florens *et al.*, 2002). A notable contribution to mass spectrometry proteomic data on PlasmoDB (http://plasmodb.org) is the merozoite proteome by Leiden Malaria Group. Other proteomes have subsequently been identified from late schizogony (Bowyer *et al.*, 2011), clinical samples (Acharya *et al.*, 2009) and phosphorylated proteins of the erythrocytic stage (Lasonder *et al.*, 2012).

In addition to the *P. falciparum* 3D7 genome, the genomes of other *Plasmodium* species, such as *P. yoelii* str. 17XNL (Carlton *et al.*, 2002); *P. vivax* Sal-1 (Zhou *et al.*, 2008) and *P. knowlesi* strain H (Pain *et al.*, 2008), have been published. The comparison of *Plasmodium* genomes with other unicellular eukaryotic species greatly improves the ability to identify orthologous proteins in order to assign functional roles to predicted *Plasmodium* proteins. This ultimately provides valuable information about the parasite's physiology and evolution. A superior model for genetic manipulation, *Toxoplasma* species has provided an important comparison for the characterization of difficult proteins among apicomplexan parasites (Kim and Weiss, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 *Bioinformatics sequence analyses*

PlasmoDB (www.plasmodb.org) was used to predict the probable protein selected for this study. This protein is encoded by the PF10_0351 gene of the *P. falciparum* 3D7 clone. The parameters for selection were (1) the presence of a signal peptide or transmembrane domain, (2) high transcriptional up-regulation during late schizogony and (3) conservation across *Plasmodium* species. These criteria are notable for invasion-related proteins.

The **I**terative **T**hreading **ASSE**mbly **R**efinement (i-TASSER) approach was used to predict protein structure and function (http://zhanglab.ccmb.med.umich.edu/I-TASSER/download/). This approach uses LOMETS (multiple threading approaches) to identify protein structural templates from PlasmoDB in order to derive the 3D model of protein function from BioLiP (ligand-protein binding database) platform. **S**imple **M**odular **A**rchitecture **R**esearch **T**ool (SMART) was also used to predict protein domains (http://smart.embl-heidelberg.de)

STRING version 10.5 database was used to predict protein-protein interactions. This platform uses genomic context predictions, high-throughput laboratory experiments, coexpression, automated text mining and previous knowledge in databases to assess interactions which cover 9,643,763 proteins from 2,031 organisms (https://string-db.org/).

3.1.2 *Prediction of peptide epitopes*

The ClustalW Multiple Alignment software was used to perform protein sequence alignments. The SignalP programme (http://www.cbs.dtu.dk/services/SignalP/) was used to confirm signal peptide predictions while the absence of transmembrane regions was verified using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). SignalP version 4.0 software was used to discriminate signal peptides from N-terminal transmembrane helices, in a manner previously described by Petersen *et al.* (2011).

The PF10_0351 peptides were designed based on the published protein sequence of *Plasmodium falciparum* (3D7 clone) available on PlasmoDB database (www.plasmodb.org). The B and T cell epitopes were predicted based on BCPreds B-cell epitope prediction server (http://ailab.ist.psu.edu/bcpred/). This computational tool offers desired epitope length and specificity threshold by comparing predictions of multiple methods such as AAP method (Chen *et al.*, 2007), FBCPred (El-Manzalawy *et al.*, 2008) and BCPred (El-Manzalawy *et al.*, 2008).

3.2.1 *Peptide design and synthesis*

The peptide design factored in multiple peptide epitopes from the same target protein to maximize the success of detection in the native protein. Therefore, three synthetic peptides were constructed from the full length of the PF10_0351 protein. These peptides represent B- and T-cell epitopes of the endogenous protein. Each peptide, comprising 14 amino acids, was given the respective names: PF10_0351-1, PF10_0351-2, and PF10_0351-3. Peptide sequences and their amino acid positions on the full-length protein sequence (566 aa) are detailed in Figure 3.2. The peptides were chemically synthesized by GenScript

Corporation, China, following standard protocols of FlexPeptide™ technology and OptimumAntigen™ design tool that combine top-grade algorithms to achieve 85-90% success rate in eliciting peptide immunogenicity. These peptides were subsequently purified by Alltima C18 HPLC LC/MS columns. Peptide selection parameters included antigenicity, surface exposure, and hydrophobicity (see Table 4).

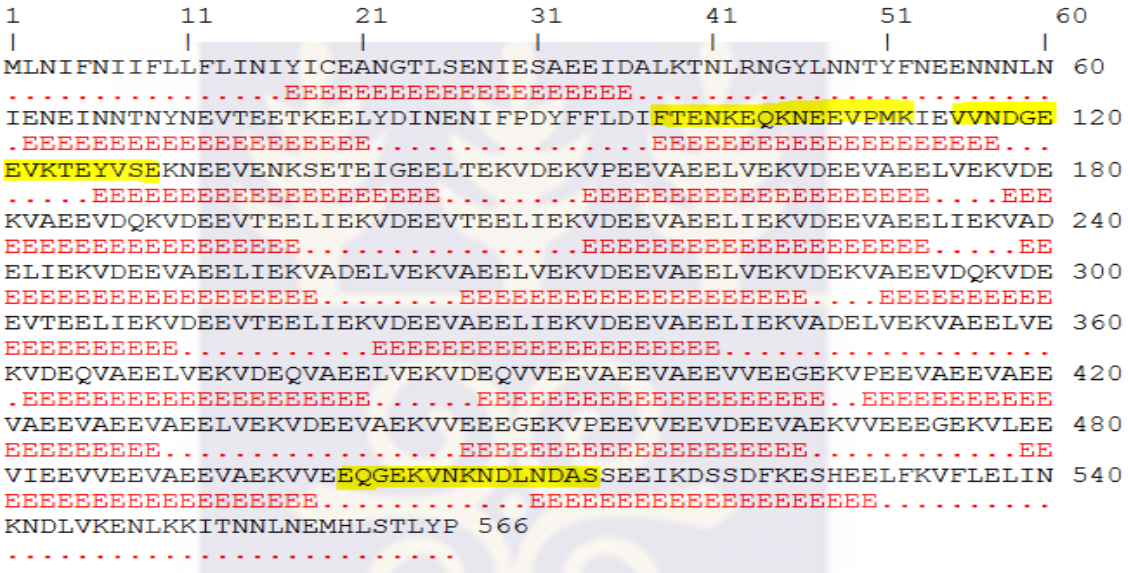


Figure 3.2: **PF10_0351 protein sequence showing epitopes** (yellow colour)

The design of the peptides took into consideration multiple peptides from the same target protein. Peptides 2 (98-101 aa) and 3 (115-128 aa) sequences are mapped to the N-terminus respectively while peptide 1 sequence (501-514) is sited towards the C-terminus. Regions of repeats of glutamic acid residues (red) recur throughout the entire length of the protein sequences. (http://ailab.ist.psu.edu/bcpred/)

3.2.2 *Polyclonal antibody production*

Preimmune rabbit antisera and polyclonal antibodies generated against the PF10_0351 peptides were outsourced to GenScript Corporation, China. The pre-immune and anti-PF10_0351 antibody working dilutions are shown in Figure 4.6 (Page 55).

3.3 *Human subjects and ethical approval*

This component of the work was approved by the Ethics Committees of the Ghana Health Service, Navrongo Health Research Centre, Kintampo Health Research Centre and Noguchi Memorial Institute for Medical Research. Parent or guardian of the children consented to the collection of samples after the details of the study were explained to them.

3.3.1 *Study sites and sample collection*

Three study sites with varying transmission intensities in different ecological zones of Ghana were chosen for the collection of blood samples. These sites were Ledzokuku-Krowor Municipal Assembly (LEKMA) Hospital in Teshie (Greater Accra Region), Navrongo Health Research Centre in Navrongo (Upper East Region), and Kintampo Health Research Centre in Kintampo (Brong Ahafo Region). While Accra has relatively low malaria transmission pattern (hypoendemic) with an entomological inoculation rate (EIR) < 50 infectious bites per annum (Klinkenberg *et al.*, 2008), Navrongo has seasonal high transmission (hyperendemic) and the rainy season has EIR = 50–250 infectious bites per annum (Kasasa *et al.*, 2013). Kintampo is holoendemic with high transmission throughout the year with EIR > 250 (Owusu-Agyei *et al.*, 2009).

Intravenous blood samples were collected between July and November 2016 during the rainy season when malaria transmission was high. Children between the ages of 2-14 years presenting with symptoms of malaria were screened using rapid diagnostic tests (RDTs). Malaria RDT-positive cases were subsequently confirmed by 10% Giemsa staining (pH 7.2, 10 min) and microscopic examination of thick and thin blood smears on slides. About 5 mL of venous blood was then drawn from each child who tested positive for both RDT and microscopy. Blood was collected into heparinized tubes and plasma was separated by centrifugation at 2,000 rpm for 10 minutes. Parasitaemia (the number of parasites per µL of blood) was estimated by counting the number of *P. falciparum* parasites per 200 WBCs on slides of thick smears as well as the WBC count of each child obtained from a haematology analyzer.

$$\text{Parasite / µL} = \frac{\text{number of parasites counted}}{200} \times \text{WBC / µL}$$

3.3.2 *Cryopreservation of laboratory and clinical parasite isolates*

Each tube of blood was centrifuged and separated into plasma, buffy coat, and erythrocytes. The parasitized erythrocytes were cryopreserved in liquid nitrogen using Glycerolyte® (Fenwal Inc., USA). The volume of the sample was measured and a volume of Glycerolyte® equal to one-third of the sample volume was added dropwise while swirling the tube after 5 minutes incubation at room temperature. These samples were then initially stored at -80ºC before they were transferred into liquid nitrogen for long-term storage.

3.3.3 *Thawing of laboratory and clinical parasite isolates*

The cryopreserved parasitized samples were removed from cold storage and thawed at 37ºC for 1 minute. Approximately 10 µL aliquots of 12% NaCl (Sigma-Aldrich, St. Loius, USA) were added to every 100 µL of the sample and incubated for 5 minutes at room temperature. Ten times the sample volume of 1.6% NaCl (Sigma-Aldrich, St. Loius, USA) was added drop by drop in the tube with gentle swirling. The sample was further centrifuged at 1500 rpm for 5 minutes at room temperature (20ºC). The supernatant was discarded and the sediment (erythrocytes) was resuspended in complete parasite medium comprising 1% (w/v) RPMI-1640 (devoid of bicarbonate) (Sigma, South Africa), 25 mL of 80 mg/mL $NaHCO_3$ solution (Sigma, Germany), 5 mL of 10 mg/mL gentamycin (Sigma, South Africa), 100 µL of 200 mM L-Glutamine (Sigma, South Africa), 100 mL of 2 mM Albumax II (Sigma, South Africa) dissolved in 900 mL sterile distilled water. This solution was filter-sterilized through a 0.22 µm filter (Millipore, USA). Twenty (20) mL of sterile normal human serum (NHS) was added only to culture medium meant for culturing of clinical isolates only.

The resultant suspension was centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was discarded and the parasitized erythrocytes were maintained in O+ erythrocytes at approximately 4% haematocrit, a parasitaemia of less than 5% and in an atmosphere of blood gas (2% O_2, 5.5% CO_2 and 92.5% N_2) in the sterile culture flask. Spent complete parasite medium was changed daily at 5% haematocrit as previously described by Jensen and Trager (1977).

3.3.4 *Parasite culture and maintenance*

The *P. falciparum* 3D7 strain and clinical isolates were maintained under intracellular growth conditions as earlier described by Trager and Jensen (1976). Parasitaemia was maintained at between 1-5% by daily replacement of spent parasite medium with fresh culture medium and equivalent proportion of erythrocyte suspensions according to the size of culture flasks. The parasitaemia was examined by Giemsa stained thin-blood smears through light microscopy.

3.3.4.1 *Synchronization of parasite cell culture*

The parasites used in this research work were obtained through alternative synchronization methods of sorbitol or 70% Percoll gradients in a continuous subculture system. Parasite synchronization was induced to investigate the different developmental stages where the protein of interest may be expressed.

Sorbitol treatment was performed as described by (Lambros and Vanderberg, 1979). Briefly, the culture flask, with >5% parasitaemia and predominantly ring forms, was centrifuged at 1500 rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended in a 15 mL Falcon tube with 5 mL of 5% (v/v) D-sorbitol in a shaking incubator at 37ºC for 10 minutes. Following centrifugation at 1500 rpm for 20 minutes, an equal volume of incomplete medium was added to the pellets which were washed thrice. Parasite culture was re-established by adding fresh uninfected erythrocytes and complete medium (5% haematocrit) to the washed pellets in a new culture flask, gassed and incubated as described earlier. Spent medium was changed on daily basis according to

guidelines by Jensen and Trager (1977). Giemsa-stained thin films were also monitored at various intervals to determine parasitaemia and stage-specific distribution of rings, trophozoites, schizonts, and gametocytes. A second sorbitol treatment was carried out after 48 hours when parasitaemia was about 10%.

To obtained highly enriched schizonts, Percoll gradient was carried out as previously described by (Rivadeneira *et al.*, 1983) and (Dluzewski *et al.*, 1984) with some modifications. This method concentrates schizonts from parasite cultures using a continuous medium of colloidal silica particles coated with polyvinylpyrrolidone (PVP) for density gradient centrifugation of cells, microorganisms, and subcellular particles. Briefly, parasite culture with >5% parasitaemia and predominantly schizonts was centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and the culture pellet was resuspended in 5.5 mL of complete medium. Ninety percent (90%) Percoll-alanine, 70% percoll and 40% percoll gradients were prepared in separate Falcon tubes. In a 15 mL Falcon tube, 3 mL of 90% Percoll-alanine was gently layered followed by 2 mL of 70% percoll and 2 mL of 40% percoll. The 5.5 mL of resuspended culture pellet was carefully layered on top of the percoll gradients. The Falcon tube was then centrifuged at 2400 rpm for 20 minutes (low acceleration and brake). Following centrifugation, the top layer of parasites (i.e the 40/70 interface) was aspirated into a 50 mL conical tube. Complete medium was slowly added to the 50 mL mark and washed thrice by centrifuging at 2000 rpm for 10 minutes. Giemsa-stained thin films were also examined to determine parasitaemia and intra-schizont stage. The supernatant was discarded and the schizonts

were resuspended in complete medium before putting the percoll purified schizonts back into culture.

