MOLECULAR CHARACTERIZATION OF RECOMBINANT *Plasmodium falciparum* PHISTB PROTEINS AS POTENTIAL TARGETS OF NATURALLY ACQUIRED IMMUNITY AGAINST MALARIA IN HUMANS

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Molecular Characterization of Recombinant *Plasmodium falciparum* PHISTB Proteins as Potential Targets of Naturally Acquired Immunity against Malaria in Humans

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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This thesis has been submitted for examination with our approval as the university supervisors

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DEDICATION

To my dear parents Vincent and Rofina Isebe for their endless love, support and instilling in me a sense of discipline and hard work that has stayed with me to date.

To my cherished daughter Victoria Rofina Wanjiku -you are such an inspiration.

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LIST OF ABBREVIATIONS AND ACRYONYMS

AP2	Activating protein 2
ATS	Acidic terminal sequence
CDC	Center for Disease Control
cDNA	Complementary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTPs	deoxy Nucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HDA	Histone deacetylase
HRPII	Histidine-rich protein II
IPTG	Isopropyl β-D-1-thiogalactopyranoside
iRBC	Infected Red Blood Cell
IRS	Indoor Residual Sprays
ITNs	Insecticide Treated Nets
IVM	Integrated Vector Management
KAHRP	Knob associated histidine-rich proteins

LSM	Larval Source Management
MAHRP1	Membrane-associated histidine-rich protein 1
OD	Optical Density
PCR	Polymerase Chain Reaction
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PHIST	*Plasmodium* helical interspersed subtelomeric
PVDV	Polyvinylidene difluoride
RESA	Ring-infected surface antigen
SBP1	Skeleton binding protein 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris Borate-EDTA
TEMED	N, N, N', N' Tetramethylenediamine
WHO	World Health Organization

ABSTRACT

Plasmodium falciparum is known to cause the deadliest form of malaria in sub-Saharan Africa. Upon infection, remodeling of infected host red blood cells (iRBCs) by parasite-exported surface proteins occurs providing a favorable niche for parasite development and maturation. Some of these molecules include products of the multi-copy family of Plasmodium helical interspersed sub-telomeric (PHIST) proteins, whose role in malaria pathogenesis largely remains unknown at present. In a recent transcriptome analysis of clinical isolates, some members of PHISTb gene family were associated with parasite adaptations to malaria transmission intensity and gametocyte development. To characterize the possibility of these proteins as potential vaccine targets, recombinant proteins were expressed in bacteria followed by ELISA-based evaluation of antibody responses using immune sera from malaria-exposed individuals. Our findings show that children and adults from malaria-endemic region recognized PHISTb proteins, Pf3D7_0532400, Pf3D7_1401600, and Pf3D7_1102500, providing a clinical evidence for the role of PHISTb antigens in immune response against *Plasmodium falciparum* infection. Antibody responses against the three recombinant PHISTb antigens were however variable. An association study of antibody responses to the different PHISTb antigens with age revealed no correlation between the age and antibody responses to Pf3D7_1102500 and Pf3D7_1401600 ($p=0.507$ and $p=0.15$, respectively, CI=95%), but a significant correlation to Pf3D7_0532400 ($p=0.009$). Furthermore, there was strong correlation of antibody responses to both the crude schizont extract and Pf3D7_0532400 ($p=0.005$), equivalent to those against Pf3D7_1102500 and Pf3D7_1401600 ($p=0.0001$). It was further established that immune responses to recombinant PHISTb proteins was varied depending on malaria transmission intensities in three different geographical sites in Kenya (Siaya, Takaungu) and The Gambia (Sukuta). Collectively, these findings empirically provide evidence of recombinant PHISTb antigens as potential targets of naturally-acquired immunity against malaria in humans and possible serological markers to *P. falciparum* infection aimed at contributing to malaria control through vaccine development.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a global health concern with 228 million cases occurring worldwide in 2018, and 93% was reported in the sub-Saharan Africa (SSA). The majority of deaths are caused by *P. falciparum* parasite accounting for 99% of estimated malaria cases in 2018 (World Health Organization, 2019). Malaria deaths occur due to complications with nearly 1 million children aged below five years affected and other groups including women in their first pregnancy and non-immune travelers being at high-risk (Kirchgatter & Del Portillo, 2005). Efforts toward drug and vaccine development for malaria control have been the focus of several studies with the target mostly being the intraerythrocytic stages that are responsible for clinical symptoms related to illness. However, the lack of commercial vaccines and rapid development of resistance to existing drugs have posed a major challenge to disease control (Bloland, 2001). In 2019, RTS, S/AS01 (RTS, S) was approved for use in young children through the national immunization program. Despite this positive step, there remains an urgent need to develop vaccines that would be instrumental in dealing with the scourge of malaria infection.

The PHIST family forms part of the large number of proteins exported by the parasite to the iRBC. The proteins are found exclusively in the genus *Plasmodium* and are greatly expanded in *P. falciparum* and *P. reichnowi* (Otto et al., 2014). The exact number of proteins in different species of malaria is not clear as publications report different counts. There are 39 PHIST proteins for *P. vivax,* but studies on the gene family Pv_fam_e shows presence of 44 *rad* genes and 21 phist genes (Sargeant et al., 2006). For *P. knowlesi,* Sargeant *et al*. report the presence of 27 PHIST proteins, but Pain et al. confirm 38 proteins, while the PlasmoDB lists 39 records (Sargeant et al., 2006). There are three PHIST subfamilies including PHISTa, PHISTb and PHISTc. PHISTa proteins

amount to 26 different proteins, and possess two conserved tryptophan residues (Warncke et al., 2016). PHISTb proteins comprise 24 members and have 300-600 residues. They have a unique C-terminal amino acid stretch following the PHIST domain (Sargeant et al., 2006). All characterized PHISTb proteins localize and interact with the human host cytoskeleton (Warncke et al., 2016). The N-terminal region and the PHIST domain confer peripheral localization, as shown for PHISTb proteins; Pf3D7_0401800 and PHISTb-DnaJ protein Pf3D7_0102200 (Tarr et al., 2014). PHIST proteins localize within the iRBCs and they are implicated in molecular and cellular activities within the iRBC. The proteins are involved in display of PfEMP1, change in cell rigidity, gametocytogenesis and celebral and pregnancy-associated malaria (Warncke et al., 2016). The PHISTc subgroup has 18 members and is diverse with the length of amino acids varying from 200-1200. The PHIST domain is located next to the C terminus of the protein, similar to PHISTa. Recently, differential expression of the PHISTb genes in high versus low malaria transmission has been revealed (Figure 1.1**)**(Rono et al., 2018)**.** Mapping of (high vs low) H-L genes reveal that up-regulated genes were localized to regions within the asexual stages in the blood and liver, while the H-L down-regulated genes were associated with sexual stages in blood and mosquito (Rono et al., 2018). Pre-immunity is a potent selection force for high rates of asexual replication resulting in reproductive output in non-immune and semi-immune hosts (Rono et al., 2018).

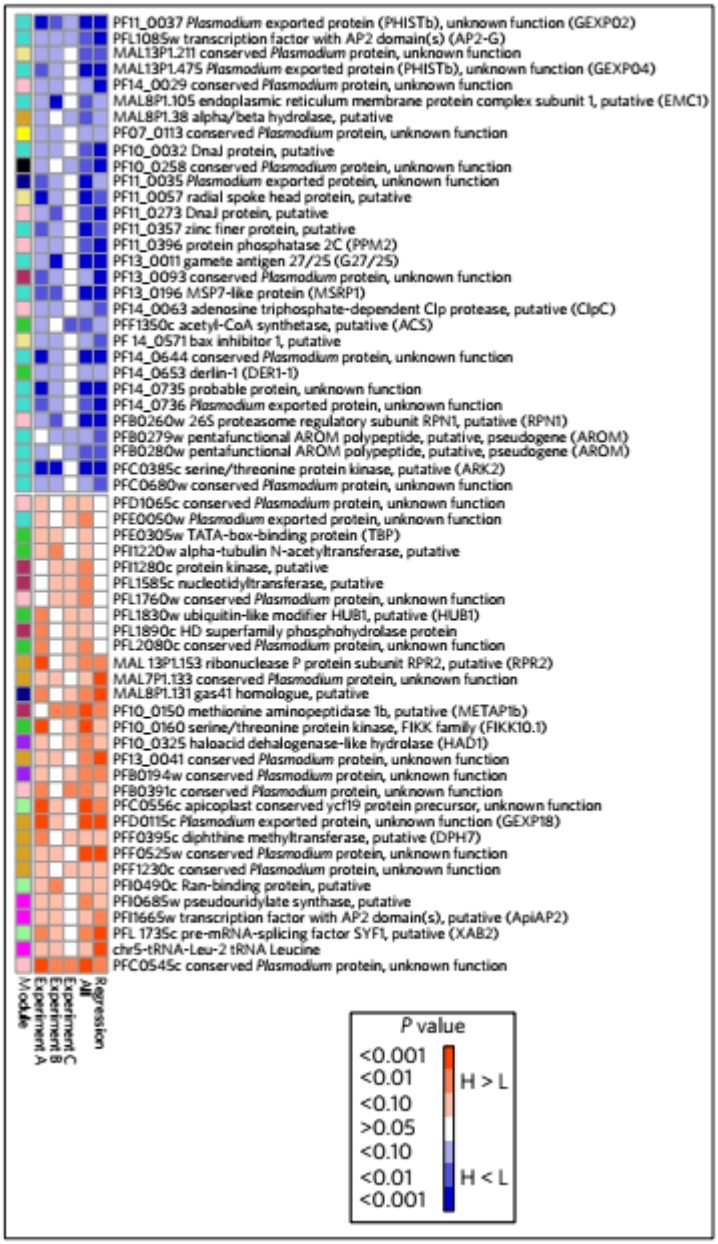


Figure 1.1: Heat map showing significance (orange-blue color legend) for genes with consistent high vs low (H-L) differences.

(Source: Rono et al., 2018)

The differences are depicted for three experiments and across experiments. The old gene ID have been used in the figure above. PHISTa, PHISTb, and PHISTc

PHISTa subgroup is short in length and has a signal sequence and PEXEL motif (Warncke et al., 2016). They exist exclusively in *P. falciparum* and consist of 6 different proteins (Warncke et al., 2016). They possess only two conserved tryptophan residues. PHISTa exists solely in *P. falciparum* (Sargeant et al., 2006). PHISTa proteins are transcriptionally silent except for Pf3D7_0402000 and PfD7_1253300. Some PHISTa genes are upregulated in pregnancy-associated malaria or cerebral malaria include; PF14_0757, PFA110w, PFI1785w, and PFB105c (Ndam et al., 2008).

PHISTb proteins comprises of 24 members and are slightly longer than the PHISTa proteins (Warncke et al., 2016). They have a long, unique C-terminal amino acid that follows the PHIST domain (Warncke et al., 2016). Previous reports showed that the PHIST domain provided a general binding motif while the C-terminus offered a specific interaction domain for Pf3D7_0532400 (Oberli et al., 2016). PHISTb proteins localize at the host cell periphery and interact with host cytoskeleton components (Warncke et al., 2016). Peripheral localization can be achieved through the N-terminal as depicted in Pf3D7_0401800 and Pf3D7_0102200. The observations could be an indication that the PHISTb proteins are associated with the cytoskeleton of an infected red blood cell (Warncke et al., 2016). The PHISTb has a *P. falciparum*, RESA, that is distinguished by a DnaJ domain (Sargeant et al., 2006). The knockout of RESA and PF14_0018 indicates an alteration in cell rigidity (Moreira et al., 2016). The DnaJ proteins could be functioning as co-chaperones with the HSP70 in folding and assembling the protein structures within an infected red blood cell (Maier et al., 2008). The PHIST domain has evolved to bind to endogenous erythrocyte proteins including the cytoskeleton. The PHIST family could present a central front in understanding parasite-host interactions which drive the colonization of human erythrocytes by the *P. falciparum* (Parish et al., 2013). PHISTb proteins have been shown to localize at the host cell periphery (Warncke et al., 2016).

PHISTc proteins are shared between *P. vivax* and *P. knowlesi* (Sargeant et al., 2006). The PHIST domain is found close to the C-terminus of the protein. They are found near

the Maurer's cleft and could be involved in trafficking of proteins (Sargeant et al., 2006). Extensive sequence divergence between the paralogs within the PHIST genes has been identified. It is an indication that the PHIST proteins diversified responding to a selective force other than the immune pressure (Moreira et al., 2016). The findings are supported by studies indicating that high and low transmission differences are not attributed to parasite response to cues from the host but, it could be due to a population-level adaptation to the environment (Rono et al., 2018).

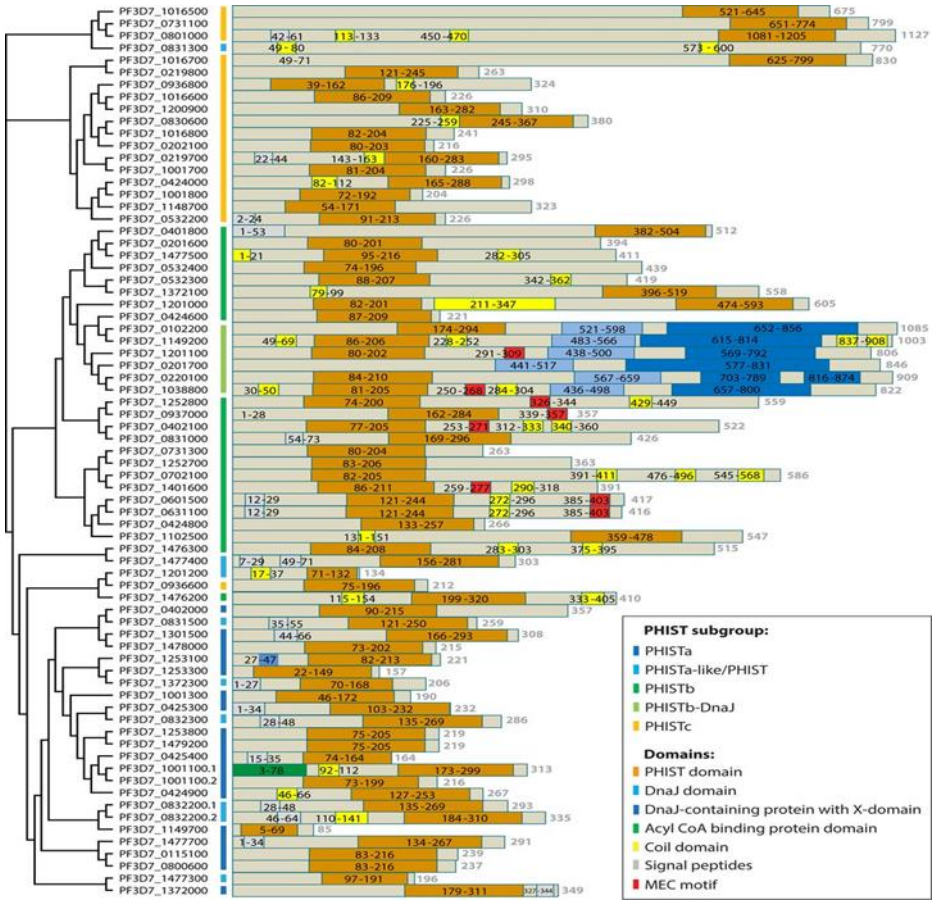


Figure 1.2: Conserved domains in PHIST family of proteins

(Source: Warncke et al., 2016).