3.5 *Invasion inhibition assay*

3.5.1 *Labelling of target RBCs and parasite DNA*

A fluorescence label/dye combination of carboxyfluorescein diacetate succinimidyl ester (CFDA SE) (Invitrogen, Paisley, UK) and Hoechst 33342 (Sigma-Aldrich, St. Louis, USA) was used for the respective staining of erythrocytes and parasite DNA. The RBCs were labelled to distinguish cells of interest (acceptor cells) from all other cells.

CFDA SE is a cell permeable dye which is cleaved by intracellular esterase to form carboxyfluorescein succinimidyl ester (CFSE), an amine-reactive product, which produces fluorescence in RBCs of the parasite culture when it binds irreversibly to lysine residues of intracellular and cell surface proteins. It has an approximately 517 nm fluorescence emission peak.

Hoechst is a bisbenzimide compound which binds the DNA of the parasite, fluorescing blue upon excitation. Its fluorescence is excited and emitted at approximately 350/461 nm respectively.

All staining procedures were carried out away from the direct source of light in a microbiological safety cabinet to maintain aseptic conditions.

Briefly, CFDA SE staining solution was prepared by dissolving 500 µg of 20 µM CFDA SE (lyophilized) in 180 µL of sterile dimethyl sulfoxide (DMSO) solution to form a 5 µM solution of CFDA. Fresh whole blood (erythrocytes) was obtained with the consent of voluntary blood donors and in accordance with established research protocols. O+ erythrocytes at 50% haematocrit were diluted to 4% haematocrit in RPMI 1640 (Sigma-Aldrich, St. Louis, USA) and centrifuged at 2000 rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended to 2% haematocrit with 20 µM CFDA SE in RPMI 1640 and incubated for 2 hours at 37°C under a shaking condition. At 2000 rpm for 3 minutes, the erythrocytes were pelleted and washed thrice with pre-warmed (37°C) incomplete medium. The cells were resuspended to 25% haematocrit in complete parasite medium and stored at 4°C for up to 24 hours.

3.5.2 *Invasion assay procedure*

To test the ability of the peptide-specific antibodies to block the parasites from invading erythrocytes, invasion inhibition assays were carried out. *P. falciparum* 3D7 strain parasites in the schizont stage (> 90%) were used to set up erythrocyte invasion assays. The parasitaemia of the percoll-purified schizonts was between 1-5%. Set up involved the seeding of the parasite culture and the CFDA SE-stained erythrocytes in a 1:1 ratio at 2% haematocrit into a 96-well titer plate. The experiment was conducted in duplicate wells. The plate was bagged, gassed (with 2% O_2, 5.5% CO_2, and 92.5% N_2) and incubated at 37ºC for approximately 20 hours. Following incubation, spent culture supernatant in each sample well was replaced and resuspended with 5 µM Hoechst 33342 to stain parasite DNA. This was done to differentiate parasitized erythrocytes from non-parasitized ones.

Flow cytometry was used to analyze erythrocyte fluorescence as a measure of antibody reduction of erythrocyte by parasites.

3.5.3 *Flow Cytometry and Data Analysis*

A 1:25 dilution was set up by adding 20 µL aliquots of resuspended parasite culture from each well to 500 µL of FACS Flow fluid (BD Biosciences) in FACS tubes. The tubes were vortexed gently. All experiments were conducted in duplicates. The stained samples were examined with a BD LSRFortessa^{TM} X-20 flow cytometer (BD Biosciences, Oxford, UK). Hoechst 33342 was excited by a UV laser and detected by a 450/50 BP filter. CFDA-SE was excited by a blue laser and detected by a 530/30 filter. BD FACSDiva 8.0.1 software (BD Biosciences, Oxford, UK) was used to collect 50,000 events for each sample. The erythrocyte population was selected by gating to distinguish cells based on properties such as forward scatter (size) and side scatter (granularity). Erythrocytes which were positive for both CFDA-SE and Hoechst 33342 dyes were determined with a dot plot and a fluorescence histogram on a logarithmic scale. The negative control value was subtracted from the test sample values, and the data were presented as the mean ± standard error of the mean (SEM). GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA) was used to plot invasion efficiency of treated and untreated erythrocytes as well as perform other statistical analyses.

3.5 *Indirect Immunofluorescence assay (IFA)*

To assess the subcellular localization of PF10_0351 protein across the schizont-stage and gametocyte-stage parasites, an indirect immunofluorescence assay (IFA) was performed

with culture mixed parasite stages or synchronized parasite culture of *P. falciparum* 3D7 strain and clinical isolate obtained from culturing procedure described earlier. To capture released merozoites or gametocytes, segmented schizonts or stressed parasites were mixed with erythrocytes, allowed to incubate for about 2 minutes before fixation for IFA procedures. Briefly, thin films of the cells were air-dried and then fixed with ice-cold absolute methanol for 5 minutes, and permeabilized in 0.1% (v/v) Triton X-100 in PBS for 10 minutes at room temperature. The slides were then blocked with 3% BSA in PBS-Tween for 2 hours at 4°C. Following blocking, the slides were washed thrice with PBS-Tween and allowed to dry. Circular marks were drawn at the backsides of the slides. Rabbit anti-PF10_0351 antibodies (GenScript, China) diluted in 1:250 with 1% BSA in PBS-Tween were applied to the surfaces of the slides (which backside were circled) and incubated in a humidified chamber for 1 hour at 37°C. The slides were washed thrice with PBS-Tween-20. A cocktail of 500 µL Alexa Fluor 488 goat anti-rabbit IgG (ThermoFisher Scientific) conjugated secondary antibody (diluted to 1:1000 with 1% BSA in PBS-Tween) and 1 µL of 0.1 ng/mL 4',6-Diamidino-2-Phenylindole (DAPI, Invitrogen) was applied to the surfaces of the slides and incubated for in a humidified chamber for 1 hour at 37°C. The DAPI was added to stain the nuclei of the parasites. The slides were washed thrice with PBS-Tween-20 and allowed to dry. These slides were thereon mounted in anti-fade reagent VectaShield (Vector Laboratories) and covered with coverslips and the edges sealed with nail polish. Preimmune rabbit antisera were used as negative controls. Rabbit antibodies raised to the merozoite surface marker (MSP1) and gametocyte surface marker (Pfs48/45) were used for co-staining schizont and gametocyte smears respectively. The slides were visualized under oil immersion using Olympus BX41 fluorescence microscope (Tokyo,

Japan) equipped with a trinocular C-mount camera (Beijing, China). Images were captured, processed and analyzed with ImageJ using the µManager software.

3.6 *Natural immunogenicity study*

To test whether the PF10_0351 peptides would react with natural antibodies in human plasma, ELISA was performed using 96-well microtiter plates (Nunc; ThermoFisher Scientific). In this experiment, preimmune rabbit sera and plasma from a malaria-naïve donor were used as negative controls. Pooled sera of malaria-positive individuals were used as positive controls, and normal human serum (NHS) was used for background subtraction. Briefly, ELISA plates were coated overnight at 4°C with 100 µL of 4 mg/mL PF10_0351 peptides using coating buffer. The plates were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). The plates were then blocked with 200 µL of 3% PBS-Tween-20 (blocking buffer) per well for 3 hours at 37°C and washed three times in PBS-Tween-20. Frozen plasma samples of malaria-positive individuals (n = 10 from each study site) were thawed and mixed gently. The test plasma (primary antibodies) were diluted to 1:640,000 with PBS after which 100 µL of the diluted plasma was added to duplicate sample wells, and incubated for 1 hour at 37°C. The plates were washed three times in PBS-Tween-20. ELISA was developed with goat anti-human IgG horseradish peroxidase-labelled rabbit antisera (1 mg/mL) as secondary antibodies. This was done by diluting the secondary antibodies to 1:10,000 with PBS, before dispensing 100 µL into each sample well, and incubating for 1 hour at 37°C. The plates were then washed three times in PBS-Tween-20 followed by two times washing in PBS only. About 100 µL of freshly prepared 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well for development. The plates were incubated at room temperature for 20 minutes in the dark.

The reaction was stopped by adding 25 µL of 0.2 M H_2SO_4 stopping solution per well. Absorbance was measured at 450 nm (OD_{450}) using the Varioskan^{TM} LUX multi-mode microplate reader (ThermoFisher Scientific). The NHS background values were subtracted from average values of the duplicate test readings as the final values which were then plotted and analyzed by *unpaired t-test* with GraphPad Prism 6.0 software. Absorbance readings above the mean OD value of malaria negative controls plus 2 SD for each response were taken as seropositive. This experiment was repeated twice, and statistically significant outcomes are presented.

3.7 *Data analysis*

The statistical analyses were performed using GraphPad Prism 6.0 software. The means and standard error of means were computed for continuous variables. For the invasion inhibition assay, the effect of peptide antibody on parasite invasion was expressed as a percentage of invasion efficiency. The levels of invasion efficiency were compared to those observed for NHS (Two-way analysis of variance with Tukey's multiple comparison test).

For the immunogenicity studies, the study participants were grouped according to their optical density, age and parasitaemia. The natural antibody responses to the peptides were expressed as optical densities, and levels (to different peptides, and from different sites) were compared using Kruskal-Wallis H test. Pairwise differences between groups were revealed using Dunn's or Tukey's multiple comparison test. Spearman's correlation analysis was used for association studies (antibody responses versus age, and antibody

responses versus parasitaemia). All values of $p < 0.05$ were considered statistically significant.

CHAPTER FOUR

4.0 **RESULTS**

4.1 *Selection of PF10_0351 protein*

The search tools utilized in PlasmoDB (www.plasmodb.org) identified PF10_0351 (PF3D7_1035900) as one of the probable *P. falciparum* proteins that may play a role in the invasion of erythrocytes. This is supported by transcriptional data from Bozdech *et al.* (2003) which suggest peak expression levels of the protein in 3D7 parasite strain around 40-hour post-invasion.

The protein consists of highly polymorphic amino acid sequences with the interspersed internal repetitive region (http://www.plasmodb.org). Located on chromosome 10 of the *P. falciparum* genome, the PF10_0351 gene is 1701 kb in length with an exon and a transcript (Figure 4.1). Characteristic of this gene are humilio homology domains (PUM-HD) and Armadillo-like alpha superhelices (http://www.uniprot.org/uniprot/). Armadillo-like repeats provide elaborate solvent-accessible surfaces for binding to proteins and nucleic acids. Located between 202 – 556 amino acid sequences and characterized by eight tandem copies of repeated 36 amino acids motif, the PUM-HD regulates translation and mRNA stability in many eukaryotes.

4.1.2 *Prediction of physical properties of the protein*

Expressed at the late stage of schizogony in the asexual blood stage, PF10_0351 consists of 566 amino acids with a predicted molecular mass of 65.2 kDa. The domains, repeats, and motifs within the protein were predicted by SMART Pfam outsourced from UniProt database (Figure 4.2). The protein sequence scans of PF10_0351 by SignalP4.0 (Petersen

et al., 2011), SignalP2.0 (Nielsen and Krogh, 1998) and TMHMM (Krogh *et al.*, 2001) algorithms indicate a signal peptide at its N-terminus (1 to 21 or 22 aa) but no transmembrane helices. The probabilities of the signal peptide and anchor region are 0.972 and 0.882 respectively. The cleavage site was predicted between amino acid positions 21 or 22 with a probability of 0.841 while the isoelectric point and charge are PI 3.9 and -163.0 respectively **(**www.genedb.org**).**

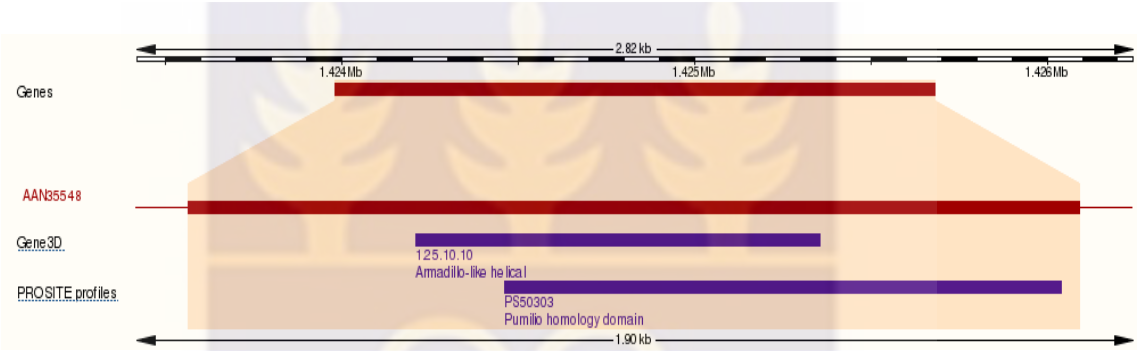


Figure 4.1: Schematic diagram of the gene encoding the PF10_0351 protein

The gene is 1701 kb in length and possesses repeats of Armidilo-like helical and humilio homology domains. (Source: PROSITE software)

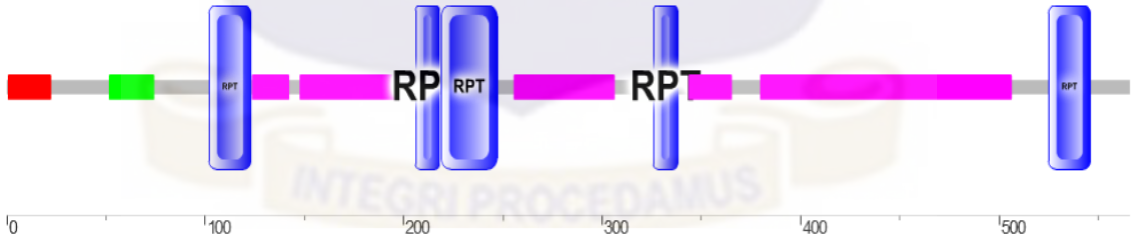


Figure 4.2: SMART diagram showing domains within PF10_0351 (*identifier*: Q8IJ49_PLAF7)

The predicted domains, repeats and motifs are colour-coded as signal peptide (red), unknown region (grey), coiled-coil region (green), internal repeats (blue) and regions of low complexity (pink). (Adapted from http://smart.embl-heidelberg.de)

4.1.3 *Orthologous PF10_0351 proteins in Plasmodium species*

The protein possesses homologues in *P. reichenowi* CDC and *P. gaboni* with conserved cysteine residues located near the N-terminus (Figure 4.3). The conserved protein sequences were predicted from PlasmoDB and the Conserved Domain Database (Marchler-Bauer *et al.*, 2011) and confirmed by NCBI BLAST tool. Alignments among the *Plasmodium* orthologs were deduced by ClustalW algorithms.

Figure 4.3: Sequence alignments of PF10_0351 gene in *Plasmodium* orthologues

The sequences of *P. falciparum* PF10_0351 protein which are homologous with *P. reichenowi* CDC and *P. gaboni* were retrieved from pairwise alignments using BLAST. The position of amino acid sequence similarity is shown in red colour. Cysteine residue aligns with all three homologs near the N-terminus.

4.1.4 *PF10_0351 protein interacts with other proteins in silico*

To predict the functional partners of the *P. falciparum* PF10_0351 protein, text mining and co-expression (score > 0.90) algorithms in STRING server (http://string-db.org/) were utilized to obtain the direct and indirect associations of PF10_0351 protein with some proteins with known 3D structure (Figure 4.4). Predicted functional partners include merozoite surface proteins (PF10_0352 and PF10_0347), conserved *Plasmodium* proteins (PF11_0277 and PF11_0193), glutamate-rich protein (PF10_0344), and erythrocyte binding antigen-181 (eba-181).

4.2 *Profiles of synthetic peptide and anti-PF10_0351 antibodies*

The peptides with >88% purity were synthesized and received in lyophilized form. The epitopes to peptides 2 and 3 were mapped towards the N-terminus while that of peptide 3 sequence was towards the C-terminus of the full-length protein. Mass spectrometry readouts clearly indicated the epitopes had high scores for peptide selection parameters such as antigenicity, surface, and hydrophilicity (Figure 4.5A). With 2 mg original concentration, each peptide had 14 amino acid sequences with a terminal cysteine residue tag to enable easy labelling or linking to resin for pull-down experiments. The LC/MS data for the peptides are shown in Figure 4.5B. Table 4 shows the peptide selection parameters and their sequences.

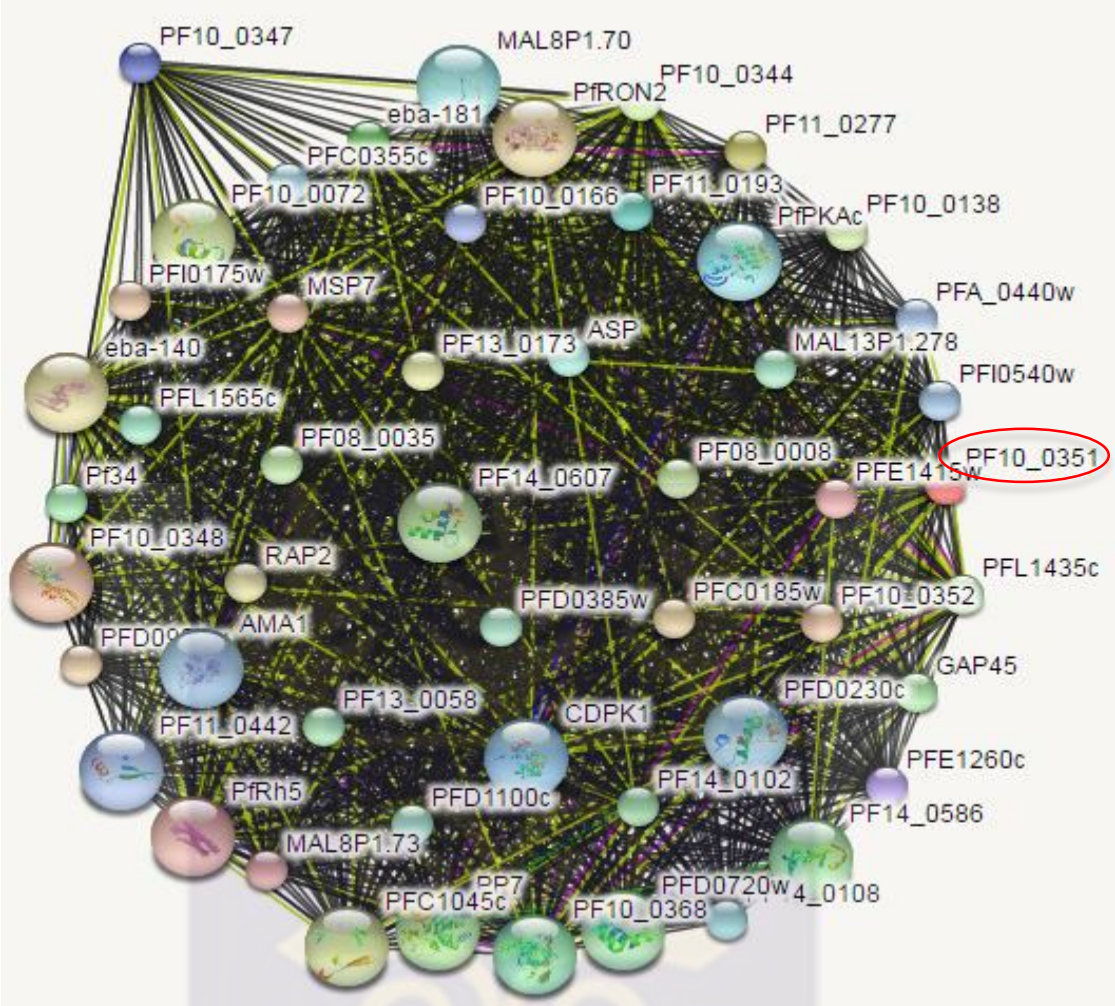


Figure 4.4: Predicted STRING functional partners of PF10_0351 protein

Evidence for direct or indirect protein-protein association is indicated by the different line (edge) colours. Network nodes represent proteins – smaller nodes represent proteins of the unknown structure while the large nodes have some known 3D structures. PF10_0351 appears to interact with merozoite surface proteins such as PF10_0352 and PF10_0347. The interactome also indicates indirect associations with asexual stage proteins such as PfRh5 and AMA-1 which are presently considered as targets for vaccine development. It interacts with Pf34 – a protein involved in the formation of the parasitophorous vacuole. (Source: https://string-db.org/)

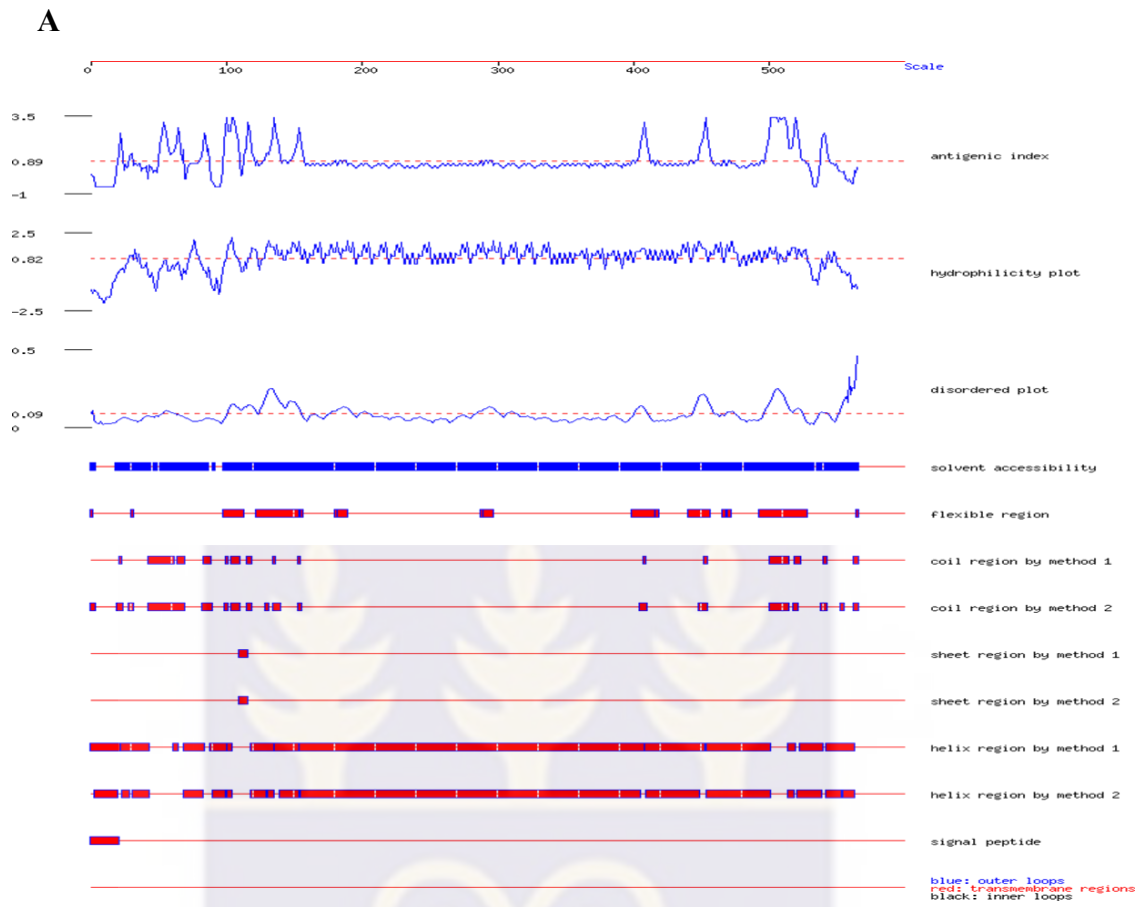


Figure 4.5: Chromatograms on peptide selection parameters and C18 HPLC analysis

(A) This figure is a chromatographic output of the peptide selection criteria. Peaks correspond to regions with high selection parameters on the full length of the protein (scale). Antigenic index, hydrophilicity and disordered plots are indicated in blue spikes while solvent accessibility is represented by blue bars. Other parameters are presented in red bars. (Source: GenScript Corporation, China)

B

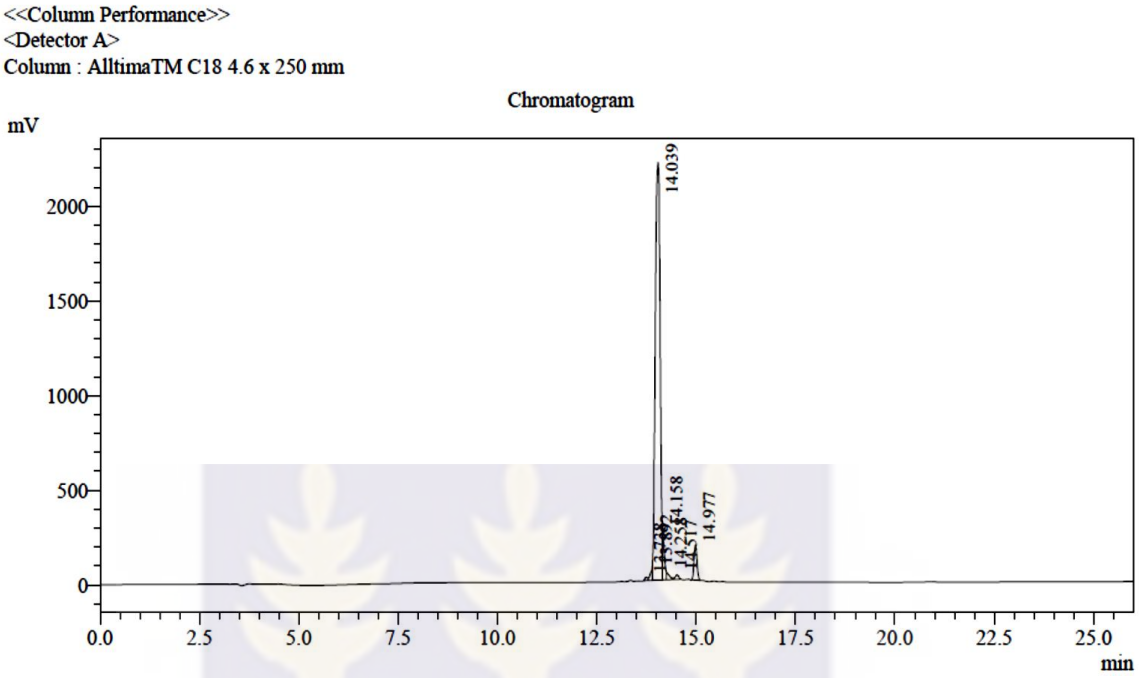


Figure 4.5 (continued): Chromatograms on peptide selection parameters and C18 HPLC analysis

(**B**) Representative chromatogram of PF10_0351 peptides obtained from C18 HPLC (LC/MS) analysis of the peptides showing absorbance (mV) on the vertical axis and retention time (min) on the horizontal axis. The peaks represent the peptides. (Source: GenScript Corporation, China)

Table 4: PF10_0351 peptide sequences and selection parameters

No	Start	Antigenic Determinant	Length	Antigenicity/Surface/Hydrophilicity	Disordered Score
1	501	CQGEKVNKNDLNDAS	14	3.21/0.93/1.05	0.2014
2	98	FTENKEQKNEEVPMC	14	2.41/0.79/1.10	0.1085
3	115	VVNDGEEVKTEYVSC	14	1.36/0.64/0.85	0.1242

4.2.1 *Optimal dilution of anti-PF10_0351 antibody*

Antibodies were generated against the peptides from the same target protein in order to increase the chance of endogenous protein detection. To determine the optimal dilution of the peptide-specific antibodies, serial dilutions of the antibodies were carried out (Figure 4.6). The antibodies were diluted from 1:1,000 to 1:512,000 in PBS. At 1 mg/mL concentration of the peptide-specific antibody, the titer is the highest dilution with Signal/Blank (S/B) ≥ 2.1. Preimmune rabbit sera were the negative controls (NC = 1:1,000).