The PHIST genes Pf3D7_1401600, Pf3D7_1102500 were down-regulated in high versus low transmission malaria (Rono et al., 2018). while Pf3D7_0532400 is involved

in presentation of PfEMP1 on the surface of infected RBCs via the ATS segment (Oberli et al., 2014). Host immunity can raise the potency of in-host selection for high virulence malaria (Mackinnon & Read, 2004). The role of PHISTb proteins in cyto-adherence to iRBCs and the differential expression of the proteins in high versus low malaria transmission areas establishes the need to study them further, for their role as potential targets of naturally acquired immunity against malaria.

1.2 Statement of the Problem

Significant milestones have been achieved as far as the fight against *P. falciparum* transmission is concerned. Antimalarial drugs have been developed targeting pathogenic blood stages of the parasite. Unfortunately, cases of drug resistance continue to be reported. It affects efforts aimed at achieving the objective of eradication of malaria. Decades of intensive research have not been successful in yielding a commercial malaria vaccine against *P. falciparum*. Currently, over 20 vaccine targets are being evaluated at clinical trials (WHO,2019). The RTS,S/AS01 is the only vaccine at the later stages of commercialization even though it does not confer complete protection against malaria (Olotu et al., 2016). Therefore, there is need for novel vaccine targets with better protection outcome or that targets different aspects of malaria parasite lifecycle are of uttermost importance to malaria control.

Pre-erythrocytic stage vaccines are being developed and target the sporozoites and liver stage parasites (Duffy et al., 2012). The development of an effective vaccine is a priority in addressing the current malaria global health program . The attempts toward malaria vaccine development are yet to realize success because few proteins with reference to Plasmodium proteome (5400 proteins) have been studied burden with even fewer making into phase I vaccine trials.

The primary focus of research groups has been on developing vaccines targeting *P. falciparum* sporozoites stage. Mainly, this has involved sporozoites recombinant proteins, DNA or viral vectored protein and attenuated vaccines which elicit malaria

reactive CD4+ and CD8+ T-lymphocyte counts and high antibody (Abs) titres (Molina-Franky et al., 2020). Despite these efforts, most advanced formulations still have limited efficacies. Nevertheless, significant developments have been attained regarding phase I, II or III trials and these have proven useful for vaccine development (Molina-Franky et al., 2020)

Studies by Rono et al. show that parasites in low transmission areas have undergone evolution enabling them to increase the rate of transmission into new hosts and less within the human host, compared to parasites in high transmission areas (Rono et al., 2018). The adaptive changes are largely attributed to variations in expression levels of proteins that encode the epigenetic and translational machinery. One of the key player is AP2-G gene a master regulator of gametocytes, the transmissible form parasites. The expression of these genes is negatively correlated with malaria transmission intensity and strongly associated with a subset of gametocyte associated genes. Notably, several genes from the Plasmodium PHIST family were upregulated in parasites from low transmission areas (Rono et al., 2018). There is limited knowledge regarding the role of the PHIST proteins, curtailing efforts toward determination of possibility of utilizing them as potential targets to disrupt parasite plasticity to adapt to changing environmental conditions and block malaria transmission.

1.3 Justification of Study

There are increased calls globally for malaria eradication. The limited number of licensed malaria vaccine and development of resistance to antimalarial drugs makes it difficult to attain the goal of elimination of malaria. The use of vaccines could play an imperative role in ensuring the elimination of *P. falciparum* (Crompton et al., 2010). In this study, the hypothesis that the attainment of such goals can be achieved upon the development of a vaccine based on plasmodial proteins exported to the infected red blood cells (iRBCs) while focusing on malaria transmission between humans and vector. PHIST proteins alter the structure of RBCs enhancing survival of the parasite (Maier et

al., 2008). These proteins interact with the intracellular segment of PfEMP1 and cytoskeletal components. PFE160w (Pf3D7_0532400), a PHISTb protein interacts with six unique PfEMP1 ATS domain variants with 2-fold differences in affinity (Oberli et al., 2016). PHISTb and PHISTc subgroups are involved in the pathophysiological processes including alteration of erythrocyte deformability, adhesion, knob formation and the display of PfEMP1 on the surface of infected red blood cells (Maier et al., 2008). This study aimed at characterizing the PHISTb proteins and determining if they are targets for naturally acquired immunity against malaria. PHISTb genes have a role in parasite adaption to transmission intensity and gametocyte production (Rono et al., 2018). The occurrence of differential expression of PHIST genes in regions of high versus low malaria parasite transmission was recently reported (Rono et al., 2018). . Specifically, Pf3D7_1102500, Pf3D7_1401600 were down-regulated. The function of these PHIST proteins is currently unknown. This study evaluated selected PHISTb genes and assess their association with malaria transmission, and their role in acquisition of naturally immunity against malaria infection. The findings will contribute to the limited body of knowledge regarding the role of PHIST proteins in *P. falciparum* infection. The findings from this study will provide fundamental information regarding the possibility of exploring the PHISTb genes as targets of naturally acquired immunity against malaria in humans. With the growing concerns of drug resistance against treatment regimens, it remains a priority to exploit other avenues for development of effective therapeutics. The PHIST family of genes provides a novel approach that can be exploited by researchers for further drug development. Moreover, with the concern of transmission dynamics, it may be beneficial to evaluate all the possible avenues that can be used to enable the attainment of better treatment against malaria infection.

1.4 Research Questions

i. What is the role of *P. falciparum* phistb genes in eliciting immune responses?
ii. Are *P. falciparum* phistb genes targets of naturally acquired immunity against malaria?

iii. How do *P. falciparum* phistb antibody responses differ in different geographical locations?

1.5 Null Hypothesis

Plasmodium PHISTb proteins are not targets of naturally acquired immunity against malaria in human infected erythrocytes

1.6 Objectives

1.6.1 General Objective

To generate recombinant PHISTb proteins and determine if they are potential targets of naturally acquired immunity to malaria in humans.

1.6.2 Specific Objectives

i. To generate PHIST gene cloning constructs to express histidine-tagged proteins.
ii. To express soluble recombinant PHISTb proteins in a bacterial expression system
iii. To test for antibody responses against the recombinant PHISTb using standard ELISA and Western blotting utilizing malaria immune sera.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Life Cycle of *Plasmodium falciparum*

The Plasmodium parasite has a complex life cycle that alternates between the mosquito vector and the vertebrate host. When an infected mosquito bites its host, approximately 100 sporozoites are injected into the dermal tissue, where they enter into the bloodstream and navigate their way to the liver (Maier et al., 2008). Here, sporozoites invade hepatocytes leading to several rounds of asexual replication. Thousands of merozoites are then released into the bloodstream leading to invasion of red blood cells and initiating the blood stage cycle (Soulard et al., 2015). Merozoites multiply in a 48-hour intraerythrocytic developmental cycle (Maier et al., 2008). Entry of merozoites into RBCs is followed by formation of a parasitophorous vacuole at the ring stage. The parasite enters the maturation phase, trophozoites phase before replication. The schizonts stage is characterized by preparation by the merozoites for reinvasion of new erythrocytes. Approximately 16-32 merozoites are released per schizonts stage during egress to reinvade the new erythrocytes, establishing a new cycle as shown in (Figure 2.1) (Maier et al., 2008). The events are characterized by remodeling of the host cell. Changes in the infected red blood cell enable intake of nutrients by the parasite, which is accompanied by modification of the structure of the host cell membrane and its rigidity (Warncke et al., 2016).

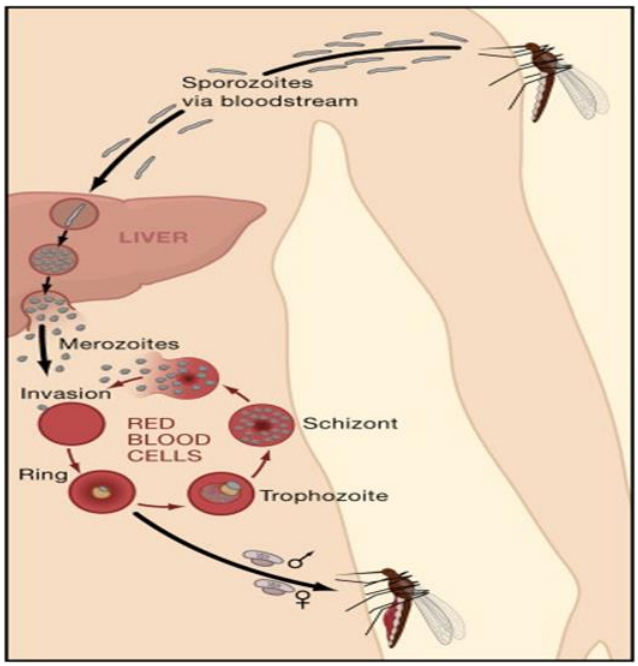


Figure 2.1: Life cycle of *P. falciparum*
(Source: Warncke et al., 2016).

Sporozoites are injected into human host's dermal tissue by an infected Anopheles mosquito during blood feeding. Sporozoites enter the bloodstream to be transported into the liver hepatocytes, where they develop forming schizonts which release thousands of merozoites into the bloodstream. Here they invade red blood cells, initiating the asexual replicative cycle. For each cycle, some of the parasites are committed to sexual cycle, and develop into gametocytes which are taken up by the female Anopheles mosquito in a blood meal initiating the reproductive cycle.

2.2 Malaria Distribution and the Disease Burden

Malaria presence is dependent on climatic factors including; temperature, humidity, and rainfall (Adeola et al., 2017). The disease is transmitted in both tropical and subtropical regions that facilitate Anopheles mosquito to multiply, and parasites to complete their life cycle in the vector (Guerra et al., 2008). The distribution of Anopheles and information regarding the variable number of vector species across different malarial

zones have been highlighted (Figure 2.2). The most significant burden of malaria disease is reported in Africa with 90% of all deaths in the world occurring at the south of the Sahara (Black et al., 2003). These deaths are primarily attributed to the presence of the deadliest form of Plasmodium species, *P. falciparum*, with 99% of deaths being reported (Hay et al., 2009). The In addition, the distribution of *P. falciparum* parasite rates is indicated (Figure 2.3) (Sinka, 2013). *P. vivax* is infectious, but outside Africa, except in Ethiopia. Also, A. gambiae is the most widespread in Africa and presents the greatest difficulty in regards to its control. Changes in distribution are likely to occur due to possible climatic variations (Sinka, 2013). The most affected individuals are the poor and the marginalized regions. Sub-Saharan Africa suffers from the highest global malaria transmission rates (Snow et al., 2012) . The disease transmission intensity is dependent on frequency with which malaria vector bites humans and the population of vector mosquitoes which have sporozoites in salivary glands (Tusting et al., 2013).

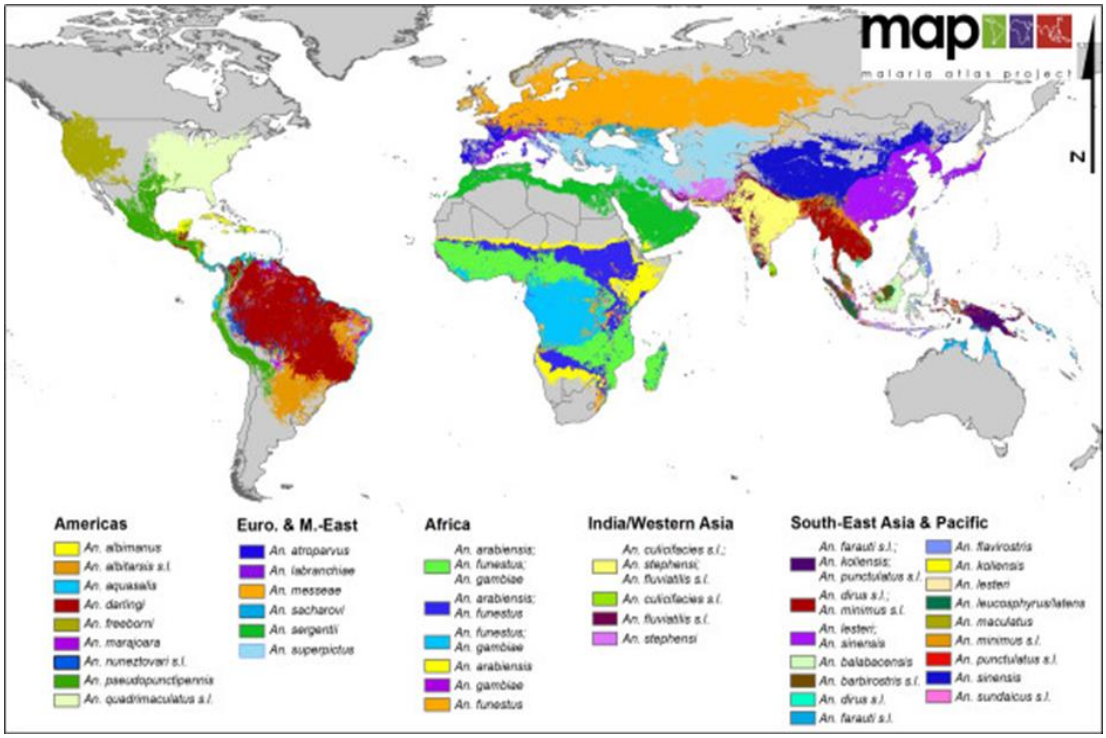


Figure 2.2: The global distribution of 34 Dominant Anopheles Vector Species

(Source: Sinka et al. 2013)

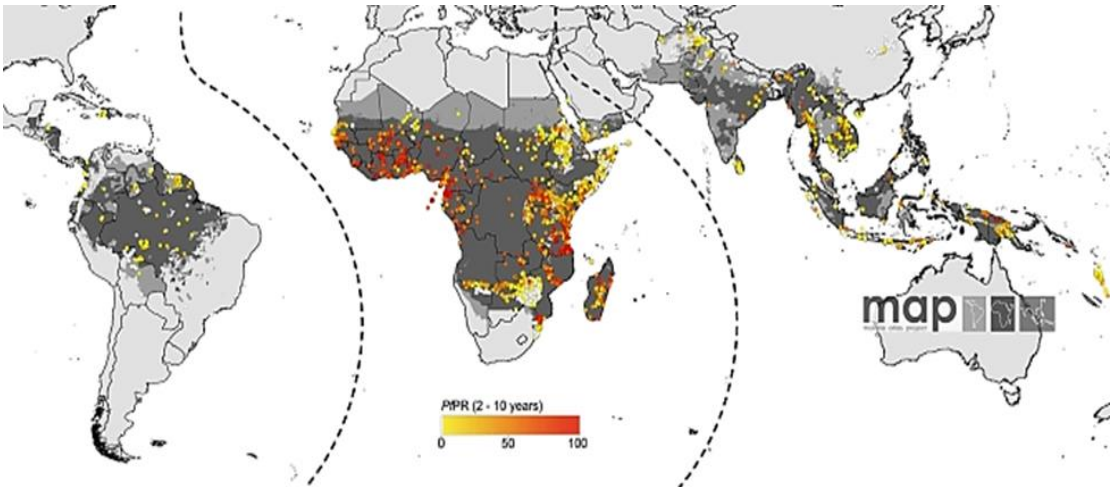


Figure 2.3: The spatial limits of *P. falciparum* malaria risk defined by PfAPI with further medical intelligence, temperature, and aridity masks
(Source: Sinka et al. 2013)

2.3 Management and Control of Malaria

The eradication of malaria is dependent upon the incorporation of different control and elimination efforts. A range of interventions have been adopted in an attempt to achieve elimination of the malaria. Success in control of *P. falciparum*-infection requires a multifaceted approach, which includes drugs and vaccines. Currently, the interventions used target both the vector and the parasite. Vector control methods include; insecticide-treated mosquito nets (ITN), larval source management (LSM), and indoor residual spraying (IRS) (Hay et al., 2009). The use of ITNs and IRS are identified to be the most effective malaria prevention strategies and have been recommended for use in sub-Saharan Africa (O'Meara et al., 2010). The effectiveness of the strategy has seen the scaling up of their use across sub-Saharan Africa in the past 10 years. The WHO Malaria Report (2012) notes that ITN ownership per household was reported at 53% while 11% of people at risk of malaria infection were protected by the IRS (West et al., 2014). LSM reduces malaria transmission by targeting the larvae and pupae of the mosquito decreasing the number that reaches adulthood (West et al., 2014). The overall effect is a reduction in transmission of *P. falciparum* species, and an eventual decline in prevalence

and morbidity (Tusting et al., 2013). In regions where there is high malaria transmission, the administration of preventive treatment, distribution of insecticide-treated nets and case management are recommended for proper control of the disease (Worrall & Fillinger, 2011). Over the years, it has become increasingly important to adopt accurate diagnosis for proper treatment of malaria (Nosten & White, 2007). Artemisinin-based compounds are currently used in the treatment of malaria. The commonly used treatment regimens include; artesunate, artemether and dihydroartemisinin (Kovacs et al., 2016). Malaria control interventions have been increased in Africa, and this has seen a significant reduction in cases of illness and decline in mortality rates by 42% in 2013 (WHO, 2014). However, these interventions have met several drawbacks mainly due to emergence and widespread drug and insecticide resistance. A lot of attention has now been directed to genomic tools to find new targets for malaria control. This will provide information regarding the different genes that can encode proteins to be explored for vaccine and drug development.

2.4 *P. falciparum* Genome

The sequence of *P. falciparum* clone 3D7 shows that the nuclear genome has 22.8 megabases that are distributed in 14 chromosomes that range in size from 0.643 to 3.29Mb (Gardner et al., 2002). The A + T composition is 80.6% and approximately 90% introns and intergenic regions. Protein-encoding genes are approximately 5300 (Gardner et al., 2002). The average length of genes in *P. falciparum* is 2.3kb. A large number of genes in *P. falciparum* encode proteins which are yet to be characterized (Gardner et al., 2002). There are two other smaller genomes, the mitochondrial and plastid with 6kb and 35kb respectively (R. F. Waller & McFadden, 2005). The genome has a full set of tRNA, but lack long tandemly repeated arrays of rRNA genes. However, there are 18S-5.8S-28S rRNA distributed in different chromosomes. Expression of rRNA is regulated developmentally, causing expression of a unique set of rRNA at different stages of the life cycle. *P. falciparum* chromosomes are varied considerably in length, with much changes in the subtelomeric regions (Gardner et al., 2002).

2.4.1 *P. falciparum* Exported Proteins to Infected Erythrocytes

Following the invasion of erythrocytes by merozoites, the parasite has to remodel the infected cells that lacks basic structures for supporting key metabolic and growth functions (Mundwiler-Pachlatko & Beck, 2013). The parasites meet this demand by exporting its own proteins into the host RBCs. A total of 300-400 proteins are exported to the host erythrocyte by *P. falciparum* (Sargeant et al., 2006). The exported proteins contain a signal sequence to facilitate entry into the endoplasmic reticulum, and a protein export element (PEXEL) signal motif which confers trafficking to the red blood cell (Boddey et al., 2016). The movement of the proteins is via the parasitophorous vacuole membrane through a translocator machine. The PEXEL binds the phosphatidylinositol-3-phosphate in the parasite endoplasmic reticulum. Some exported proteins lack the protein export element motif; their transport pathway is unknown (Heiber et al., 2013).

Proteins for export are trafficked through the parasite's vesicular pathway. Multiple checkpoints and multiple partially redundant signals exist which ensure selective export of proteins that are destined to iRBCs (Heiber et al., 2013). Sargeant et al. developed an algorithm for prediction of exported proteins. The findings depict that ExportPred predicted 797 sequences which are exported with many representing overlapping gene predictions and annotations. Further sorting and protein trafficking occurs in the Maurer's clefts. The exported proteins including. *P. falciparum* erythrocyte membrane protein 1(PfEMP1), PfEMP3, RESA and KAHRP except MESA interact with spectrin, an erythrocyte skeleton protein (K. L. Waller et al., 2010). Remodeling of the infected cells leads to significant morphological and structural changes, altering its physical properties. Knob structure found on the surface of iRBCs contain PfEMP1 a sticky parasite molecule that confers cytoadherence by binding to host endothelial receptors (Moxon et al., 2011). The PfEMP1 variants have different domains that mediate a range of interactions with different host cell molecules (Claessens et al., 2012). Studies have shown the existence of erythrocyte surface antigens recognized by prevalent antibodies

and PfEMP1 could be the likely target because of the diverse agglutination profiles demonstrated (Bull et al., 1999).

2.4.2 Plasmodium exported proteins encoded by multigene family

FIKK kinases {(phenylalanine (F) – isoleucine (I) – lysine (K) – lysine (K)} represent a class of putative protein kinases recently reported (Schneider & Mercereau-Puijalon, 2005). The FIKK kinase family is involved in remodeling of the erythrocyte membrane skeleton proteins. The FIKK member targets a protein at varying time points of the asexual blood cycle (Nunes et al., 2010). Specifically, FIKK12 targets a 80kDa protein during the trophozoites stage while the FIKK7 targets a 300 kDa protein at the schizonts stages (Nunes et al., 2010). The FIKK proteins could have different functions in the iRBCs including trafficking, adhesion and antigenic variation. They are expanded in *P. falciparum* lineage with at least 6 paralogs. They have a kinase domain that is not characterized, depicting the possibility of using them for drug design or vaccine development (Osman et al., 2015). The *P. falciparum* erythrocyte membrane protein-1 is part of the parasite exported proteins. PfEMP-1 binds to spectrin. The protein has a conserved Acidic Terminal Sequence (ATS) across all the variants. The ATS binds to erythrocyte components including spectrin-actin-band 4.1 complex (Cutts et al., 2017). The binding of PfEMP1 to the iRBCs is strengthened by indirect interactions. The ATS domain of PfEMP1 associates with the band 3 protein through the parasite's PHIST protein Pf3D7_0532400 which binds the band and the ATS-C. The PfEMP-1 variants which exhibit the strongest direct binding to spectrin, have the weakest affinity for Pf3D7_0532400, thus, the weakest indirect association to the cytoskeleton (Cutts et al., 2017). Rifins represent a class of variant proteins that are also expressed on iRBCs. Members of this protein family are transcribed at the asexual stage, expressed on the surface of iRBCs and are phenotypically variable. The rifins act as accessory molecules in resetting or could be vital for resetting in parasites that fail to express the rosetting-type PfEMP-1 variant (Newbold et al., 1999). Stevor proteins have been shown to play a role in rosetting and invasion of iRBCs (Niang et al., 2014).

2.4.3 PHIST Proteins

Sargeant et al. identified a new protein family, the PHIST proteins, characterized by a domain of about 150 amino acids forming four alpha helices. Some members of this family have the PEXEL motif while others contain the DnaJ domain. Three primary classes of the family have so far been identified, PHISTa, PHISTb, and PHISTc. A total of 89 PHIST proteins have been identified in Plasmodium (Sargeant et al., 2006). The PHIST proteins comprise approximately 14% of proteins exported to infected red blood cells, and about 2% of the *P. falciparum* proteome (Warncke et al., 2016).

CHAPTER THREE

MATERIALS AND METHODS

The study was conducted as indicated in this flow chart.

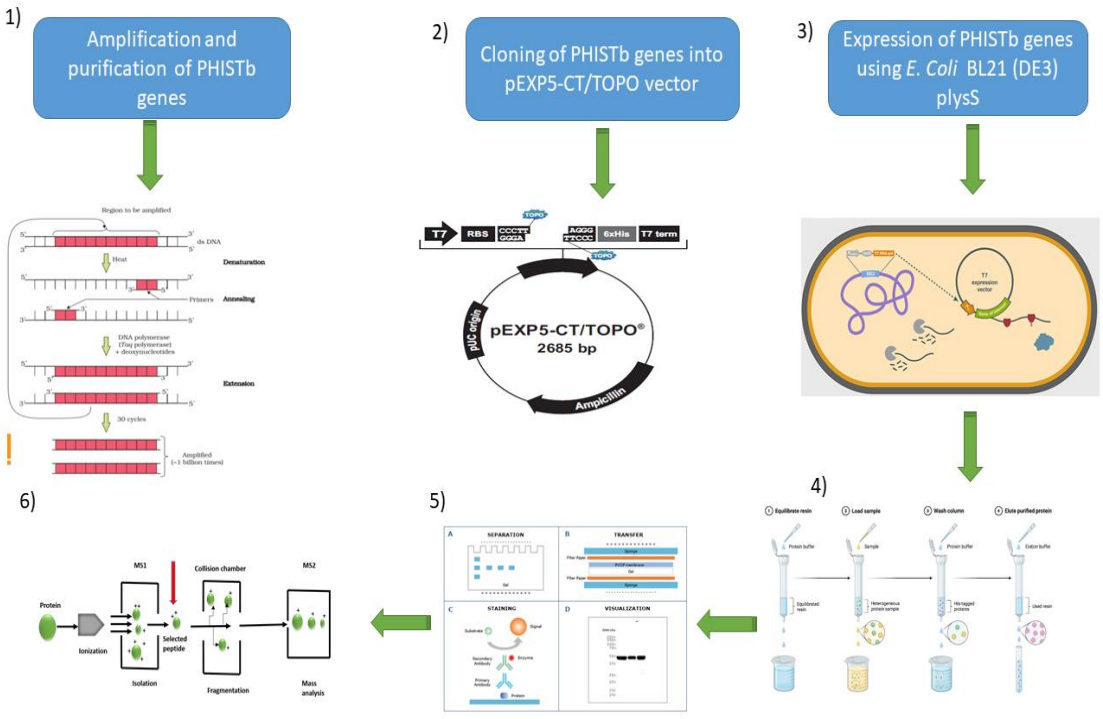


Figure 3.1: Flow chart of the methods used in the study

3.1 Ethical Statement

Ethical approval was sought from the KEMRI Scientific and Ethics Review Unit. Written informed consent was obtained from participants or respective parents/guardians before the collection of samples.

3.2 Sample Size

For this work, 544 samples were utilized, as guided by Chadha's formula (Chadha, 2006). As such all available archived samples from Kenya (Takaungu and Siaya) and The Gambia (Sukuta) were used to evaluate immune responses toward purified PHISTb antigens (Snow et al., 1997)

$$n = N*X / (X + N - 1),$$

Where,

$$X = Z\alpha/22 *p*(1-p) / MOE2,$$

$Z\alpha/2$ is the critical value of the Normal distribution at $\alpha/2$ (e.g. for a confidence level of 95%, α is 0.05 and the critical value is 1.96), MOE is the margin of error, p is the sample proportion, N is the population size.

3.3 Sample Collection and Preparation

Samples used in this study had previously been obtained from a cross-sectional survey of children exposed to malaria over time (1991-1995) between regions of high and low malaria transmission was done in Kenya (Siaya and Takaungu) and The Gambia (Sukuta) respectively. The samples were archived and were present in the KEMRI-Wellcome Trust Research Programme Biobank.

3.4 Characteristics of the Study Population

The study used human sera from children (n = 544) previously collected samples from individuals residing in malaria endemic regions in Africa namely Sukuta (Latitude: 13.4070° Longitude: 13.41033 -16.70815) in the Gambia, Takaungu (Latitude: -3.6667 Longitude: 39.7500), and Siaya (Latitude: -0.0833 Longitude: 34.2500) in Kenya. The data was obtained from a cross-sectional survey conducted among children aged below the age of nine years during periods of low and high malaria transmission (Figure 3.1).