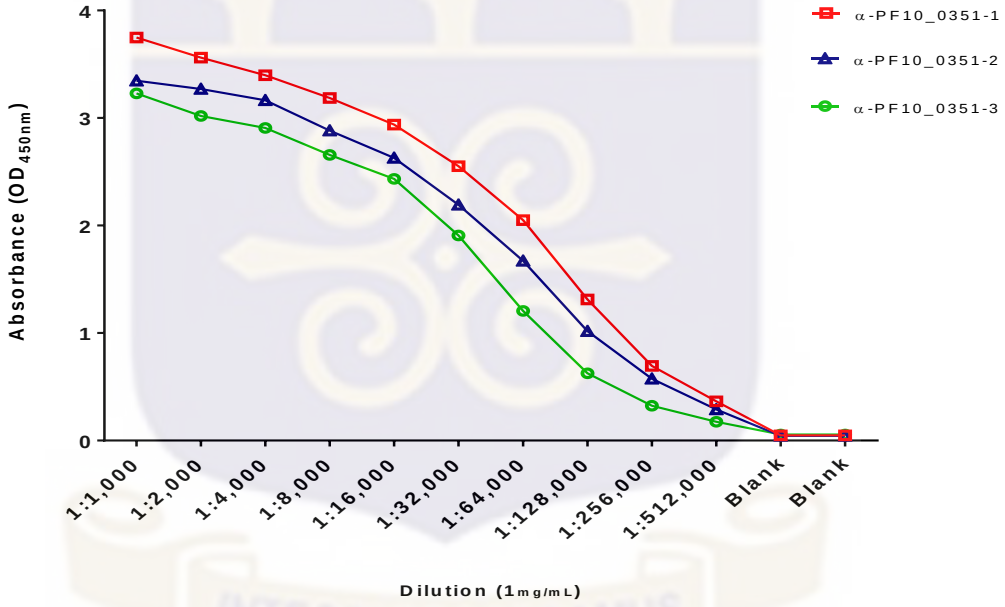


Figure 4.6: Dilutions of antibodies generated to the PF10_0351 peptides

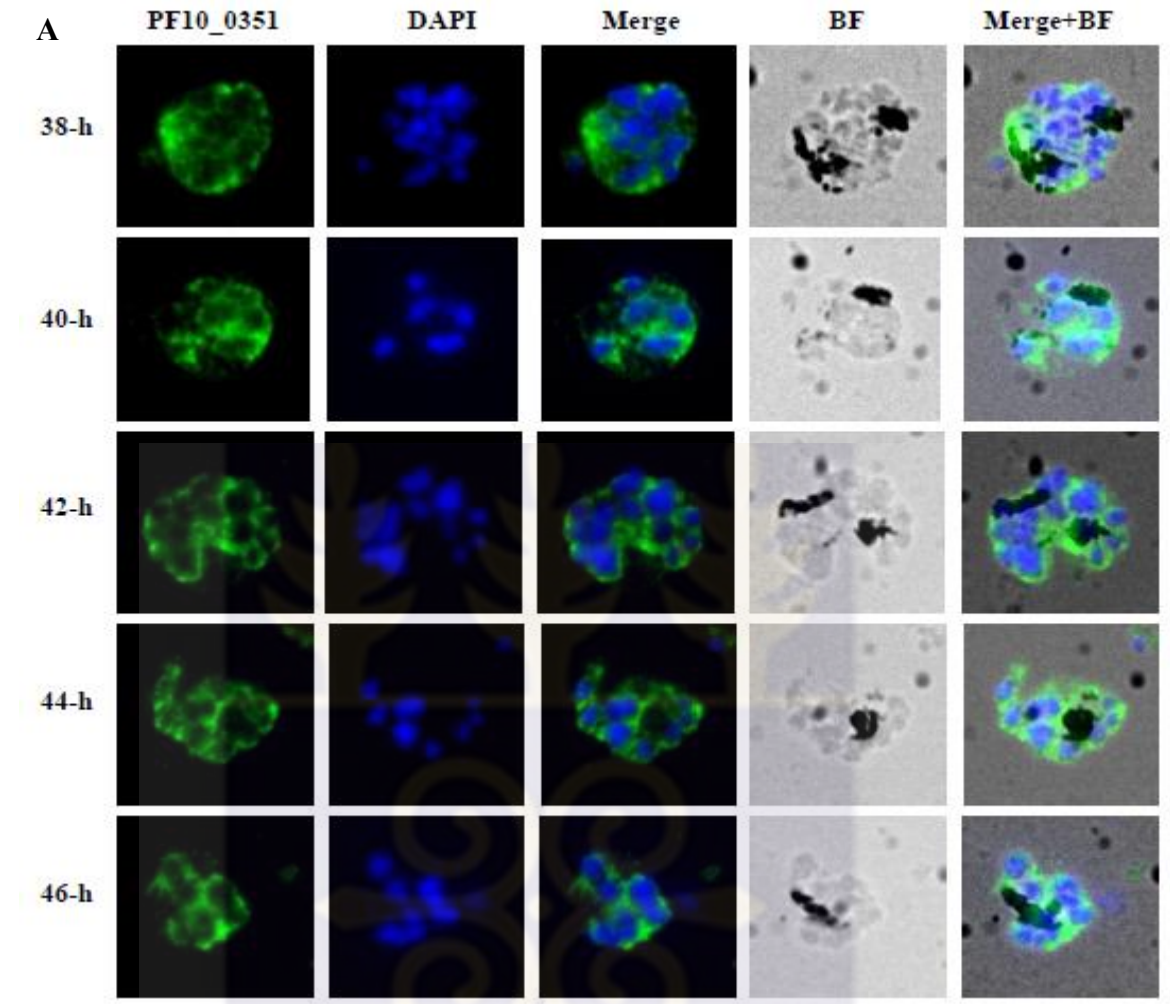
The dilutions of the peptide antibodies are represented by α-PF10_0351-1 (red squares), α-PF10_0351-2 (blue triangles) and α-PF10_0351-3 (green circles). The titer is the highest dilution with Signal/Blank (S/B) ≥ 2.1. Preimmune sera are the negative controls (NC = 1:1,000). The mean absorbance of the peptide-specific antibodies was: α-PF10_0351-1 = 0.073; α-PF10_0351-2 = 0.075; α-PF10_0351-3 = 0.101.

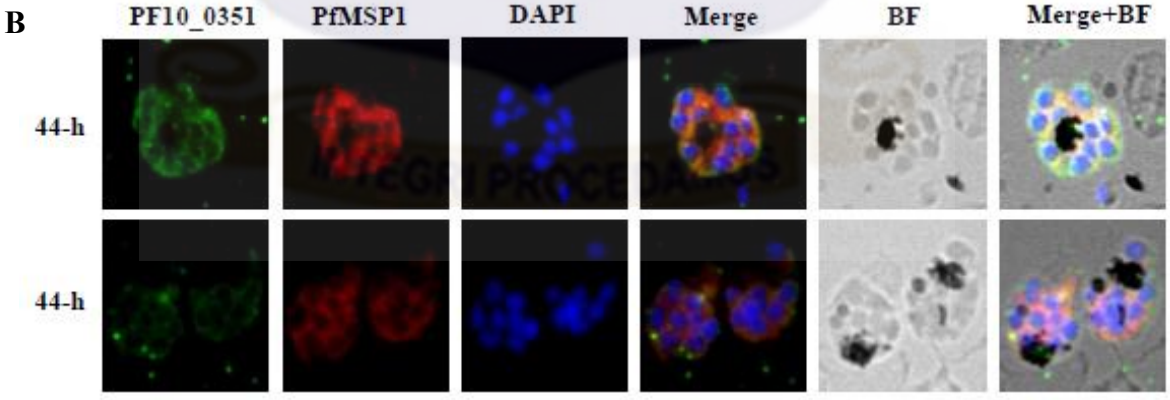
4.3.1 *PF10_0351 protein localizes to the surface of schizonts and merozoites*

Indirect IFA experiments were carried out on schizont stage parasites and released merozoites using the antibodies raised against the PF10_0351 peptides (Figure 4.7). Fluorescence was strongly observed on the surface of the schizonts and merozoites, indicating that the results were positive. This pattern of fluorescence can readily be seen in the segmented schizonts in panel (A) which shows time points from 38-h to 46-h post-invasion. The merger of PF10_0351 and DAPI also shows distinct staining of both peripheral surface and nuclei respectively.

To confirm the localization of PF10_0351 protein, antibody to the merozoite surface marker, PfMSP1, was used to co-stain the PF10_0351 protein. As expected, the PfMSP1 partially colocalized with the PF10_0351 protein on the surface of schizonts (Figure 4.7B). Although a yellow spectrum was expected from the merger of the green and red fluorescence, some faint traces of red could still be observed. The inability of neighbouring cells to pick the stain reinforces the specificity of the staining. There appears, however, to be a weak fluorescence pattern in the second 44-h image which subsequently impacts on the outlook of the Merger+BF in panel (B).

Noticeably, the PF10_0351 protein appears to be localized near or around the nuclei of three separate released merozoites (see arrows in Figure 4.7C). This observation may give an indication that the FP10_0351 protein may temporarily reside within the ER which is also known to surround the nucleus.





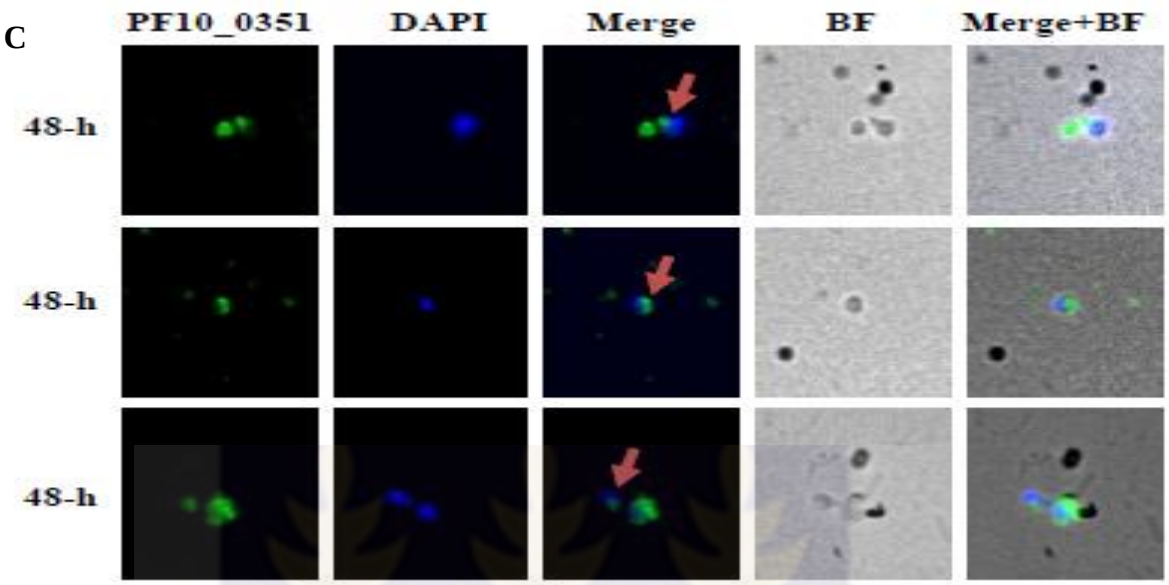


Figure 4.7: **Localization of PF10_0351 protein in late schizonts and released merozoites of *P. falciparum* 3D7**

Indirect IFA was used to detect the presence of endogenous PF10_0351 protein in thin smears of synchronized parasite culture of (A) early to near late schizonts as shown for the 38 to 46-h time points. This was confirmed by the partial colocalization with merozoite surface marker (PfMSP1) shown in panel (B). In panel (C), PF10_0351 appears to localize to the surface of free merozoites as shown for the 48-h time point. Then smears were fixed; incubated with anti-PF10_0351 rabbit antisera (primary antibody) and Alexa Fluor 488 secondary antibody; and observed by fluorescence microscopy. The protein is shown in green with the parasite nuclei stained with DAPI (blue). Arrow points to the proximity of detected protein to the nuclei. The bright field (BF) shows black spot representing hemozoin crystals. The merged images show a pattern of fluorescence probably corresponding to surface staining.