Details of the study population are described elsewhere (Snow et al., 1997). Sera obtained from 3 blood samples of malaria naïve adults (volunteers from United Kingdom and Sweden) were used as negative controls. Pooled hyper-immune sera from Malawian adults was included as positive controls (Murungi et al., 2016).

	Sukuta, The Gambia	Kilifi North, Kenya	Kilifi South, Kenya	Siaya, Kenya
P. falciparum parasite prevalence among children 1–9 years old [4]	37%	49%	74%	83%
P. falciparum parasite prevalence among infants aged 3–11 months [4]	17%	26%	43%	59%
Estimate of force of transmission, *h*, no. of new infections/infant/year	0.41	0.60	1.12	2.11
Seasonality of disease index (months)	73% (September–November)	44% (June–August)	34% (May–July)	33% (May–July)
Period of hospital surveillance*	1/1/92–31/12/94 (4 years)	1/11/90–31/10/95 (5 years)	1/6/92–31/5/96 (4 years)	1/1/92–31/12/92 and 1/11/94–31/10/96 (3 years)
Cumulative incidence of malaria admission by age 10 years	247.48	241.79	154.04	164.38
Incidence of malaria admissions/1000 infants aged 1–11 months/year (no./total)	23.3 (66/2830)	59.5 (318/5342)	79.0 (407/5152)	84.6 (374/4420)
95% confidence interval	17.7–28.9	53.0–66.1	71.3–86.7	76.1–93.2

NOTE. From cross-sectional surveys among infants aged 3–11 months, force of infection (*h*) was estimated by using simple constant risk catalytic conversion model: $X(a) = 1 - e^{-ha}$, where $X(a)$ denotes proportion of persons of age *a* that have been exposed to parasite (i.e., evidence of asexual *P. falciparum* parasites in peripheral blood and/or evidence of total *P. falciparum* IgM in sera). Seasonality index represents proportion of cases seen over total period of surveillance presenting during 3 highest consecutive months. Cumulative sums of annual age-specific (single-year) rates of malaria admission for each area were derived to provide lifetime risk of malaria admission by 10th birthday.

* Given as day/month/year.

Figure 3.2: Characteristics of the Study Population

(Source: Snow et al., 1997)

3.5 Cloning and Expression of Selected PHIST Genes

3.5.1 RNA Extraction and Quality Assessment

Total RNA was extracted from 100 µL of pelleted iRBCs from culture-adapted *P. falciparum* 3D7 parasites at the trophozoite stage using Trizol reagent (Life Technologies, Thermo Fisher Scientific, USA) kit according to manufacturer's instructions.

3.5.2 cDNA Synthesis

Prior to cDNA synthesis, 2µL of the sample (RNA) was treated with DNase I for 20 minutes at 37˚C. Reverse transcription of the DNase treated RNA was performed using a

cDNA synthesis SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Following cDNA synthesis, the samples were preserved at -20^{o}C until use.

3.5.3 Primer Design

Three Pf3D7 gene sequences for Pf3D7_1401600, Pf3D7_1102500, and Pf3D7_0532400 were retrieved from PlasmodiumDB (plasmodb.org/plasmo/). Primers were designed manually based on the DNA sequence. The primers targeted and amplified the C-terminal region. Restriction sites for *Bam*H1 and *Sal*1 were added at both the 5' ends of the forward and reversed primers (Table 3.1). The best primer parameters were selected using the DNA calculator (www.sigma-genosys.com). The parameters included the appropriate concentration and melting temperatures.

3.5.4 PCR Amplification

2 µL of the cDNA was subsequently used as template to amplify PHISTb genes by PCR employing gene-specific primers in a 20 µL final reaction volume that had 1mM GoTaq master mix (Promega Corporation, USA) (10µL) 0.5mM Foward primer, 0.5mM Reverse primer, supplemented with 2.5mM $MgCl_2$. under the following thermocycler condition; initial denaturation at 95˚C for 3 minutes, 35 cycles of denaturation at 94˚C for 15 seconds, annealing at 50˚C for 30 seconds, extension at 68˚C for 2 minutes, and a final elongation step of 68˚C for 7 minutes (Table 3.1). The PCR products were electrophoresed through Red Safe (iNtRON Biotechnology, South Korea) stained 1% TBE agarose gel for 1 hour at 110V (Bio-Rad Model Powerpac Basic Power Supply, Bio-Rad Laboratories, Inc., USA) and cloned into pEXP5-CT TOPO vector (Invitrogen) as per manufacturer's instructions**.**

The PCR reaction included a negative control lacking the cDNA template. The lack of template was to test for contamination of PCR reagents. A negative control without the template was added to test for DNA contamination within the cDNA sample. Thermocycler (Applied Biosystems, Foster City, California, United States) was used for

PCR amplification under defined thermocycling conditions: Initial denaturation 94^0C for 15 minutes, 35 cycles of initial denaturation at 94^0C for 3 minutes, 50^0C for 30 seconds, 68^0C for 2 minutes and a final denaturation of 68^0C for 7 minutes.

Table 3.1: Gene-specific PCR primers for amplifying PHIST genes from cDNA and recombinant plasmid containing specific PHISTb gene inserts

Gene-targeted	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Expected Sizes (bp)
Pf3D7_1401600	ATGAAAGTTGAAGGAAGTGTTCATGT	TATGGTCACATATCTTTGGAC	1437
Pf3D7_1102500	ATGGAAGAAAATACATATTCAAGAGA	TATATAAGGAGCTTCTGTAATACT	1875
Pf3D7_0532400	ATGAATGTTTGTATGAGGGAGAGA	TCCAAGATTTGTCCTTTGTATTTTCAGT	1587

Table 3.2:Preparation of PCR master mix for use in gene amplification.

Reagent	Volume (µl) for 1×	Volume (µl) for 5×
GoTaq Master Mix	10	50.0
25 mM $MgCl_2$	1.2	6.0
Gene-specific forward primer	1.0	5.0
Gene-specific reverse Primer	1.0	5.0
Nuclease-free water	4.8	24.0
Template (cDNA)	2.0	10.0
Total volume	20	100

3.5.5 Analysis of PCR Products by agarose gel electrophoresis

The quality and quantity of PCR products was checked by using prepared agarose gels. Preparation of 1% agarose gels was done by mixing 1g of agarose powder (Promega, Madison, Wisconsin, USA) with 100ml of 1× TBE buffer in a conical flask. The mixture was dissolved by heating in a microwave oven for 3 minutes and left to stand in order to

cool just enough to allow the addition of 5 µl of RedSafe nucleic acid staining solution (iNtRON Biotechnology, South Korea) and cast in a tray containing gel combs of appropriate size (10-20 wells). The gel was allowed to set for 30 minutes before use. 20 microlitres of each sample was loaded into individual wells in the polymerized gel. A 3µl volume of 1 kilobase pair molecular weight marker (hyperladder I, Bioline, UK) was used to estimate the sizes of the PCR products. The loaded gels were run at constant voltage of 100 volts for 70 minutes and then viewed using digital gel imager when the run was completed (BioRad Molecular Imager, USA). The products were visualized to check for the size of bands by comparing with the molecular weight marker. The bands obtained for the target genes were analyzed for size and quantity.

3.5.6 Recovery and purification of the PCR products from agarose gel

In order to use high quality PCR products for cloning and other downstream experiments, the target amplicons were cleaned by resolving the products through 1% agarose gel to remove components such as; remaining primers, dNTPs, and buffers followed by recovery of specific DNA bands by gel purification. For this 20µl of PCR products were purified by separation in a 1% agarose gel, a band of interest excised from the gel under UV and stored at 4˚C awaiting subsequent analysis. DNA was recovered using appropriate commercial kits for extraction and purification of DNA from agarose gels according to the manufacturer's recommendations (QIAquick Gel Extraction Kit). Gel excision was done as follows; the DNA band was excised from the agarose gel. Excess agarose gel was removed to reduce the size of the gel slice. The gel slice was then placed in a colorless pre-weighed 1.5mL Eppendorf tube and the weight recorded. Gel Extraction was done according to the manufacturer's protocol (QIAquick, Gel Extraction Kit).

3.6 Gene Cloning

3.6.1 Preparation of LB-agar plates

Prior to starting the cloning process, all the media required was prepared. LB agar was prepared by dissolving 20g of LB (Lauria Bertani) broth powder (LB; 10g Bacto tryptone, 5g Bacto yeast extract, 5g NaCl, pH adjusted to 7.0 with NaOH) and 32g of agar in 1 liter of distilled water and autoclaved at 121^{o}C for 15 minutes. This media was cooled in a water bath to 50^{o}C and 1ml of ampicillin was then added and mixed gently to achieve a final concentration of 100 µg/ml. The media was dispensed into Petri dishes (25ml per petri dish) and left overnight to settle. The plates were preserved at refrigeration temperatures 4^{o}C. LB broth was prepared as described above except that agar was not added. More LB broth media was also prepared and supplemented with chloramphenicol (34µg/ml) and both ampicillin (100µg/ml) and chloramphenicol (34µg/ml). This media was preserved in aliquots of 250 ml at room temperature (22-25^{o}C) in sterile airtight glass bottles until use.

3.7 Ligation and Transformation

3.7.1 Preparation of Competent Cells

TA cloning was used to introduce PCR products of the targeted genes into a cloning vector. TA cloning takes the advantage of the lack of 5'-3' proofreading activity in DNA polymerase used for PCR and hence introducing an adenosine triphosphate residue to the 3' ends of the double-stranded PCR product. Such PCR amplified product can be cloned into a linearized vector with complementary 3' T overhangs. To clone the PCR products, pEXP5-CT/TOPO vector (Invitrogen) was used.

3.7.2 Cloning of PHISTb genes into pEXP-CT/TOPO Vector

The reaction was performed according to the manufacturer's instructions. The freshly prepared PCR products were cloned as stipulated shown in Table 3.5. The cloning was based on this plasmid map.

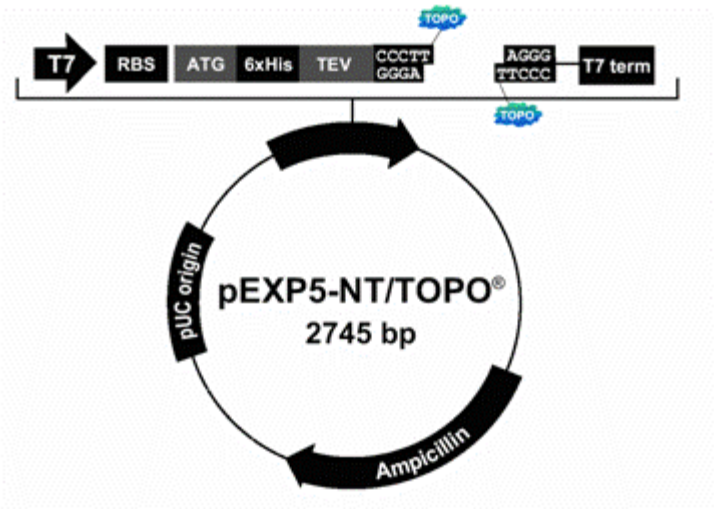


Figure 3.3: Plasmid map for cloning

Table 3.3: TOPO Cloning Reaction

Reagent	Chemically Competent *E. coli*
Fresh PCR product	0.5-4µL
Salt solution	1µL
Dilute Salt Solution	--
Water	added to a final volume of 5µL
Vector	1µL

Final Volume	**6µL**

The reaction was mixed gently and incubated for 45 minutes at room temperature 25°C.pEXP5-CT/TOPO vector was used since it clones PCR products directly into expression cells hence by-passing sub-cloning steps often associated with other TA cloning vectors. The reaction was placed on ice awaiting transformation to TOP10 Competent *E. coli.*

3.7.3 Transformation of One Shot (Top 10*) E. coli* cells

To express PHIST proteins, 2 µl of the TOPO cloning reaction previously explained (**Table 3.5**) above was added into a vial of 50 µL of chemically competent *E. coli* cells thawed in ice and mixed gently. The reaction was incubated on ice for 30 minutes, heat-shocked for 30 seconds at 42^{o}C then placed on ice 2 minutes to transform the competent cells with the gene-vector construct. The transformed cells were allowed to recover in 250 µl of S.O.C (Super Optimal broth with Catabolite repression) medium with gentle shaking (200rpm) at 37 ^{o}C for 1 hour in shaking incubator (INNOVA 4000, New Brusnswick Scientific). Meanwhile, the LB-agar plates (containing ampicillin) were placed at room temperature inside a hood for warming up. 100 µl from each transformation reaction was spread on a pre-warmed LB agar plate supplemented with ampicillin and incubated at 37^{o}C overnight.

After overnight incubation, single colonies were picked up from the plates using a sterile glass colony picker and subcultured in sterile culture tubes containing 2ml LB media with ampicillin for approximately 18h. The colonies were screened by colony PCR using gene-specific primers to check that the inserts were successfully ligated to the vector and using the second set of primers consisting a T7 forward primer and a gene-specific reverse primer to screen for the colonies in which the genes had been integrated into the plasmid in the correct orientation for protein expression (see Table 3.1 for the primer sets used). The PCR conditions to be used are shown in Table 3.2. The positive insert

clones on LB-agar plates was cultured in LB broth, supplemented with 100µg/mL ampicillin. Recombinant plasmids were purified using PureYield^{TM} plasmid miniprep system (Promega Corporation, Madison, WI) according to the instructions of the manufacturer as described below.

3.8 Plasmid Isolation

The bacterial cell pellet was resuspended in 600µL of TE in a 1.5mL microcentrifuge tube, followed by addition of 100µL of Cell Lysis Buffer (Blue) (Promega Corporation, Madison, USA) and mixed by inverting six times. This was followed by addition of 350µL of cold (4-8˚C) Neutralization solution and mixed thoroughly by inverting. The lysate was centrifuged at maximum speed in a microcentrifuge for 3 minutes at 16060×g at room temperature. This would pellet the bacterial chromosomal DNA and other cell components while the plasmids remain at the supernatant. The supernatant was transferred using a pipette to a PureYield minicolumn placed on a collection tube. The contents of the tube were centrifuged at 16060×g for 15 seconds at room temperature. The flow through was discarded and 200µL of Endotoxin Removal Wash added to the minicolumn and centrifuged under the same conditions stated previously. The next step was the addition of 400µL of the Column Wash Solution (CWC) to the minicolumn and centrifuged again. The minicolumn was transferred to a 1.5 mL microcentrifuge tube followed by addition of 30 µL of Elution Buffer directly to the minicolumn matrix. This was allowed to stand for 1 minute at room temperature, and centrifuged for 15 seconds to elute the plasmid DNA. The purified plasmids were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and sequenced.

3.9 Expression of Cloned PHIST Genes

3.9.1 Preparation of BL21 (DE3) plysS Chemically Competent Cells for Protein Expression

The *E. coli* cells for protein expression were prepared from the commercial stock of One Shot® BL21 (DE3) pLysS Chemically Competent *E. coli* (Invitrogen). 2 µL of the stock cells was used to inoculate 4ml of sterile LB broth supplemented with chloramphenicol. This culture was incubated overnight at 37^{o}C in shaking incubator. This overnight culture was used to inoculate 20ml of sterile LB broth supplemented with chloramphenicol, incubated at 37^{o}C in a shaking incubator and the OD_{600nm} monitored until it reaches 0.4. The suspension was centrifuged at 4000 rpm at 4^{o}C for 10 minutes. The bacterial pellet was re-suspended on ice in 5ml of ice-cold 100mM $CaCl_2$ and the suspension left on ice for 30 minutes before centrifugation at 4000rpm, 10 minutes at 4^{o}C.The bacterial pellet was again re-suspended in 2 ml volume of ice-cold 100mM $CaCl_2$. 200µl aliquots of the cells was frozen at -80^{o}C. For quality control, a comparison of the transformation efficiency between the prepared and the commercial cells was performed.

3.9.2 Transformation of BL21 (DE3) plysS cells with Pf3D7_1401600, Pf3D7_1102500, Pf3D7_0532400-pEXP5-NT plasmids for protein expression

200µl of BL21 (DE3) plysS competent cells were transformed with 2 µl of plasmid constructs of Pf3D7_1401600, Pf3D7_1102500, and Pf3D7_0532400-pEXP5-CT which contain gene fragments to be expressed as recombinant proteins with a histidine tag on the N-terminal region of the protein.

3.9.3 Induction of Protein Expression

The optimal expression conditions for the PHISTb proteins was done. A starter culture was prepared by inoculating 2mL of the LB broth with recombinant *E. coli* clone of BL21 (DE3) plysS competent cells. After transformation, the bacteria were grown

overnight, and the following day, pure colonies from the plates were subcultured at 37^{o}C in a shaking incubator (200rpm) in 4ml LB broth with 50µg/mL ampicillin overnight. These cultures were then expanded in 200 ml of the same sterile media with scheduled analysis of the optical density (OD) of the bacteria culture. At an OD_{600nm} range of 0.4-0.6, an aliquot of 1ml volume from each tube was picked, centrifuged and the pellet frozen at -20^{o}C. The aliquots picked was used later to analyze for protein expression as the negative reference (no induction). The remaining amount of culture was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1mM for protein expression and incubated at 37^{o} C until the OD_{600nm}had reached 2. After 1 hour of incubation, 1 ml aliquot of the culture was made and the pellet recovered by centrifugation (this served as the positive case-induced to analyze for protein expression). The bacterial pellet from the remaining amount of culture was recovered by centrifugation at 4000xg for 20 minutes and preserved at -20^{o}C (for protein extraction). The sample was collected at 1-hour interval for 6 hours and the last collection was taken after an overnight incubation.

3.9.4 Extraction of recombinant PHISTb proteins Pf3D7_1401600, Pf3D7_1102500, Pf3D7_0532400 his and analysis by SDS

Previously pelleted bacterial cells that had been induced using IPTG and grown to an OD of 2 were re-suspended in 5 volumes of bugbuster protein extraction solution buffer (Invitrogen) and benzonase nuclease added to a final concentration of 1mM. The mixture was incubated at 4^{o}C for one hour with gentle agitation and then centrifuged at 4000 x g at 4^{o}C for 20 minutes. The supernatant was aspirated from the tube, and both the pellet and supernatant was analyzed by SDS-PAGE. A 1:1 dilution of the supernatant and protein denaturing buffer (Laemli buffer with mercaptoethanol) was mixed, heated at 95^{o}C for 10 minutes and resolved on an SDS gel. Both the pellet and supernatant were run on the gel to identify the fraction the proteins contained. Fifteen microliters of each sample was separated on a 12% polyacrylamide gel. Initially, the electrophoresis was set at 80V until the samples enter a resolving gel, and then it was

raised to 120V for 1 hour. To visualize the proteins separated in the gels, Coomassie Brilliant Blue staining (20% methanol, 1%acetic acid and 0.15% R-250 dye) was employed. The gel was stained overnight with gentle shaking. The next day, the gel was transferred into a destaining solution to wash off the excess dye. Kimwipes were placed in the solution to absorb the excess dye released from the gel. A band of increasing intensity at regions of the expected size range was seen for the recombinant PHISTb proteins, and this was confirmed by performing Western blot to check for reactivity.

3.10 Proteomic Analysis by Western Blot and mass spectrometry

The identity of the recombinant PHISTb proteins was confirmed by Western Blot (Burnette, 1991). The recombinant PHISTb proteins will be expected to carry a 6×His-tag at the C-terminal region, thus detection was done using the C-terminal anti-His antibody. The protein samples were separated on a 12% SDS-PAGE and then electrophoretic ally blotted onto a PVDF membrane (ThermoFisher Scientific, USA). This was achieved after an overnight incubation at 4˚C in transfer buffer (0.2M zwitterionic glycine, 25mM Tris base, 20% [v/v] methanol) at 30 V. The membrane was blocked in 5% [w/v] non-fat dry milk in 1×PBS (137mM NaCl, 2.7Mm KCl, 100Mm Na_2HPO_4, 2mM KH2PO4, pH 7.4) for 3 hours, followed by incubation in 1% w/v non-fat dry milk in 1×PBS containing the HisProbeTM-HRP (1:5000) for 1 hr with shaking. The membrane was washed three times for 5 minutes in PBS-Tween (1× PBS pH 7.4 0.05% [v/v] Tween 20). This was followed by three more washes each done at 1 minute in 1×PBS. Hydrogen peroxide was added to the developer, mixed quickly and added to the blot. The blot was left shaking for a few minutes to allow the development for the reactive protein. The reaction was left for 2 minutes to let the membrane air dry, then viewed under the digital imager. The transfer buffer to be used to transfer the proteins from the gel to a PVDF membrane was prepared by mixing 200 ml of methanol and 100ml of 10x Tris Glycine in 700ml of cold double distilled water to make 1L of working solution. TBST was prepared by dissolving 6.05g of Tris and 8.76g of NaCl in 800ml of distilled water, pH was adjusted to 7.6, and the buffer topped up to a liter using

distilled water. Tween 20 was then added to this buffer to a final concentration of 0.1%. The two buffers were kept at 4^{o}C until use. In-gel digestion was done for mass spectrometry analysis using the protocol outlined in Kessler lab-proteomics protocols

Following the western blot and mass spectrometry confirmation, the protein was purified under denaturing conditions to recover it from the inclusion bodies. A Qiagen protein extraction kit was used for purification, and the buffers used in the kit contained 8M urea as the denaturing agent. Methods of getting rid of the urea were done to recover the pure proteins as it would interfere with down-stream assays, especially the ELISA.

3.11 Dialysis against Decreasing Urea Concentrations

The purified protein was re-suspended in a buffer containing 8M urea, NaCl, and Tris. It was dialyzed against buffers containing 50 mM NaCl, 20 mM Tris and Urea in decreasing concentrations (from 6M to 0M). This reduced salts concentration, got rid of urea and refolded the protein to its normal conformation. Dialysis was done for two days to get rid of all urea completely.

After dialysis, the protein was quantified using Bradford's and Bicinchoninic acid (BCA) protein quantification assays. BCA ensures no salts and urea at high concentrations exist which interfere with down-stream analysis on proteins. 4 ml was quantified and the protein concentration determined.

3.12 Sample Preparation, LC-MS/MS analysis, and Antigen Validation

PHISTb antigens were validated via mass spectrometry. Aliquots of 10 µg of the purified PHISTb proteins (antigens) were separately denatured in 50 mM Tris-HCl (Sigma-Aldrich, United States) containing 8 M urea (Sigma-Aldrich, United States), pH 8. Subsequently, the denatured proteins were reduced with 40 mM Dithiothreitol (Sigma-Aldrich, United States) at room temperature with shaking for 1 hour and alkylated in the dark for 1 hour with 80 mM iodoacetamide (Sigma-Aldrich, United States). Proteins were precipitated with four times the sample volume of cold acetone for

1 hour at -20°C and the protein pellet obtained after discarding the supernatant following centrifugation for 10 minutes at 14,000 ×g at room temperature. Proteins were resuspended in 15 µL of 6M urea in 50mM Tris-HCL (pH 8) buffer and digested with trypsin/Lys-C mix (Promega) according to the manufacturer's instructions using the two step in-solution digestion. Peptides obtained were desalted using C18 Spin columns according to manufacturer's instructions (Thermo Scientific), dried in a Speedvac concentrator and re-suspended in 15 µL of resuspension solvent (99% H_2O, 1% acetonitrile, 0.1% formic acid).

Five microliters of peptides were loaded using a Dionex Ultimate 3000 nano-flow ultra-high-pressure liquid chromatography system, UHPLC (Thermo Scientific, United States) on to a 75 µm × 2 cm C18 trap column (Thermo Scientific, United States). Chromatographic separation of peptides was carried out on a reverse-phase 25 cm-long column maintained at 40°C over a 40-minutes elution gradient (2 to 30% of mobile phase B; 80% acetonitrile with 0.1% formic acid) at a flow rate of 0.3 µL/minutes. The resolved unique peptides were measured using LC instrumentation consisting of a Dionex Ultimate 3000 nano-flow UHPLC interfaced via a nano-electrospray ion source to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, United States). The ms^1 settings were: Resolution, 70000; Automatic gain control (AGC) target, 3e6; maximum injection time, 100 ms; scan range, 380-1600 m/z; while the ms^2 settings were: Resolution, 17500; AGC target, 5e4; maximum injection time, 100ms; isolation window, 1.6 m/z. The top 10 most intense ions were selected for ms^2 and fragmented with higher-energy collision fragmentation using normalized collision energy and were subsequently excluded for the next 20s. Mass spectrometry raw files for expressed recombinant PHISTb antigens (Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600) were searched on Proteome Discoverer software version 1.3.0.339 using the Mascot server (Matrix Science) using 3D7 *P. falciparum* protein FASTA sequence database. Cysteine carbamidomethylation was set as a fixed modification and deamidation of asparagine or glutamine and methionine oxidation as variable modifications. The false

discovery rate (FDR) was set to 0.01 for both proteins and peptides and a maximum of two missed cleavages were allowed in the database search. A minimum of two unique peptides for a protein was considered a positive identification.

3.13 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was done to test the purified protein against sera from individuals exposed to malaria to determine if antimalarial antibodies recognized each of Pf3D7_1401600, Pf3D7_1102500, Pf3D7_0532400 antigens. Samples obtained from a previously cross-sectional study was adopted. A sample size of 544 was used. Standard ELISA protocol was used to test the protein against serially diluted MIG (Malaria Immune Globulin) (pooled sera from Malawian donors exposed to malaria), PHIS (pooled hyper-immune sera) obtained from adults previously exposed to malaria from Kenya. Sera from UK donors who have not been exposed to malaria were used as a negative reference for this test. Crude schizont extracts used in the study were obtained from *P. falciparum* cultures. Results were expressed as OD ratio=OD sample/OD naïve serum pool as described (Koffi *et al*., 2017).

3.14 Data Analysis

3.14.1 Bioinformatics

A search to identify the protein sequences for the PHISTb genes was done using the tBLASTx algorithm of the NCBI GenBank and PlasmoDB (Plasmodium database).

3.14. 2 Statistical Analysis

Statistical analysis was conducted using R. The antibody levels in different groups were compared using the Mann-Whitney signed rank test, Spearman rank correlation test for the non-normally distributed paired data and fisher exact test. Normality of the data was tested using the Shapiro-Wilks test. For data that does not normalize after

transformation, Kruskal-Wallis rank sum test was used to verify the differences between the groups. P-values which are <0.05 was considered as significant.

CHAPTER FOUR

RESULTS

4.1 Amplification of PHISTb genes

PHISTb genes were amplified and DNA purified from agarose gels for cloning and transformation. The expected band size for Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 was 1587bp, 1437bp and 1875bp respectively (Figure 4.1). A PCR positive amplicon was included as positive control, while negative control included a reaction without the template. The 1kb hyperladder (Bioline, Germany) served as a marker to confirm the expected band sizes for each amplified gene.

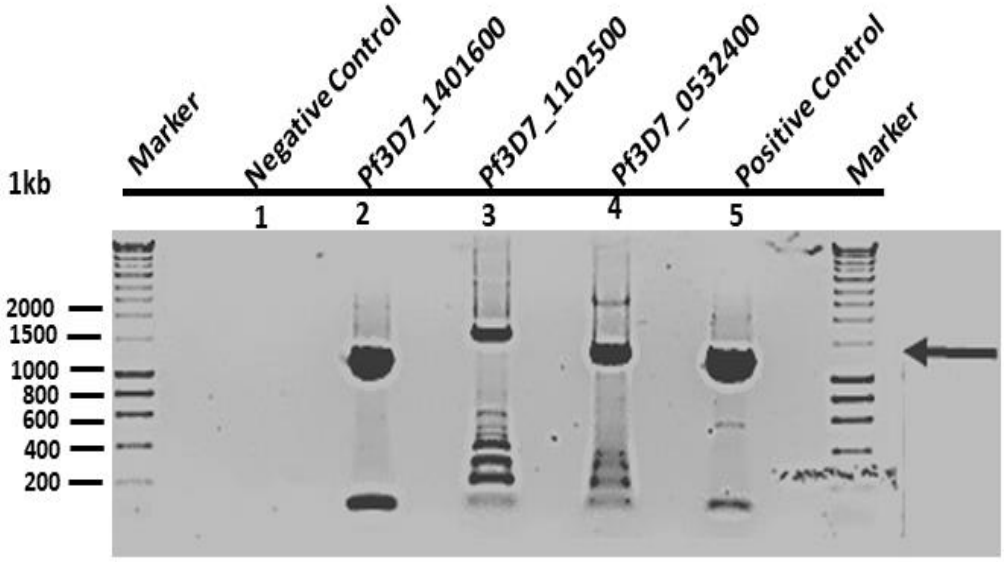


Figure 4.1: Amplification of PHISTb genes by PCR.*PHIST* genes Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600 were amplified from cDNA.