4.3.2 *Co-localization of PF10_0351 in gametocytes of 3D7 strain and clinical isolate*

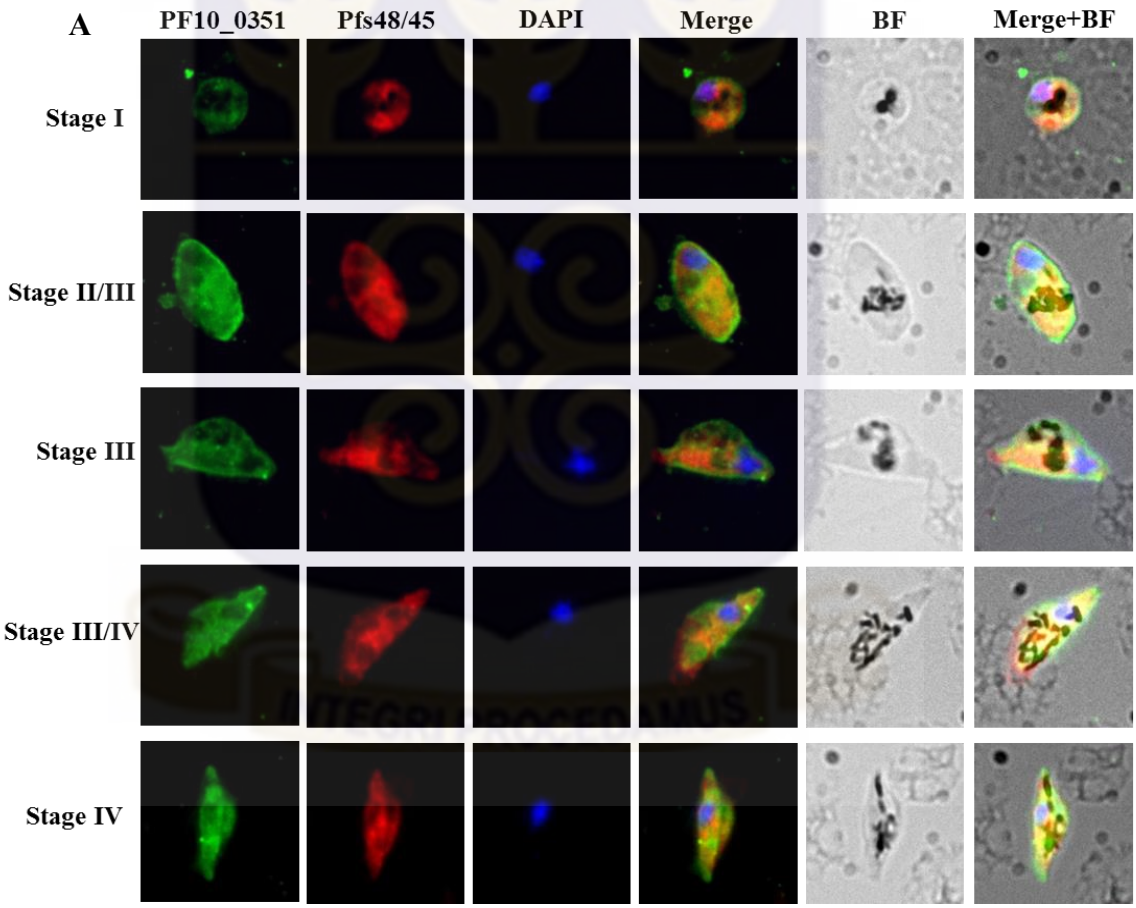
The preliminary observation that the PF10_0351 gene product may be resident in the ER, and by extension, may be expressed throughout the life cycle of the parasite, led to the labelling of synchronized gametocyte stage-specific smears with the peptide antibody to detect the presence of the protein in 3D7 clone by indirect IFA. The synchronized gametocyte cultures were therefore treated with 50 mg/mL N-acetylglucosamine for 48 hours to enhance development (Gupta *et al.*, 1985).

Immunofluorescence analyses of the gametocyte stage clearly showed positive results for the staining. The localization of PF10_0351 to the surfaces on the 3D7 gametocytes was confirmed by co-staining with antibodies to the gametocyte surface marker, Pfs48/45 (Figure 4.8A). It can be observed that the fluorescence patterns vary as the various stages of gametocytogenesis progressed. The stage I gametocyte showed weak fluorescence compared to stages II - V gametocytes. Moreover, the Merger+BF image in the stage I gametocyte does not show distinct yellow spectrum as compared to the other stages.

To determine whether the PF10_0351 protein can be detected in natural infections, additional co-immunofluorescence assays were performed with clinical parasite isolates obtained from a human patient diagnosed with *P. falciparum* malaria. As observed earlier, Pfs48/45 co-localized with the PF10_0351 protein to the peripheral membrane on gametocytes (Figure 4.8B). Again, the stage II gametocyte showed stronger fluorescence than the stage I gametocyte. However, the expected yellow spectrum from Merger+BF

appears to diminish as the gametocytogenesis progressed through stage II gametocyte to stage V.

In both 3D7 and clinical isolates, the morphological characteristics of the gametocyte stages are clearly shown. The stage I gametocyte is round-shaped, and indistinguishable from trophozoite, whereas the stages II – V gametocytes take crescent forms.



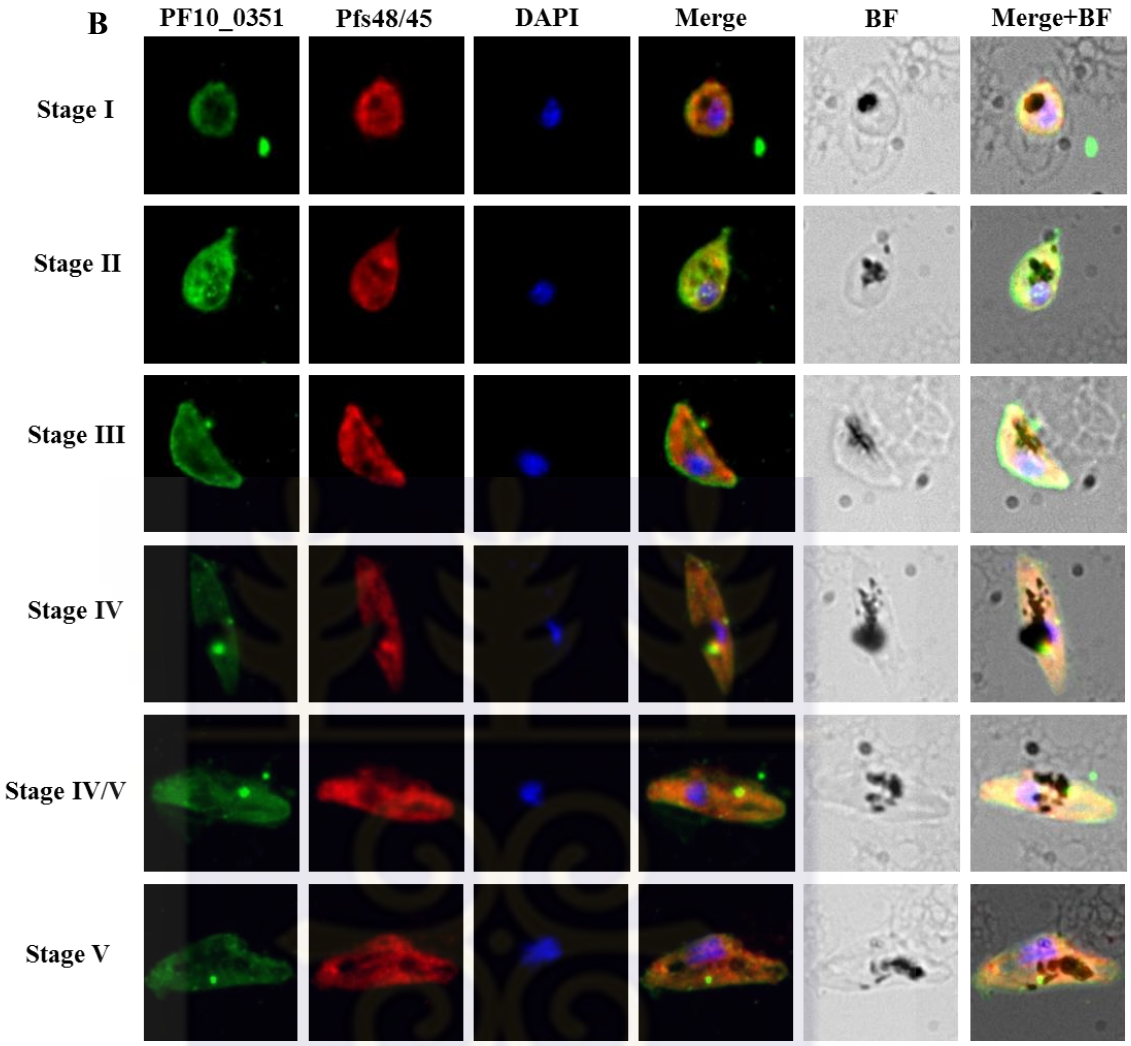


Figure 4.8: *P. falciparum* PF10_0351 protein co-localizes with Pfs48/45 to the peripheral surface in gametocytes

Immunofluorescence microscopy (IF) detected the presence of endogenous PF10_0351 protein in thin smears of synchronized asexual gametocyte stages in parasite culture. IF analysis suggests surface co-staining in gametocytes of (A) 3D7 laboratory strain and (B) clinical isolate. All the five stages of gametocytes were positive for the antisera albeit low fluorescent intensity can be observed in the round-shaped stage I gametocytes. PF10_0351 is detected by secondary antibody goat anti-rabbit IgG conjugated with Alexa Fluor 488 (green) and the parasite nuclei by DAPI (blue). The bright field (BF) shows black spots indicating haemozoin crystals. The merged images show a pattern of fluorescence probably corresponding to surface staining.

4.4 *PF10_0351 peptide-specific antibodies inhibit erythrocyte invasion*

To evaluate the ability of the peptide antibodies to block parasites from invading erythrocytes, invasion inhibition assay was performed on *P. falciparum* 3D7 parasite culture. A dose-dependency experiment was performed for each anti-PF10_0351 antibody. Erythrocytes were treated with increasing concentrations (31.25, 62.5 125, 250, and 500 µg/mL) of the antibodies on 1% parasitaemia. The ability of the antibodies to inhibit erythrocyte invasion of the parasites was expressed as percentage invasion efficiency.

Results obtained from the FACS analyses showed that there was a reduction in parasitaemia in treated cells (Figure 4.9). The assay revealed that all three antibodies generated against the peptides blocked invasion in a dose-dependent manner (Figure 4.10). Peptide-specific antibody invasion inhibitory effect was most effective at 500 µg/mL concentration. At 1 mg/mL concentration of anti-PF10_0351 antibodies (1, 2 and 3), parasite invasion efficiency was reduced by 42%, 53%, and 90% respectively, compared to the normal human sera (NHS) which showed parasite levels as an untreated control.

When compared with the NHS control, each peptide antibody showed a significant difference in their invasion-inhibitory effect (Tukey's multiple comparison test, $p<0.0001$). The same statistical significance was observed when the invasion inhibitory effect of each peptide antibody was compared with another.

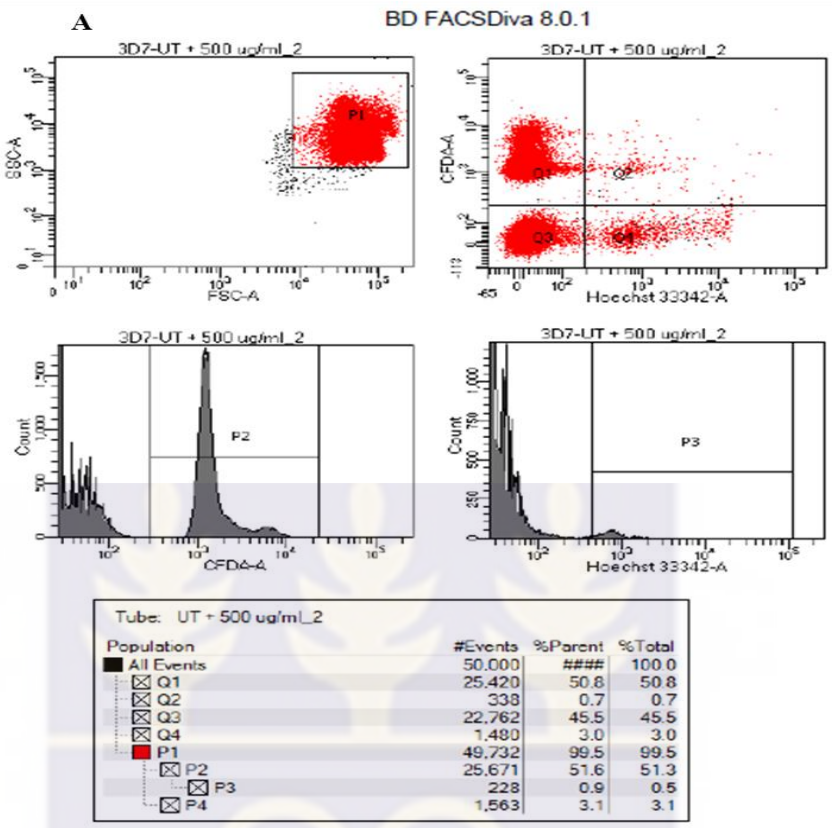


Figure 4.9: Representative flow cytometer plots of invasion inhibition assay

The plots show parasite densities in cell populations of treated and untreated erythrocytes. CFDA SE and Hoechst 33342 dyes were used to stain erythrocytes and parasite nuclei respectively. Cells were treated with varying concentrations of peptide antibodies. Controls included pre-immune rabbit antisera and NHS. Specifically, the panel represents an inhibition of erythrocyte invasion by peptide 3 antibody at a concentration of 500 µg/mL. FACS plot of infected RBCs shows that at this concentration, anti-PF10_0351-3 peptide antibody reduced *P. falciparum* 3D7 parasitaemia in treated cells to 0.9 compared with 3.3 parasitaemia in untreated erythrocyte control experiment (not shown here).