PCR products were separated on a Red-safe-stained 1% agarose gel at 60V for 1hr. The expected band sizes were compared against the 1kb hyperladder (Bioline, Germany).

Both a negative and a positive control (known positive amplicon) were included for quality control.

4. 2 Screening Colonies for Successful Transformants

PHIST genes amplified were cloned into pEXP5-CT TOPO vector, screened for positive colonies, and subsequently transformed into Top 10 chemically-competent *E. coli* cells (Figure 4.2). Colony PCR used to identify successful transformants. Plasmids with correct insert were sub-cloned into BL21 (DE3) competent cells for protein expression. The cells were cultured overnight at 37˚C. Recombinant PHISTb expression was successful using the *E. coli* BL21 (DE3) cells and these were confirmed using SDS-PAGE and Western Blot assays before purification and subsequent confirmation using Mass spectrometry.

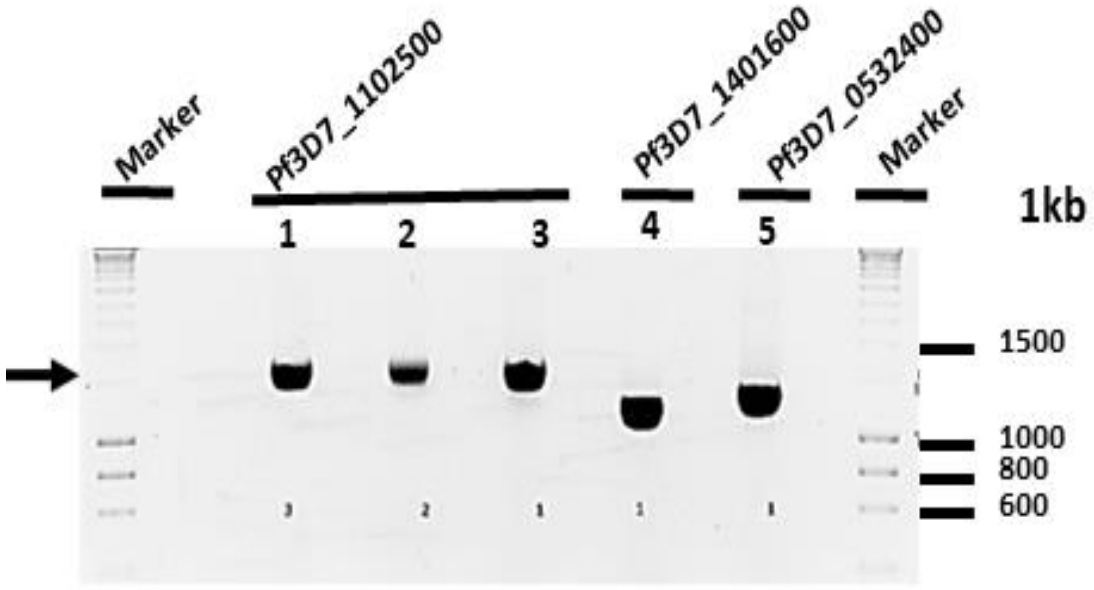


Figure 4.2: Screening of bacterial colonies by colony PCR.

Three colonies were confirmed PCR positive for Pf3D7_1102500, and one colony selected for either Pf3D7_1401600 or Pf3D7_0523400.

4.3 Sequence analysis of PHISTb Plasmid Constructs

The purified recombinant plasmids were sequenced. Sequences were edited and analysed through BLAST of the National Center for Biotechnology Information GenBank (http://www.ncbi.nih.gov/).

4.4 SDS/PAGE Western Blot analysis

The expressed recombinant PHISTb antigens were confirmed using SDS-PAGE. The expected molecular weight was 55.4kDa, 62.5kDa and 46.5kDa for Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600 respectively. The *E. coli* BL21 (DE3) cells were lysed and purified. SDS-PAGE gels are shown for each antigen (Figure 4.3). The Rainbow molecular marker (Sigma-Aldrich, Germany) was used and is marked M for SDS-PAGE (Figure 4.3). Washes were done for each antigen prior to five elution. The expressed PHISTb antigens were transferred from the gels to the nitrocellulose membrane for western blot using Anti-6X His tag® antibody (Abcam, USA). The bands indicate the expected sizes for each antigen and arrows show the molecular weight according to the marker (Figure 4.4).

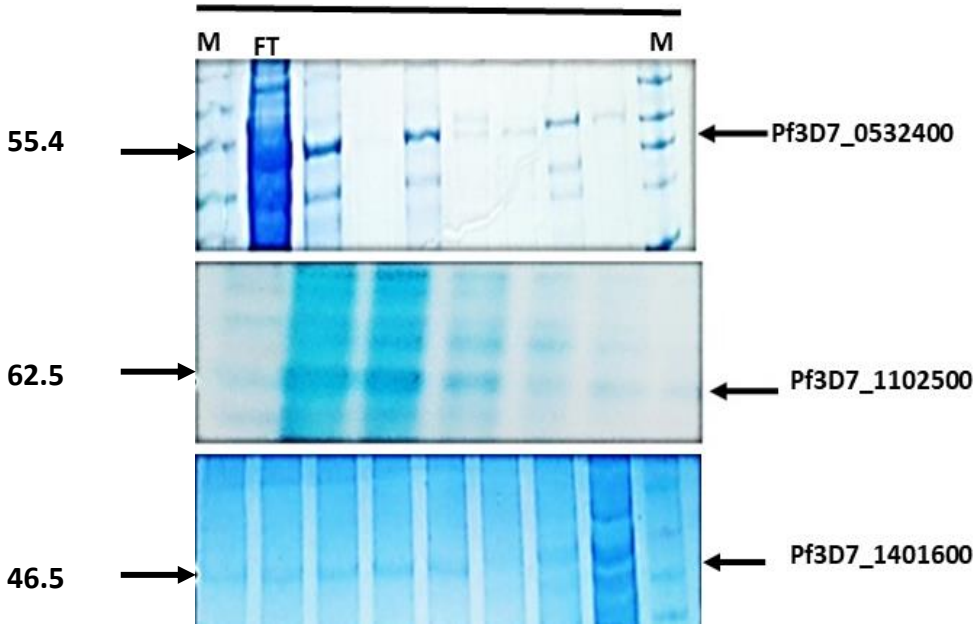


Figure 4.3: SDS-PAGE for expressed recombinant PHISTb proteins.

Arrows indicate the expected molecular weight of each antigen in line with the marker. FT is the flow through, M is the molecular marker. Corresponding molecular weights of each antigen are indicated

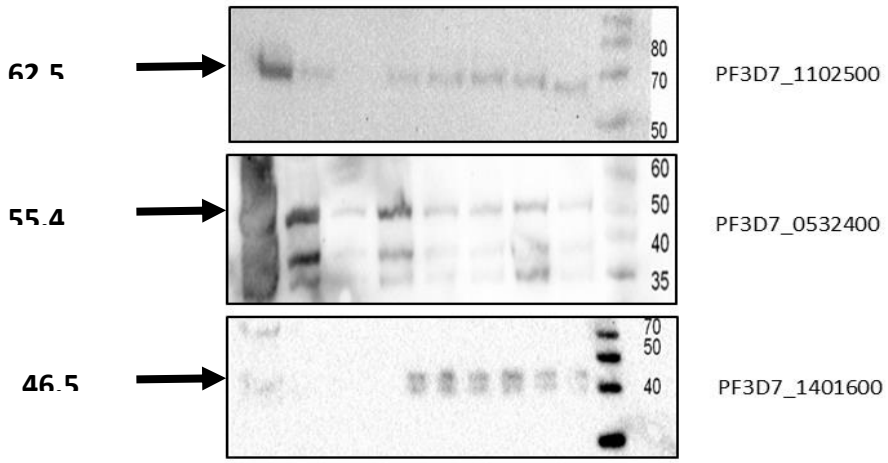


Figure 4.4: Western blot to confirm identity of recombinant PHISTb antigens.
The identity of the recombinant PHISTb proteins was confirmed by western blot. Recombinant PHISTb antigens were expressed in a bacterial expression system and purified under denaturing conditions for subsequent functional assays. The expressed proteins were confirmed by 12 % SDS-PAGE by the presence of protein bands at molecular weights 55.4kDa, 62.5kDa and 46.5kDa corresponding to Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600 respectively.

4.5 Mass Spectrometry Analysis

In order to confirm the identity of each PHISTb antigen used prior to ELISA, mass spectrometry was performed. Briefly, proteins were identified based on the analysis of peptides generated following proteolytic digestion using trypsin.

Table 4.1: Mass spectrometry of target antigens

Target Antigen	Antigens Identified by Mass Spectrometry	Unique Peptides
Pf3D7_0532400	Pf3D7_0532400 (PHISTb)	35
Pf3D7_1102500	Pf3D7_1102500 (PHISTb)	18
Pf3D7_1401600	Pf3D7_1401600 (PHISTb)	28

Table 4.2: Characteristics of Antigen Pf3D7_0532400

Sequence (Identified peptides)	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	Exp Value
TYGQSTNPqVYR	3	1	1	PF3D7_0532400	Q9(Deamidated)	7.17599E-10
IDLGADIIENGLNK	3	1	1	PF3D7_0532400		4.56302E-09
KNPEmLTENQINEEENENTK	4	1	1	PF3D7_0532400	M5(Oxidation)	2.85942E-08
TYGQSTNPQVYR	5	1	1	PF3D7_0532400		2.53667E-08
kIDNLDLYDENIIDSMK	3	1	1	PF3D7_0532400	K1(Carbamyl)	1.84631E-07
KNPEMLTENQINEEENENTK	2	1	1	PF3D7_0532400		1.8443E-07
KNPEMLTENQINEEENENTkVGGANTK	6	1	1	PF3D7_0532400	K20(Carbamyl)	1.81162E-07

Table 4.3: Characteristics of Antigen Pf3D7_1102500

Sequence (Identified peptides)	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	Exp Value
HImEYDNSENTIDDLSEEENYNQLFNLLYR	2	1	1	PF3D7_1102500	M3(Oxidation)	1.62405E-10
HImEYDNSENTIDDLSEEENYnQLFNLLYR	6	1	1	PF3D7_1102500	M3(Oxidation); N22(Deamidated)	1.25938E-07
HIMEYDNSENTIDDLSEEENYNQLFNLLYR	2	1	1	PF3D7_1102500		1.23968E-07
DLSNVSIWDEYELDLYNVK	1	1	1	PF3D7_1102500		2.66143E-07
GVKPNVYENIEVEEDPNEK	3	1	1	PF3D7_1102500		7.30209E-07
YGEQLNNDFMGNINEQEYITGK	2	1	1	PF3D7_1102500		2.14856E-06

Table 4.4: Characteristics of Antigen Pf3D7_1401600

Sequence (Identified peptides)	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	Exp Value
KEEEEEEEEEKGVDVAEDEDALK	13	1	1	PF3D7_1401600		2.55334E-12
GVDVAEDEDALKDSTASGDSSTSR	4	1	1	PF3D7_1401600		4.8916E-09
TSDYIDAQDSDALVEDEVTEEENISDESVK	3	1	1	PF3D7_1401600		2.16697E-07
DGDGcYSTGPNGQYNEcMK	3	1	1	PF3D7_1401600	C5(Carbamidomethyl); C17(Carbamidomethyl)	7.51939E-08
SSTLSQGNYSNFK	3	1	1	PF3D7_1401600		7.45625E-07
KYNVDNSFSEK	2	1	1	PF3D7_1401600		8.32018E-07

Recombinant PHISTb antigens were positively identified from the 3D7 *Plasmodium falciparum* protein FASTA sequence database. As indicated, a minimum of two unique peptides for each protein was considered as positive. The samples were subsequently utilized for evaluation of antibody responses against PHISTb antigens through enzyme-linked immunosorbent assay (ELISA)

4.6 Recombinant PHISTb Antigen Responses to Pooled hyper-immune sera (PHIS) and Sera from European malaria naïve adults (UK06, EU35, SWE03)

In the current study, pooled hyper-immune sera (PHIS) was used as the positive controls and sera from malaria naïve adults as the negative control. Immune recognition was determined following the formula (mean + 3 Standard Deviation) of all the negative controls used. Human sera was considered positive if the OD_{492nm} values fell above the calculated cut-off (Figure 4.5). This was also used to determine the antigen coating concentration for subsequent ELISA assays.

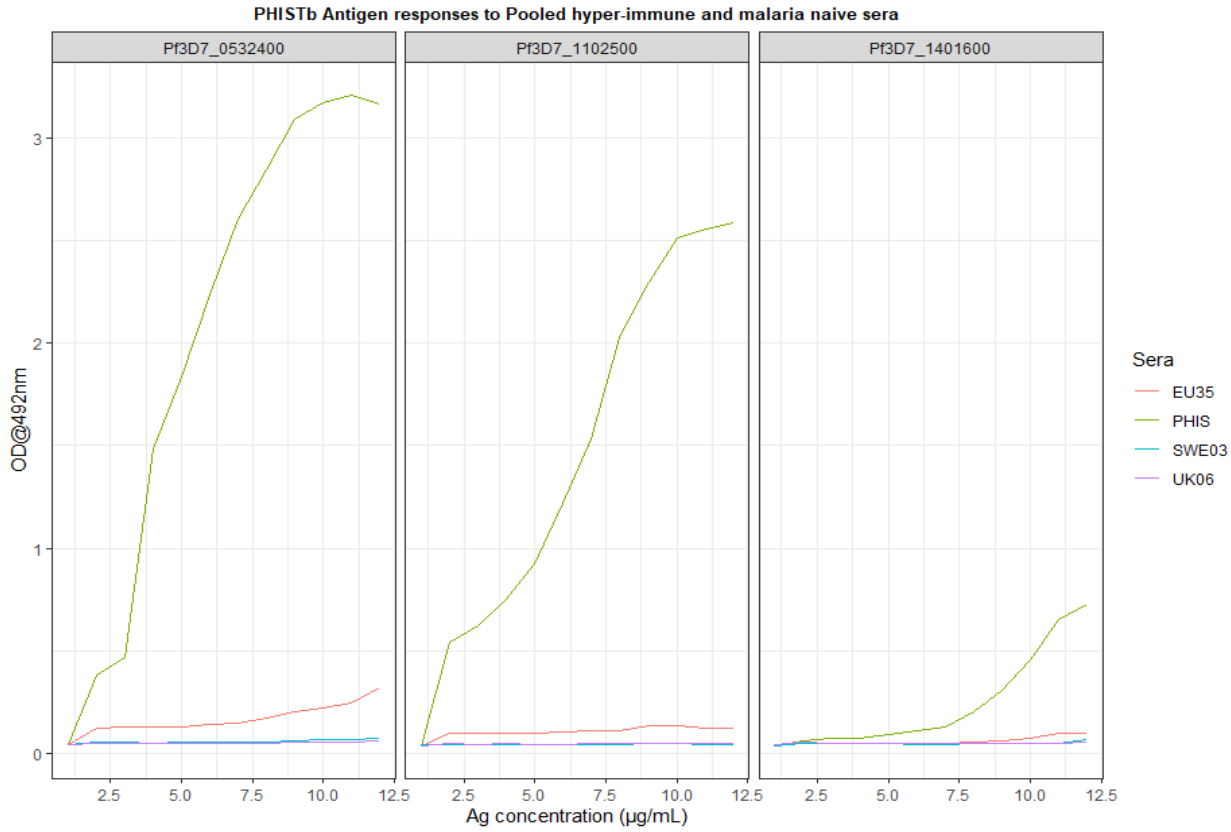


Figure 4.5: Antibody responses against PHISTb antigens (Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600.