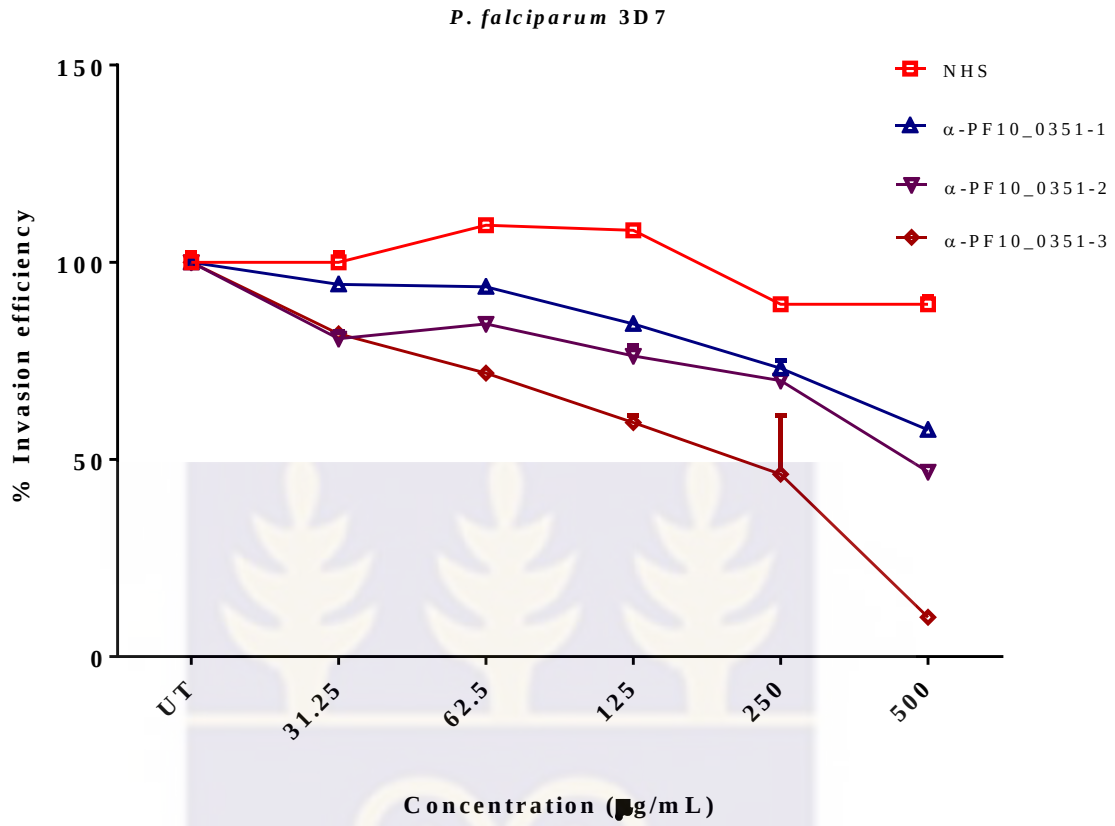


Figure 4.10: Testing the invasion inhibitory property of anti-PF10_0351 antibodies in *P. falciparum* 3D7 parasite culture

Invasion inhibition assays were set up in duplicate in a 96-well plate to assess the efficacy of anti-PF10_0351 antibodies in blocking merozoites from invading new erythrocytes. RBCs and parasite nuclei were labelled with CFDA-SE and Hoechst 33342 stains respectively. Data (including parasitaemia) was acquired and analyzed by Flow cytometer BD FACSDiva 8.0.1. The graph is presented as a percentage mean of invasion efficiency relative to untreated erythrocytes (rabbit pre-immune sera or NHS). All three anti-PF10_0351 antibodies inhibited parasite invasion of erythrocyte in a dose-dependent manner at a concentration of 1 mg/mL. The invasion was efficient at lower concentrations of the peptide-specific antibodies. This efficiency was statistically significant by Tukey's multiple comparison test ($p<0.0001$). Error bars represent SEMs. NHS is represented by red squares; α-PF10_0351-1 blue triangle; α-PF10_0351-2 by violet inverted triangle and α-PF10_0351-3 by brown squares.

4.5 *Natural antibodies in human plasma recognize PF10_0351 peptides*

To determine whether the chemically synthesized PF10_0351 peptides will react with natural antibodies present in human plasma, an indirect ELISA was performed. The results show that the peptides were recognized by the human plasma (Figure 4.11). Plasma antibody responses to the peptides were expressed as optical densities. Generally, the median optical densities were highest in plasma samples from Kintampo. Plasma recognition of the peptides appears to increase from Accra through Navrongo to Kintampo. This trend was observed for all the three peptides.

Peptides 1 and 2 showed a significant difference in their reactivity to the natural antibodies in the human plasma (Dunn's multiple comparison test, $p<0.0001$) (Figure 4.11A). This trend was also observed between peptides 1 and 3. However, the level of plasma recognition between peptides 2 and 3 was not statistically significant.

4.6 *Age and parasite density do not significantly affect PF10_0351 peptide recognition*

To assess the effect of age and parasitaemia on the ability of human plasma to recognize the PF10_0351 peptides, a Spearman's correlation was conducted to detect the association between peptide recognition by plasma antibodies and age or parasite density (see Table A5 in Appendix). No significant association was observed between age and plasma antibody responses to all the three peptides (Figure 4.12A). Similarly, no significant association was observed between parasitaemia and the plasma recognition of the peptides (Figure 4.12B).

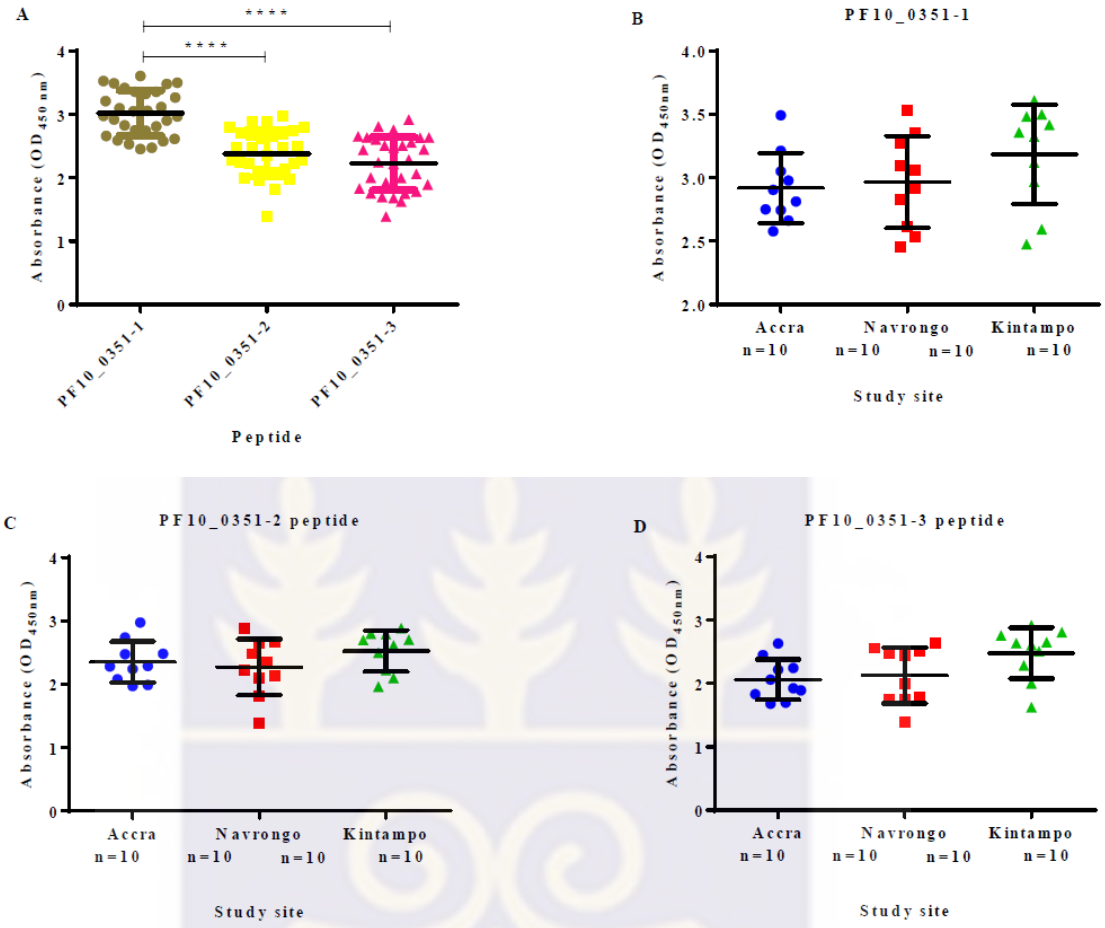


Figure 4.11: PF10_0351 peptide recognition by natural human plasma

Panel **A** compares plasma recognition among the peptides 1 (hazelnut), 2 (yellow) and 3 (hot pink). No significant difference was observed in the reactivity of peptides 2 and 3 with plasma from the three study sites. In panels, **B**, **C** and **D**, peptides 1, 2 and 3 did not show significant differences in their reactivity to plasma from the study sites: Accra (blue), Navrongo (red), and Kintampo (green). An across group was done using Kruskal-Wallis test, ****** *p*<0.0001** with Dunn's multiple comparison test, ****** *p*<0.0001**.

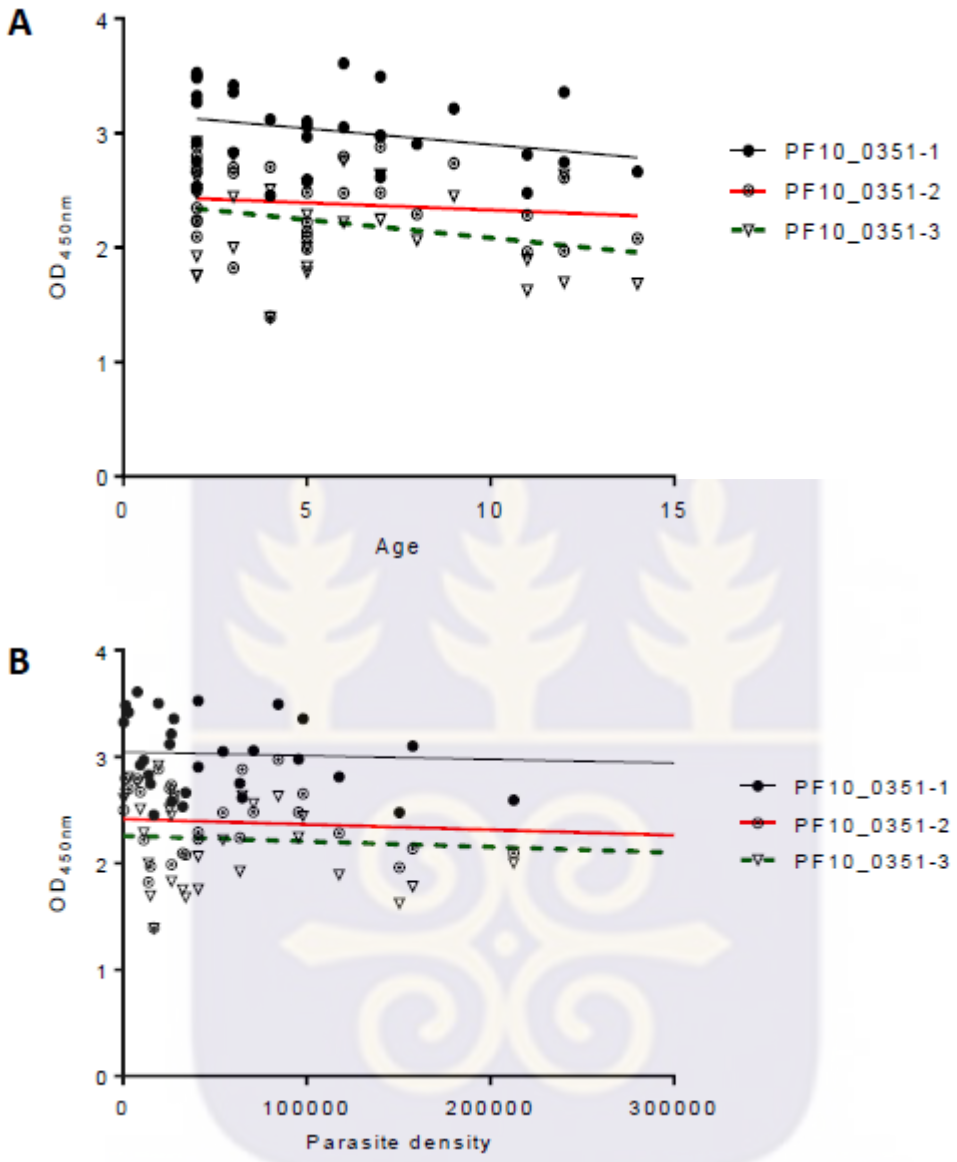


Figure 4.12: **Correlation of natural human plasma titers with age and parasite density across the four endemic areas of Ghana**. The reaction of PF10_0351-1 (closed circles), PF10_0351-2 (dotted circles) and PF10_0351-3 (open triangles) with natural antibodies in human plasma was correlated with the (**A**) ages and (**B**) parasite density of study participants. The black line, red line and green dash lines represent the linear regression of peptides 1, 2 and 3 respectively. Each peptide was analyzed in relation to 30 plasma samples from all three study sites. When compared across the three study sites, there was no significant correlation with age and parasite density by Spearman's correlation test.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 *Discussion*

Merozoite surface proteins remain attractive research targets for interventions against malaria owing to their ability to interact directly or in a complex with the human red blood cells for invasion. More importantly, studies have shown that these proteins are capable of inducing an immune response against malaria (Hisaeda *et al.*, 2002; Oeuvray *et al.*, 1994). However, there appears to be a repertoire of uncharacterized invasion-related proteins which need to be studied to improve our knowledge of the biology of the *P. falciparum* parasite and aetiology of malaria. This study sought to characterize and validate the functional role of one of the probable proteins (PF10_0351) predicted to play a role in erythrocyte invasion.

The gene product of PF10_0351 is a probable invasion-related protein classified as a peripheral surface antigen with similar ORF with MSP3 albeit it does not contain alanine-rich heptad repeats (Pearce *et al.*, 2005). Rather, the gene encoding the PF10_0351 protein contains Armadillo-like helices and pumilio homology domains for binding on surfaces. Both MSP3 and PF10_0351 genes are located on chromosome 10 and share some common sequence homology. In addition, both have predicted signal peptide and the N-terminus but lack a transmembrane domain. Also, proteomic and transcriptional data on PF10_0351 protein suggest that it is highly expressed at late schizont (Bozdech *et al.*, 2003).

These claims were established by carrying out immunolocalization studies on late schizonts and released merozoites. IFA clearly showed that the protein is localized to the peripheral surfaces of schizonts and merozoites. Co-staining with the merozoite surface marker, PfMSP1, also showed a partial colocalization to the peripheral surface of schizonts, however, additional work may have to be carried out to confirm this observation. Deloron *et al.* (2013) have also reported surface localization of the PF10_0351 gene product with confocal microscopy and IFA. This observation is further highlighted by studies by Pearce *et al.* (2005) which indicated that the protein does not localize to the rhoptry.

An important observation from this present IFA experiment is that the PF10_0351 protein appears to reside near or around the nucleus. Since the ER surrounds the nucleus, and secretory proteins first transit the ER (Foth *et al.*, 2003), it is suggestive that the biosynthesized PF10_0351 protein may first reside within or around the ER before part of it is trafficked through the Golgi apparatus to the peripheral surface of the merozoite. Of note, the ER fundamentally persists throughout the life cycle of the *Plasmodium* parasite.

This preliminary observation that the PF10_0351 gene product may first be resident in the ER, and by extension, may be expressed throughout the life cycle of the parasite, led to the labelling of synchronized gametocyte stage-specific smears with the peptide antibody to detect the presence of the protein in 3D7 clone by IFA. It has already been established that *Plasmodium* NF54 and 3D7 clones are reliably adapted to gametocyte formation *in vitro* although they may lose their ability to produce gametocytes over time (Ponnudurai *et al.*, 1986; Ponnudurai *et al.*, 1982).

The round-shape stage I gametocyte seem to show low fluorescent intensity compared with the fluorescence observed in stages II-V of crescent-shaped gametocytes. This difference in fluorescence intensity may be biological or artefact. For the latter reason, the fixing or permeabilization steps of IFA could have altered cellular architecture such that artefacts could be interpreted as false positive signals. Of note, indirect IFA is subject to high variability for fluorescence intensity (Dahle *et al.*, 2004). Photobleaching – the substantial decrease in fluorescence emission with time – of intracellular fluorophores could also lead to artefacts (Diaspro *et al.*, 2005; Wustner *et al.*, 2014). Again, dark and light bands of artefacts or stripes of intensity could alternate in the axial or vertical direction of biological imaging by fluorescence microscopy, irrespective of the methods of deconvolution used (Markham and Conchello, 2001). Biologically, the difference in intensity may be due to delayed initiation of PF10_0351 protein biosynthesis at the asexual-sexual transition or during early gametocytes, as earlier suggested by Lanfrancotti *et al.* (2007). Moreover, the trend could be attributable to an upregulation of the gene encoding the PF10_351 protein. This thinking is supported by studies by Silvestrini *et al.* (2000); Silvestrini *et al.* (2005); Young *et al.* (2005); and Eksi *et al.* (2005) which have suggested that there may be limited upregulation of some genes during early gametogenesis than in later stages. It can further be reasoned that the increased fluorescent intensity may be linked to the requirement of the protein for events or mechanisms leading to gamete specialization, considering that the PF10_0351 protein showed a similar pattern of staining with Pfs48/45. The *pfs48/45* gene has been implicated as a determinant in male gamete fertility in *P. falciparum* and *P. berghei* (van Dijk *et al.*, 2001). In a complex with Pfs230, Pfs48/45 directly associates with gametocyte plasma membrane by GPI anchorage (Alano, 1991). The importance of this

gametocyte surface marker has also been realized in a study where monoclonal antibody raised against the Pfs48/45 protein was able to inhibit transmission (Targett *et al.*, 1990). Findings from the immunolocalization experiment suggest that the PF10_0351 protein is expressed in schizonts, merozoites, and gametocytes of the *P. falciparum* parasite, and that it may be localized to the surfaces where it associates with other proteins for erythrocyte surface attachment as has been earlier reported by (McColl *et al.*, 1994; Oeuvray *et al.*, 1994) in studies involving other merozoite surface proteins.

The peptide-specific antibodies used in the invasion inhibition assay showed a dose-dependent inhibitory activity. This trend of effectiveness at higher concentrations (250-500 µg/mL) of the peptide-specific antibodies is corroborated by earlier works on clinical isolates (Persson *et al.*, 2008) which suggested that higher antibody levels are required for neutralizing parasites. In the absence of continuous physiological systems, static cultures may play little role in aiding antibody mobility for parasite neutralization.

Though erythrocyte invasion by released merozoites is thought to be a brief process allowing for parasite escape from immune recognition (Gilson and Crabb, 2009; O'Donnell and Blackman, 2005), the peptide antibodies were effective in reducing parasite invasion of the erythrocyte. One reason for the incomplete inhibition of merozoite entry and growth in the treated cells could be that the invading merozoites may have switched to an alternative invasion pathway to avoid the inhibitory effects of the antibodies. Findings from the invasion inhibition assay suggest that FP10_0351 peptide antibodies were effective in

inhibiting merozoites from invading erythrocytes *in vitro* and that they may be useful in designing intervention strategies against *P. falciparum* malaria.

Numerous studies have characterized the importance of merozoite proteins, such as MSP, EBL and RfRH, for erythrocyte invasion (Jaskiewicz *et al.*, 2010; Malpede and Tolia, 2014; Tham *et al.*, 2012) but the exact parasite protein or cocktail of proteins which could be most important targets of naturally acquired clinical immunity remains unclear. Resolving this challenge may need the evaluation of the immunogenicity of other parasite proteins predicted to play a role in invasion. An indirect ELISA was, therefore, performed to determine whether PF01_0351 peptides would react with natural antibodies present in human plasma. The results show that the peptides were recognized by the human plasma, consistent with a similar immunological characterization involving adult participants from malaria-endemic areas in Ghana where recombinant proteins of the PF10_0351 gene was recognized in sera by ELISA (Deloron *et al.*, 2013). The surface location of these peptide epitopes, as observed in IFA experiments, may have reasonably offered better accessibility to antibodies in the human plasma for easy recognition. This finding also complements the results obtained from invasion inhibition assay where peptide 2 and 3 antibodies were much more effective in inhibiting parasite invasion of erythrocyte than peptide 1 antibody. Some other studies on natural antibody response against merozoite antigens such as EBA-140 ligand, carried out in individuals living in malaria-endemic areas, also observed positive outcomes (Ford *et al.*, 2007; 2013; Richards *et al.*, 2010; Stanisic *et al.*, 2015; Stanisic and Good, 2016). Notably, both peptides 2 and 3 were mapped towards the N-terminus where glutamic acid motifs may enhance the ability of this region to associate with positively

charged interacting partners. These regions may also have high surface exposure and contain B-cell epitopes akin to hydrophilic regions in endogenous proteins. This may have probably enhanced the easy recognition of peptides 2 and 3 by the naturally-induced antibodies in the human plasma. Interestingly, a similar model of acidic amino acid residues at the N-terminus of MSP-3, -6, -7 and -9 has been proposed by others (Li *et al.*, 2004; McBride and Heidrich, 1987).

The differences in peptide recognition by natural human plasma from the various study sites were also analyzed. These differences were expected because the transmission intensity of Navrongo (hyperendemic) is between the levels in Accra (hypoendemic) and Kintampo (holoendemic). Mensah-Brown *et al.* (2015) have earlier reported that transmission intensity in Accra, Navrongo and Kintampo affects the progression of malaria in the four study sites.

The ages and parasite densities of the study participants did not influence the ability of their plasma to recognize the peptides. This trend was expected because although immunity to *P. falciparum* malaria may develop gradually in children residing in endemic areas, older children could be asymptomatic for parasitaemia. Additionally, frequent exposure may be required to maintain immunity to clinical malaria because sterile immunity is unattainable (Pavli and Maltezou, 2010). It has also been established that parasite density is not a determinant of malaria severity, implying that the quantum of immune effectors likely to be present in the human plasma does not necessarily correspond with the level of parasitaemia.

It has also been suggested that antibodies may establish clinical immunity by diminishing blood-stage parasitaemia in order to prevent severe outcomes of the infection (Bloland *et al.*, 1999; Marsh and Kinyanjui, 2006). However, from this study, there was no strong association between parasite densities and natural human plasma recognition of the peptides. It is likely other immune effectors may be acting together with the neutralizing antibodies. Oeuvray *et al.* (1994) have demonstrated that anti-MSP3 antibodies are capable of reducing *Plasmodium* parasite load by inducing ADCI effects, mediated by blood monocytes. Additionally, immune effectors, such as interferon gamma, could be interacting with B-cells (Udhayakumar *et al.*, 1995) to bring about protection against malaria infections. The longevity of these effectors in natural human plasma was, however, not readily estimated from this study.

The advantage of using multiple peptide antigens from a single target protein in immunogenicity studies does not only increase the success rate in detection of endogenous protein, it also offsets the difficulty in the synthesis of whole protein as well as avoiding the occurrence of cross-reactivity in studies which use full-length of the protein of interest for assaying. Any intent to consider the PF10_0351 gene product for inclusion in a multi-component blood-stage vaccine design may have to take into consideration a combination with other equally potent merozoite proteins that could elicit highly effective antibodies with a much-enhanced erythrocyte invasion inhibitory effect (Healer *et al.*, 2013; Pandey *et al.*, 2013; Richards *et al.*, 2013). Findings from the ELISA experiment, therefore, suggest that the peptides 2 and 3 appear to be highly immunogenic and antigenic as indicated by

their recognition by natural antibodies in the plasma of patients diagnosed with malaria. This reaffirms the fact that PF10_0351 protein can be detected in natural infections, and as a result, *P. falciparum* merozoite antigens are potential targets for human immunity.

5.2 *Conclusion*

Three major conclusions may be drawn from this work. First, PF10_0351 synthetic peptides could be recognized by natural plasma from malaria patients residing in malaria-endemic areas with different transmission intensities. The notable plasma recognition of peptide 2 and 3 epitopes which were mapped towards the acidic regions of the N-terminus of the full-length protein suggests that further characterization of these epitopes may provide insight into the natural protective immunity elicited from this antigen.

Second, preliminary flow cytometric data from the invasion inhibition assay suggest that all three peptide antibodies effectively reduced invasion efficiency in a dose-dependent manner at higher concentrations (µg/mL).

Finally, the analyses of immunofluorescence images appear to localize the PF10_0351 protein to the surfaces of schizonts, merozoites, and gametocytes. Of note, the detection of the protein in all five stages of the sexual stage gametocyte suggests that PF10_0351 may be associated with gamete specialization in *P. falciparum*.

Taken together, these findings provide support for further characterization of PF10_0351 protein in order to understand its role in *P. falciparum* parasite's life cycle and plausible antigen target for vaccine prospects.

5.3 *Recommendation*

In spite of the data presented in this work, further studies need to be carried out to evaluate the essentiality of PF10_0351 in other developmental stages of the parasite's life cycle. This may help elucidate the functional interactions of PF10_0351 at different stages in defining the biological function of the gene.

Also, for the natural immunogenicity aspect of this work, a cross-sectional study with a larger plasma sample size should be conducted to examine longitudinal antibody responses to the peptides.

A combination of anti-PF10_0351 antibodies and other peptide-specific antibodies should be considered in a further study to establish an increased antibody inhibitory effect against *P. falciparum* parasite invasion.

Lastly, an assessment of antibody responses to the full length, correctly folded PF10_0351 protein may give suggestions to the possibility of antibodies to some additional conformational epitopes.

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APPENDIX

A) Materials

- Flat–bottomed 96 well plate
- Latex gloves
- Alcohol swabs
- 70% Ethanol
- Permanent markers
- Sample rack
- Sterile Falcon tubes (15mL and 50mL)
- Sterile Needles
- L-Alanine
- FACS Flow Fluid
- FACS tubes

B) Preparation of Working Solution

i. Albumax

- 2 L RPMI 1640
- 100 g Albumax II
- 400 mg Hypoxanthine

ii. Parasite Wash Medium (PWM)

- 500 mL RPMI 1640
- 5 mL Gentamicin (10 mg/mL)
- 50 µL L-Glutamine (200 mM)

iii. Complete Parasite Medium (CPM)

- 500 mL RPMI 1640
- 5 mL Gentamicin (10 mg/mL)
- 50 µL L-Glutamine (200 mM)
- 50 mL Albumax

- 10 mL Normal Human Serum (NHS) for field isolates only.

iv. 90% Percoll-Alanine by sterile filtering

- 225 mL of Percoll
- 7.33 g of L-Alanine
- 25 mL of 10X RPMI 1640 medium

v. X mL of 70% Percoll by mixing

- (7/9)* X of 90% percoll
- (2/9)*X of 1X RPMI

vi. Y mL of 40% Percoll by mixing

- (4/9)*X of 90% Percoll
- (5/9)*X of 1X RPMI.

vii. 5% Sorbitol

- Weigh 2.5 g of D-sorbitol into a 50 mL Falcon tube
- Add 50 mL PBS and mix thoroughly
- Sterile-filter and store at 4 ºC

viii. 80 mg/mL $NaHCO_3$

- Measure 500 mL of dH_2O into a receiving bottle
- Weigh 40 g of $NaHCO_3$ powder and dissolve in the dH_2O
- Sterile-filter and store at 4 ºC