Antibody responses against PHISTb antigens illustrated. Pooled hyper-immune sera (PHIS) (positive control) and serum from malaria naïve adults from Europe (UK06, EU35 and SWE03) (negative control) Antibody seropositivity determined as the cutoff above the mean plus 3 standard deviations of 3 malaria naïve adult sera. The cut-off was +/-0.25. Antibody responses measured at a wavelength of $OD_{492nm.}$

4.7 Evaluation of trend in antibody responses

Children were categorized into four classes (1-2, 3-5, 6-8, 9-11) to evaluate trend in antibody responses against recombinant PHISTb antigens (Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500) and crude schizont extract obtained from *P. falciparum* culture. Children aged below 2 years had low antibody titres but there was no relation between the responses and age (Figure 4.6)

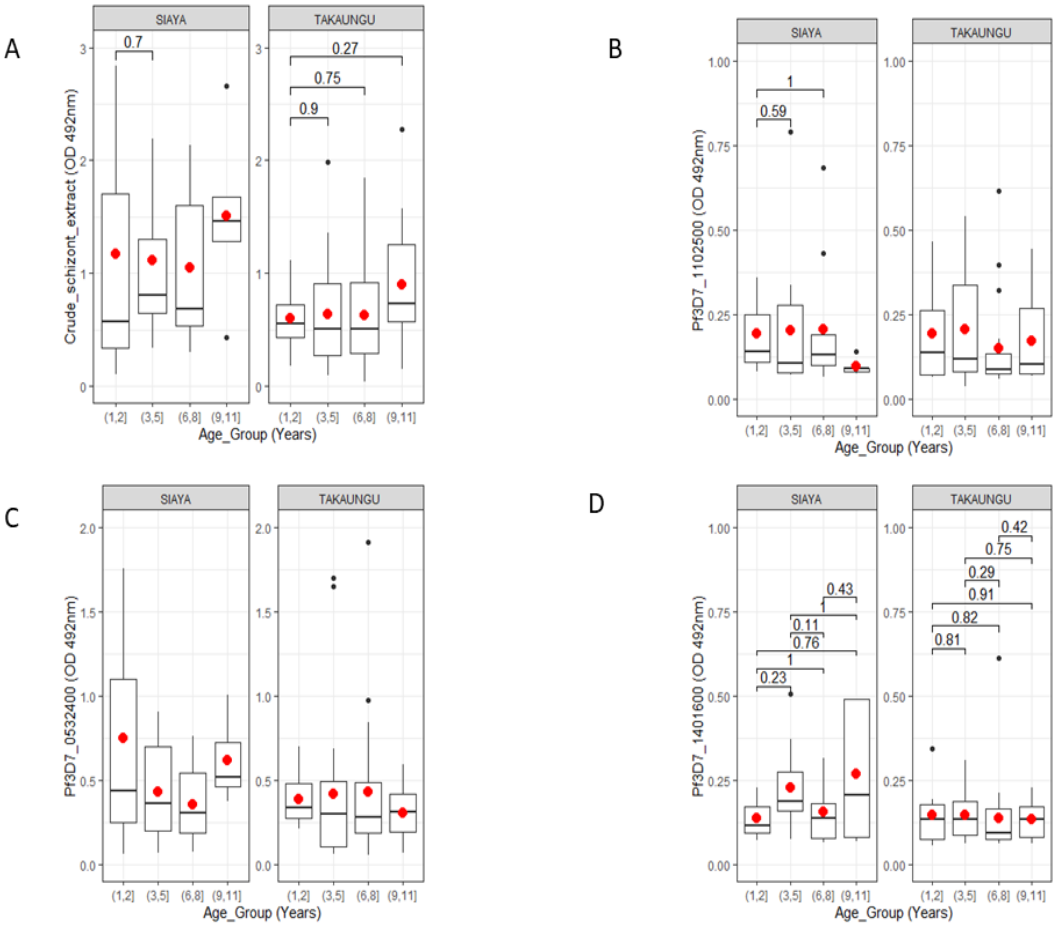
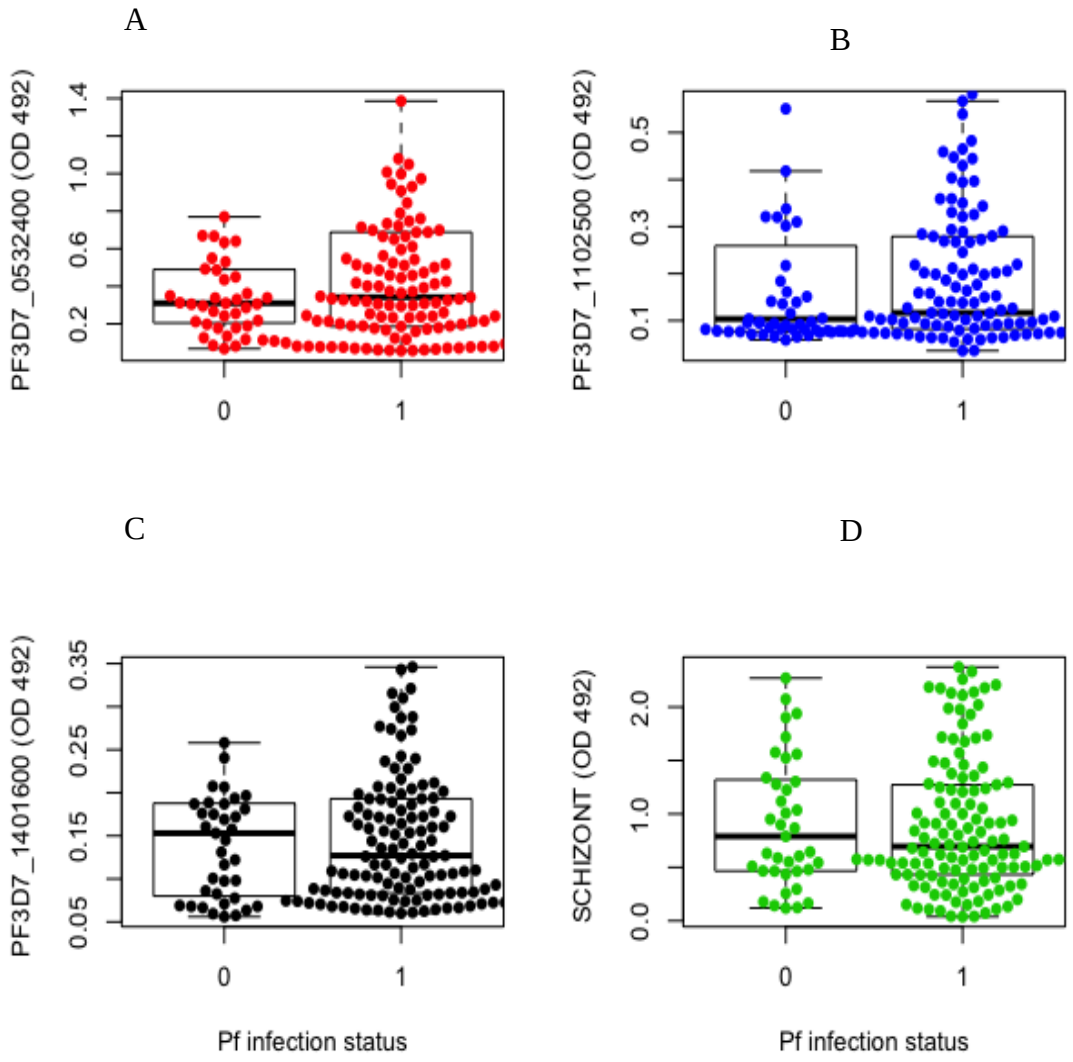


Figure 4.6: Antibody responses in children by age classes (1-2,3-5,6-8,9-11) against crude schizont extract (A), and recombinant PHISTb antigens, Pf3D7_1102500 (B), Pf3D7_0532400 (C) and Pf3D7_1401600 (D).

Antibody responses were measured in OD@492nm. Boxes indicate median and interquartile ranges. The dots indicate the mean antibody response against antigens.

A correlation analysis was performed to determine association between crude schizont extract and recombinant PHISTb antigens with age. There was a negative association between age and antibody responses to both Pf3D7_0532400 and Pf3D7_1102500 but this was not significant in both cases (p-value=0.5941 and p-value=0.6685 respectively). There was a positive association between the crude schizont extract and Pf3D7_1401600 with age, but this was not significant (p-value=0.591 and p-value=0.938 respectively).

4.7.1 Antibody levels between children who tested positive and negative for parasitemia



Antibody levels between children who had tested positive for *P. falciparum*, and those who were negative was compared (Figure 4.7). There was an increase in the level of responses to various antigens in infected children relative to those who tested negative. However, the difference between these two groups was not significant (A; p=0.9, B; p=0.83, C; p=0.33, D; p=0.40).Figure 4.7: Antibody levels between children with (1) and without (0) parasitemia.

Boxes indicate the median and interquartile ranges, whiskers indicate maximum and minimum values and filled circles show outliers. Pf indicates *P. falciparum* infection status. No significant difference is evident in individuals tested positive and negative for *P. falciparum* infection across all tested antigens (A-D). *P. falciparum* schizont extract is included to cater for confounding outcomes.

4.7.2 Evaluation of correlation of antibody responses to recombinant PHISTb antigens and schizont extract.

Correlations in antibody responses for different antigens was evaluated. There was a positive correlation in antibody response between the crude schizont extract and Pf3D7_0532400, and this was significant (p-value = 0.005816). There is a stronger correlation in antibody responses between Pf3D7_0532400 and Pf3D7_1102500 (p-value = 4.412e-07) compared to Pf3D7_1401600 (p-value = 0.07991). There is a stronger correlation between Pf3D7_1401600 and Pf3D7_1102500 (p-value < 2.2e-16).

Table 4.5: Spearman's rank correlation test was determined between schizont extract and PHISTb antigens.

	Crude schizont extract	**Pf3D7_0532400**	**Pf3D7_1102500**	**Pf3D7_1401600**
Crude schizont extract	1			
Pf3D7_0532400	0.65	1		
Pf3D7_1102500	0.03	0.08	1	
Pf3D7_1401600	0.09	0.02	0.6	1

The data presented below shows a correlation in antibody response in PHISTb antigens and the schizont extract. Statistically significant values P value of<0.05 are shown in boldface.

There was a positive correlation between Pf3D7_0532400 and schizont extract (p=0.005), a similar outcome depicted for Pf3D7_1102500 and Pf3D7_1401600 (p-value < 2.2e-16).

4.7.3 Antibody responses across different geographical localities with variations in malaria transmission levels

Antibody responses to schizont extract and recombinant PHISTb antigens were compared across different geographical locations with varying transmission intensities. Siaya exhibit high transmission intensities, while Takaungu and Sukuta have moderate and low malaria transmission respectively (Figure 4.8). The sample numbers used in the study were 201, 190 and 153 for Siaya, Takaungu and Sukuta respectively. There was a difference in antibody responses across the three geographical locations (p=0.0001). Our data suggests an association between antibody levels and transmission intensities. In Pf3D7_0532400, there is a significant difference in antibody response between the high, moderate and low malaria transmission areas (p=0.00071 and p=0.0034). Antibody responses to Pf3D7_1102500 vary with transmission intensity but this is not statistically different (p=0.13). However, there is a significant difference in antibody responses between Siaya and Sukuta (p=0.0023). A high antibody response is recorded for both schizont extract and Pf3D7_0532400, compared to Pf3D7_1102500 and Pf3D7_1401600 which did not show significant differences in antibody responses against different transmission intensities (p=0.13 and p=0.57 respectively). A high antibody response is recorded for both the schizont extract (A) and Pf3D7_0532400 (D) in Siaya compared to Takaungu and Sukuta. Low antibody levels recorded for Pf3D7_1102500 (B) and Pf3D7_1401600 (C). There is no statistical difference in antibody levels for Pf3D7_1401600 between Siaya and Takaungu (p=0.2).