C) Sequences of PF10_0351

Predicted Protein Sequence

566 aa

MLNIFNIIFLLFLINIYICEANGTLSENIESAEEIDALKTNLRNGYLNNTYFNEENNNLNIENEINNTNYNEVTEETKE
ELYDINENIFPDYFFLDIFTENKEQKNEEVPMKIEVVNDGEEVKTEYVSEKNEEVENKSETEIGEELTEKVDEKVPEEV
AEELVEKVDEEVAEELVEKVDEKVAEEVDQKVDEEVTEELIEKVDEEVTEELIEKVDEEVAEELIEKVDEEVAEELIEK
VADELIEKVDEEVAEELIEKVADELVEKVAEELVEKVDEEVAEELVEKVDEKVAEEVDQKVDEEVTEELIEKVDEEVTE
ELIEKVDEEVAEELIEKVDEEVAEELIEKVADELVEKVAEELVEKVDEQVAEELVEKVDEQVAEELVEKVDEQVVEEVA
EEVAEEVVEEGEKVPEEVAEEVAEEVAEEVAEEVAEELVEKVDEEVAEKVVEEEGEKVPEEVVEEVDEEVAEKVVEEEG
EKVLEEVIEEVVEEVAEEVAEKVVEEQGEKVNKNDLNDASSEEIKDSSDFKESHEELFKVFLELINKNDLVKENLKKIT
NNLNEMHLSTLYP

Predicted RNA/mRNA Sequence (Introns spliced out)

1701 bp

ATGTTGAATATTTTTAATATAATTTTCTTGTTGTTTTTAATAAACATATATATATGTGAAGCCAATGGAACACTCTCTG
AAAATATTGAAAGTGCTGAAGAGATAGATGCTTTAAAAACGAATTTAAGAAATGGATATTTAAATAATACTTATTTTAA
TGAAGAAAACAATAATTTAAATATAGAAAATGAAATAAATAATACAAATTATAATGAAGTAACAGAAGAAACTAAAGAA
GAATTATATGATATAAATGAAAATATTTTCCCTGATTATTTTTTTCTTGATATCTTTACTGAAAATAAAGAACAAAAAA
ATGAAGAAGTACCAATGAAAATAGAAGTAGTAAATGATGGAGAAGAAGTAAAAACAGAATATGTATCTGAAAAAAATGA
GGAAGTAGAAAATAAATCGGAAACTGAAATAGGTGAAGAATTAACTGAAAAAGTAGATGAAAAAGTACCTGAAGAAGTA
GCTGAAGAATTAGTTGAAAAAGTAGATGAAGAAGTAGCTGAAGAATTAGTTGAAAAAGTAGATGAAAAAGTAGCTGAAG
AAGTAGATCAAAAAGTAGATGAAGAAGTAACTGAAGAATTAATTGAAAAAGTAGATGAAGAAGTAACTGAAGAATTAAT
TGAAAAAGTAGATGAAGAAGTTGCTGAAGAATTAATTGAAAAAGTAGATGAAGAAGTTGCTGAAGAATTAATTGAAAAG
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AGCTGAAGAAGTAGATCAAAAAGTAGATGAAGAAGTAACTGAAGAATTAATTGAAAAAGTAGATGAAGAAGTAACTGAA
GAATTAATTGAAAAAGTAGATGAAGAAGTTGCTGAAGAATTAATTGAAAAAGTAGATGAAGAAGTTGCTGAAGAATTAA
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GAAGAAGTAGCTGAAGAAGTAGTTGAAGAAGGTGAAAAAGTACCTGAAGAAGTAGCTGAAGAAGTAGCTGAAGAAGTAG
CTGAAGAAGTAGCTGAAGAAGTAGCTGAAGAATTAGTTGAAAAAGTAGATGAAGAAGTAGCTGAAAAAGTAGTTGAAGA
AGAAGGTGAAAAAGTACCTGAAGAAGTAGTTGAAGAAGTAGATGAAGAAGTAGCTGAAAAAGTAGTTGAAGAAGAAGGT
GAAAAAGTACTTGAAGAAGTAATTGAAGAAGTAGTTGAAGAAGTAGCCGAAGAAGTAGCTGAAAAAGTAGTTGAAGAAC
AAGGTGAAAAAGTAAACAAAAATGATTTAAATGATGCATCTTCCGAGGAAATTAAGGATTCTAGTGATTTTAAAGAATC
TCATGAGGAATTATTTAAAGTTTTCCTGGAGTTAATTAATAAAAACGATTTAGTTAAAGAAAATTTAAAAAAGATTACA
AACAATTTAAATGAAATGCATTTAAGCACTTTATATCCATAA

Genomic Sequence

1701 bp

ATGTTGAATATTTTTAATATAATTTTCTTGTTGTTTTTAATAAACATATATATATGTGAAGCCAATGGAACACTCTCTG
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TGAAGAAAACAATAATTTAAATATAGAAAATGAAATAAATAATACAAATTATAATGAAGTAACAGAAGAAACTAAAGAA
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ATGAAGAAGTACCAATGAAAATAGAAGTAGTAAATGATGGAGAAGAAGTAAAAACAGAATATGTATCTGAAAAAAATGA
GGAAGTAGAAAATAAATCGGAAACTGAAATAGGTGAAGAATTAACTGAAAAAGTAGATGAAAAAGTACCTGAAGAAGTA
GCTGAAGAATTAGTTGAAAAAGTAGATGAAGAAGTAGCTGAAGAATTAGTTGAAAAAGTAGATGAAAAAGTAGCTGAAG
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AGCTGAAGAAGTAGATCAAAAAGTAGATGAAGAAGTAACTGAAGAATTAATTGAAAAAGTAGATGAAGAAGTAACTGAA
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CTGAAGAAGTAGCTGAAGAAGTAGCTGAAGAATTAGTTGAAAAAGTAGATGAAGAAGTAGCTGAAAAAGTAGTTGAAGA
AGAAGGTGAAAAAGTACCTGAAGAAGTAGTTGAAGAAGTAGATGAAGAAGTAGCTGAAAAAGTAGTTGAAGAAGAAGGT
GAAAAAGTACTTGAAGAAGTAATTGAAGAAGTAGTTGAAGAAGTAGCCGAAGAAGTAGCTGAAAAAGTAGTTGAAGAAC
AAGGTGAAAAAGTAAACAAAAATGATTTAAATGATGCATCTTCCGAGGAAATTAAGGATTCTAGTGATTTTAAAGAATC
TCATGAGGAATTATTTAAAGTTTTCCTGGAGTTAATTAATAAAAACGATTTAGTTAAAGAAAATTTAAAAAAGATTACA
AACAATTTAAATGAAATGCATTTAAGCACTTTATATCCATAA

Table A1: List of Reagents

Reagent	**Source**
Albumax II	Invitrogen
CFDA SE	Invitrogen
Gentamicin (10mg/mL)	Invitrogen
Hoechst 33342	Sigma-Aldrich
L-Glutamine	Sigma-Aldrich
L-Alanine	Sigma-Aldrich
Percoll®	Sigma-Aldrich
Phosphate Buffered Saline, pH 7.4	Sigma-Aldrich
RPMI 1640 medium (with L-Glutamine)	Sigma-Aldrich
FACS flow Fluid	BD Biosciences
PF10_0351 peptides (2 mg/mL)	GenScript Corporation
Anti-PF10_0351 rabbit antibodies (5 mg/mL)	GenScript Corporation
Pre-Immune rabbit IgG (5 mg/mL)	GenScript Corporation

Table A2: Extra details on PF10-0351 peptides

Peptide	**PF10_0351-1**	**PF10_0351-2**	**PF10_0351-3**
Concentration	2 mg	2 mg	2 mg
Peptide order ID	U6393BK290-1	U4514BK290-1	U4110BK290-1
Peptide sequence	***C***QEKVNKNDLNDAS	FTENKEQKNEEVPM***C***	VVNDGEEVKTEYVS***C***
Purity (%)	92.7	95.2	88.5
MW	1634.73	1826.02	1670.80
Antibody ID	U1491BJ210-32	U1491BJ210-35	U1491BJ210-38

Table A3: List of antibodies

Antibody	**Type**	**Animal**	**Target**	**Supplier**
α-PF10_0351	Polyclonal (primary)	Rabbit	PF10_0351	GenScript
α-MSP1	Polyclonal (co-stain)	Rabbit	MSP1	ThermoScientific
α-Pfs48/45	Polyclonal (co-stain)	Rabbit	Pfs48/45	ThermoScientific
Alexa Fluor 488	Polyclonal (secondary)	Goat	Rabbit	Invitrogen
α-human IgG HRP	Polyclonal (secondary)	Goat	Human IgG	Invitrogen

Table A4: Parasite density, age, and absorbance (OD) of study participants

Sample ID	Parasite density	Patient's age	Peptide 1 OD_{450}	Peptide 2 OD_{450}	Peptide 3 OD_{450}
EIMA058	26,318	05	2.577	1.988000	1.8310
EIMA060	95,531	07	2.978	2.480000	2.2450
EIMA067	54,405	06	3.051	2.476000	2.2190
EIMA076	84,464	07	3.493	2.975000	2.6300
EIMA084	63,597	02	2.751	2.240000	1.9240
EIMA089	34,109	14	2.663	2.080000	1.6810
EIMA093	14,941	12	2.745	1.972000	1.6980
EIMA133	40,924	08	2.905	2.290000	2.0630
EIMA160	117,623	11	2.812	2.283000	1.8930
EIMA054	26,291	09	3.213	2.737000	2.4480
EIMN056	9,373	02	2.921	2.670000	2.5070
EIMN059	403,918	02	3.270	2.347000	2.4740
EIMN066	13,924	03	2.829	1.823000	1.9980
EIMN070	40,950	02	3.527	2.228000	1.7560
EIMN079	16,737.5	04	2.454	1.392000	1.3870
EIMN080	64,896	07	2.616	2.882000	2.6340
EIMN093	157,777.5	05	3.100	2.138000	1.7830
EIMN120	32,508	02	2.531	2.095000	1.7530
EIMN200	98,040	03	3.357	2.652000	2.4430
EIMN212	70,933	05	3.059	2.483000	2.5600
EIMK002	25,291	04	3.119	2.702000	2.5080
EIMK015	19,300	02	3.500	2.885000	2.9190
EIMK019	7,800	06	3.609	2.793000	2.7580
EIMK028	1,326	02	3.482	2.798000	2.6520
EIMK072	360	02	3.324	2.499000	2.6050
EIMK098	3,013	03	3.418	2.698000	2.8090
EIMK135	212,660	05	2.592	2.097000	2.0010
EIMK153	150,480	11	2.476	1.961000	1.6250
EIMK491	11,207	05	2.967	2.220000	2.2860
EIMK497	27,729	12	3.357	2.611000	2.6350

Parasite density is presented in parasite/µL. Age is presented in years. OD_{450} denotes absorbance value (measured at 450 nm) after NHS background subtraction. EIM=Erythrocyte Invasion Mechanism. A=Accra, N=Navrongo, K=Kintampo.

Table A5: Correlation between peptide recognition by plasma antibodies and age or parasite density

Peptides	Age		Parasitaemia	
	R^2 (95% CI)	*P value	R^2 (95% CI)	*P value
PF10_0351-1	0.080 -0.065 to 0.009	0.131	0.006 -2.230e-006 to 1.216e-006	0.672
PF10_0351-2	0.015 -0.053 to 0.027	0.525	0.013 -2.003e-006 to 1.310e-006	0.553
PF10_0351-3	0.073 -0.076 to 0.012	0.150	0.011 -2.483e-006 to 1.436e-006	0.588

***P** analyzed by Spearman correlation with R^2 (95% CI) = R square (95% Confidence Interval), at < 0.05 level of statistical significance, using GraphPad Prism 6.0

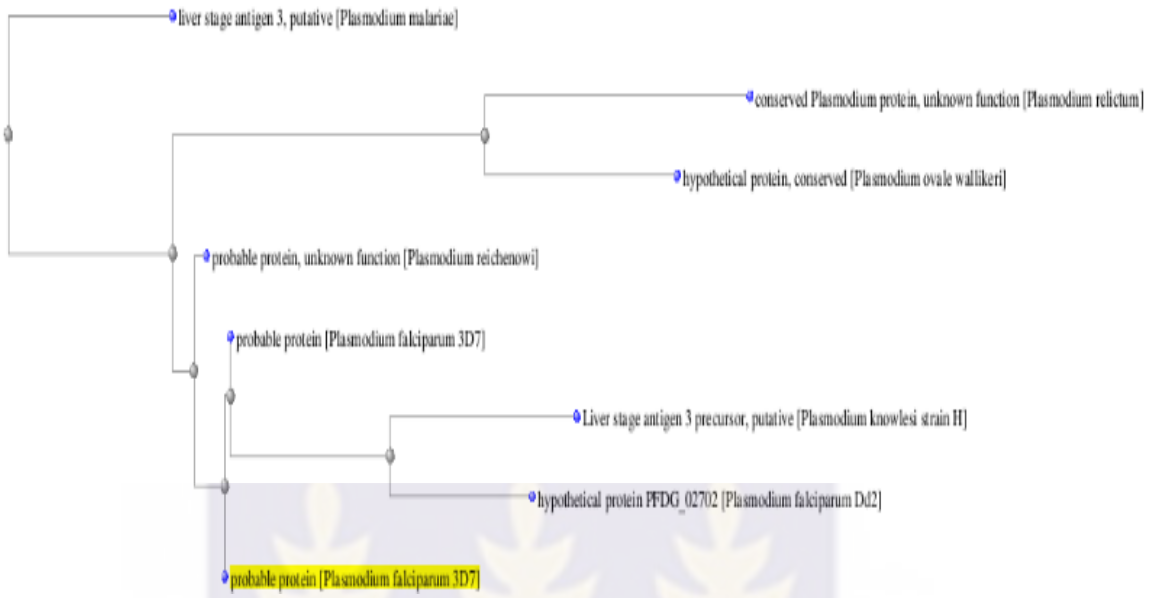

Figure A1: **Phylogenetic tree of *Plasmodium* species showing the relatedness of PF10_0531 sequence homology**.

The tree was constructed using BLAST pairwise alignments and neighbor joining. The yellow colour indicates the query. The blue nodes represent apicomplexans. The grey nodes indicate the linkage between the related species.