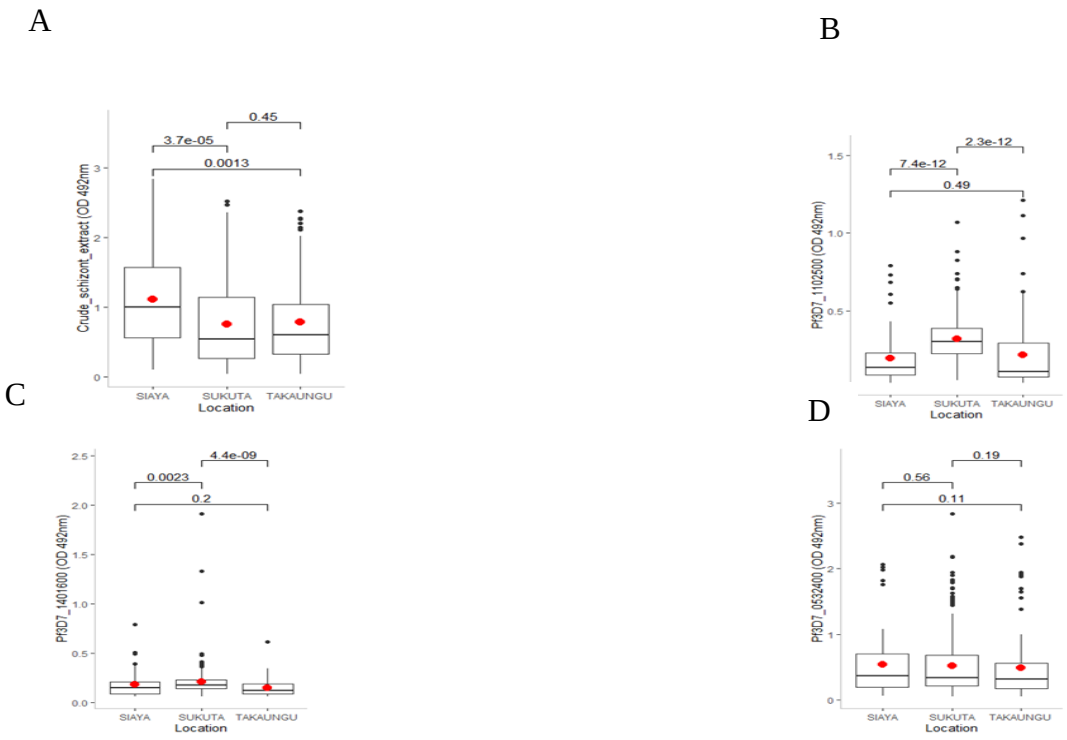


Figure 4.8: Pattern of antibody responses across different transmission intensities.

The figure shows antibody responses to schizont extract (A) and PHISTb antigens (B-D) across three different geographical locations with varying malaria transmission intensities. Sukuta in The Gambia is predominantly a low malaria transmission area, while Takaungu and Siaya have moderate and high transmission intensities respectively. On the y-axis is the antibody response for each recombinant PHISTb antigen and schizont extract, while the x-axis shows the geographical location. Boxes indicate the median and interquartile ranges and the mean antibody responses is shown in a circle.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Although a majority of studies on naturally-acquired immunity against malaria have focused on other blood stage antigens, few studies have evaluated the role of *P. falciparum* PHISTb antigens in eliciting immune responses in humans. Merozoite surface proteins as well as those expressed by mature liver schizonts and late schizont and merozoite-stage of blood stage Plasmodium parasites are the most commonly studied antigens (Fowkes et al., 2010). PHIST proteins are known to play a role in mammalian host cell remodeling of infected red blood cells. In this study, recombinant PHISTb antigens can be recognized by individuals previously exposed to malaria. Antibody levels were low in children, with Pf3D7_1401600 reporting the lowest immune response compared to the schizont extract, Pf3D7_0532400 and Pf3D7_1102500, a finding that is consistent with other studies focusing on recombinant *P. falciparum* antigens (Abdel-Latif et al., 2002: Murungi et al., 2016). A previous study reported low antibody levels for Pf3D7_1401600, with the immune responses associated with protection as evidenced by reduced odds of clinical episodes of malaria (Kamuyu, 2017). The finding suggests need for further evaluation of Pf3D7_1401600 as a possible vaccine candidate as it has been shown to provide potential protection against malaria infection. The findings presented here offer crucial insight regarding malaria immunity in children.

In this study, PHISTb antigens can elicit an immune response against *P. falciparum* infection. Our findings with hyper-immune sera indicates variable antibody responses with recombinant PHISTb antigens suggesting variation in levels of immune responses that are elicited by individual proteins. Antibody responses to PHISTb antigens were

low in children below two years, compared to those in adults, and this was to be expected. Children aged between 3 to 5 years previously exposed to malaria presented higher antibody responses to both schizont and recombinant PHISTb antigens, except in Pf3D7_1102500 and Pf3D7_1401600 compared to those below the age of three. However, decline in antibody responses occurred beyond five years of age for schizont extract, Pf3D7_1102500 and Pf3D7_1401600. These findings are indicative of age as an important factor in malaria infection and pathogenesis. These variations are attributed to the differences in host immune factors and conformational differences in individual recombinant PHISTb antigens. The impact of the young host age and declining cases of malaria intensity could equally account for the reduced levels of antibody responses (Murungi et al., 2016).

Next, the study investigated if there was a relation between age and antibody responses against PHISTb antigens. There was no relationship between age and antibody responses against the investigated PHISTb antigens. This may be due to different reasons. First, it could be due to poor immunogenicity of PHISTb antigens that were investigated. Low antibody responses could be the result of poor memory which is generated by the PHISTb antigens. Studies conducted by Boudin et al. showed no relation between age and transmission-reducing activity in antigens investigated (Boudin et al., 2004). Here, there is no direct evidence of age and acquisition of antibody responses. Secondly, it could be associated with the dynamics linked with development of antibodies. In Premawansa et al. it has been shown that development of antibodies could be affected by frequency of infection. Studies focusing on Pfs230 and Pfs48/45 showed no correlations of age with induced antibody responses (Premawansa et al., 1994).Therefore, the development of an immune response against PHISTb antigens could be influenced by rates of re-infection.

There is an association of age, augmented by malaria infection to increase in levels of antibody responses to schizont extract and a recombinant PHISTb antigen (Pf3D7_0532400). Also, the study provides evidence of co-acquisition of antibodies to

PHISTb antigens. These findings suggest that the antibody responses to Pf3D7_1401600 and Pf3D7_1102500 could be co-acquired as previously reported in other *P. falciparum* antigens (Murungi et al., 2016)Young children living in an area with limited malaria exposure reported low antibody responses, but this could be a biomarker of past exposure and possible risk of malaria infection (Stanisic et al., 2015). Siaya has a higher proportion of children with malaria parasites compared to Takaungu and Sukuta. A comparison of antibody responses to schizont extract and recombinant PHISTb antigens confirms an increase in levels for parasite positive individuals; however, the difference is not statistically significant. Whether or not the responses to PHISTb antigens are associated with protection against *P. falciparum* infection, is a factor that should be probed further. There is limited knowledge regarding the function of antibodies required in the mediation of immunity or which serve as a correlate of immunity (Stanisic et al., 2015). Age is a critical factor in determining protection against clinical malaria (Daou et al., 2015)

5.2 Conclusion

This study reveals that recombinant PHISTb antigens are targets of naturally acquired immunity against malaria. Evaluation of antibody responses in three locations with varying malaria transmission intensities indicates variable levels of immunity. An assessment with pooled hyper-immune sera shows PHISTb antigens respond with different antibody levels, as compared with serum obtained from malaria naïve individuals. The responses further show that PHISTb antigens could serve as potential serological markers for *P. falciparum* infection. The structural function of PHIST as a multigene family in infected erythrocytes has previously been shown, indicating they could be critical following infection by the parasite. Based on the role played by PHISTb antigens in the remodeling of iRBCs, anti-PHISTb antibodies could have an important function in abrogating survival and normal development of Plasmodium parasites in the human host. Antibody responses to PHISTb antigens are a confirmation of risk of malaria infection among young children. High antibody responses may mirror an

increased *P. falciparum* exposure as shown in varying levels in different geographical locations. Responses to recombinant PHISTb antigens showed impact of transmission intensity on levels of antibodies. Further studies are needed to elucidate the role of PHISTb antigens in *P. falciparum* infection, and the determination of the role they play in the modulation of immunity against human malaria infection that depends on the age and levels of exposure that influence antibody responses to recombinant PHISTb antigens. Moreover, it is critical to conduct studies on other proteins within the PHIST gene family and ascertain the role that they may have in the fight against malaria infection. As it stands, there are limited drugs or licensed vaccines which are used in the treatment and prevention of malaria infection. The findings from these study establish an interesting area of research to determine the role of PHISTb antigens in mitigating malaria infection. Key questions arise including whether or not these antigens can prevent transmission of malaria to new hosts. There is need to attempt to address these questions as it will be fundamental in mitigating the fight against malaria by increasing the pool of available vaccine candidates. There is evidence that recombinant PHISTb antigens are potential targets of immunity against malaria as they can induce an antibody response.

5.3 Recommendation

The fight against malaria remains to be a challenge globally. There remains an urgent need to come up with better preventive and treatment approaches to mitigate adverse outcomes due to the disease. Key among this approach is the development of effective vaccines to prevent infection. The current study identifies PHISTb antigens as potential vaccine candidates to prevent malaria transmission among humans. With the findings of this research, there are key proposals for various stakeholders involved in the fight against malaria.

Key among them are:

i. To investigate these antigens as possible vaccine candidates. In this study, the focus was on three PHISTb antigens, PF3D7_0532400, PF3D7_1102500 and PF3D7_1401600. The PHIST family of genes has numerous candidates that can be exploited for evaluation as possible vaccine targets.

ii. The findings of this research can be used to build efforts aimed at identifying other PHISTb genes as targets of naturally acquired immunity against malaria transmission in humans.

iii. The study shows the potential role of PHISTb antigens as serological markers of infection by *P. falciparum*. The findings establish an avenue to study these proteins in detail and determine if they can be exploited as a serological marker of malaria infection among children.

iv. There is need to determine if the antibody responses against PHISTb antigens can confer protection against malaria infection among humans. This follows from studies by Kamuyu and colleagues who show that antibodies against Pf3D7_1401600 can confer protection among infected children.

v. There is need to investigate other PHIST proteins within the PHIST gene family to ascertain if they can be utilized as vaccine targets

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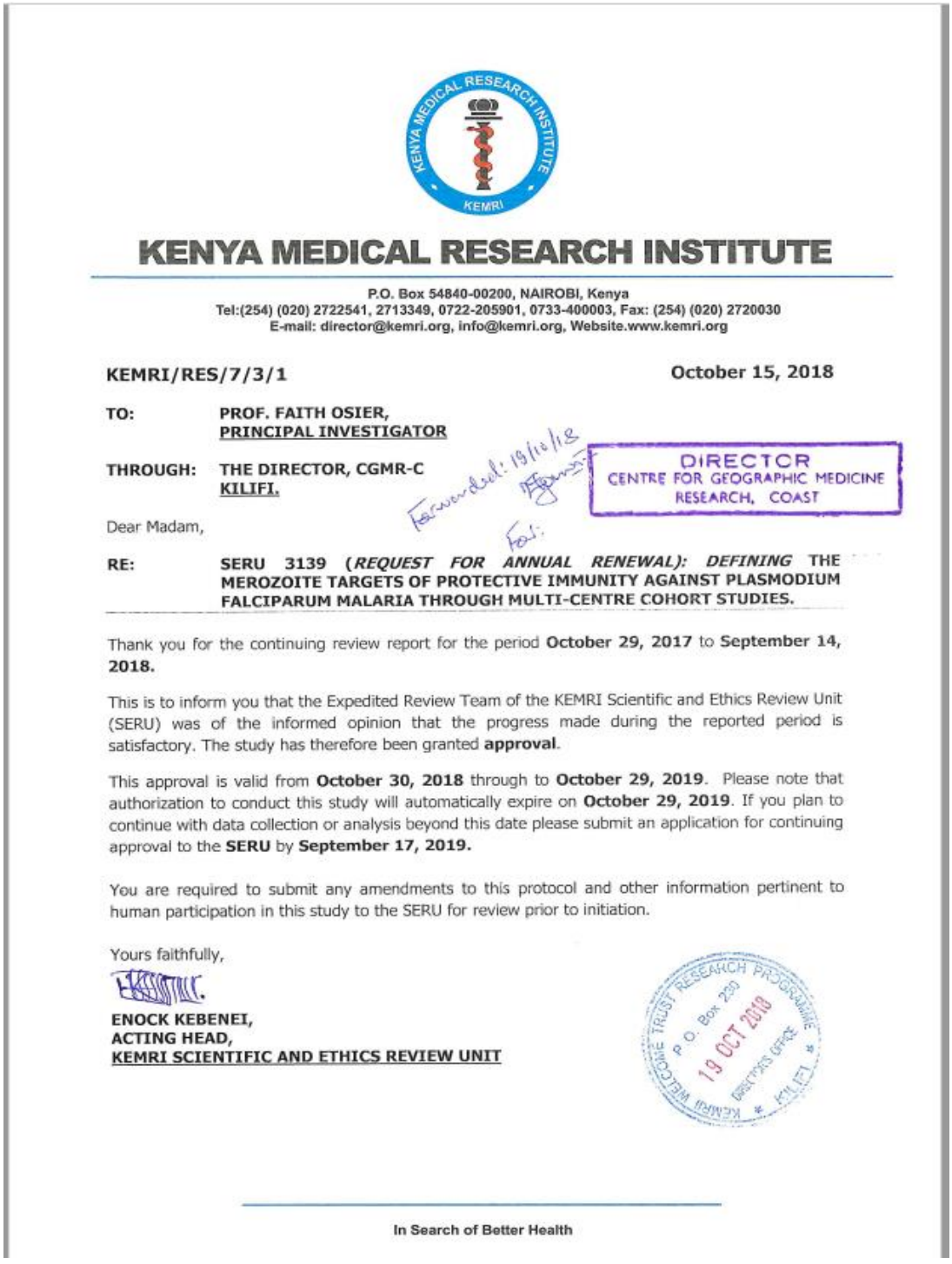
APPENDICES

Appendix I: Ethical Clearance



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KEMRI/RES/7/3/1 **October 15, 2018**

TO: **PROF. FAITH OSIER,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGMR-C**
KILIFI.

Received: 19/10/18

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE
RESEARCH, COAST

Dear Madam,

RE: **SERU 3139 (*REQUEST FOR ANNUAL RENEWAL): DEFINING* THE MEROZOITE TARGETS OF PROTECTIVE IMMUNITY AGAINST PLASMODIUM FALCIPARUM MALARIA THROUGH MULTI-CENTRE COHORT STUDIES.**

Thank you for the continuing review report for the period **October 29, 2017** to **September 14, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **October 30, 2018** through to **October 29, 2019**. Please note that authorization to conduct this study will automatically expire on **October 29, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **September 17, 2019.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

KEMRI WELLCOME TRUST RESEARCH PROGRAMME
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Appendix II: Publication

Wellcome Open Research

Wellcome Open Research 2021, 5:136 Last updated: 24 MAY 2021

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RESEARCH ARTICLE

REVISED Molecular characterization of *Plasmodium falciparum* PHISTb proteins as potential targets of naturally-acquired immunity against malaria

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