Receptor-based identification of novel peptides against blood stage Plasmodium falciparum

A thesis submitted by Huizhuo Liu

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Abstract

Malaria is a devastating infectious disease that affects millions of people worldwide, particularly in developing countries. It is a leading cause of morbidity and mortality, and its impact on global health cannot be underestimated. The severe disease is caused by *Plasmodium falciparum*, which invades human erythrocytes through ligand-receptor interactions. Despite extensive research, there are still several unknowns regarding the mechanism of *P. falciparum* invasion of human erythrocytes. To fill this gap in knowledge, we conducted a project where we screened a phage display cDNA library of *P. falciparum* using both enzyme-linked immunosorbent assay and erythrocytes as baits for phage display by using enzyme-linked immunosorbent assay as baits, antibodies 7899 were used to against constructed phage display. We identified several clones as potential and promising binders to receptors on human erythrocytes. Among these RBC-bound peptides, we focused on two members of the PHISTc protein family, PHISTb and PHISTc, to elucidate the basic mechanism of PHISTb-RBC and PHISTc-RBC interactions and understand their roles in malaria pathogenesis. Our research aims to contribute to the development of a vaccine against human malaria. By understanding the mechanism and function of parasite-derived binding proteins to red blood cells, we can design more effective interventions to prevent the spread of malaria and ultimately save lives. This research sets the foundation for future studies that can lead to the development of effective malaria vaccines and other interventions to combat this global health threat.

Acknowledgements

First and foremost, I would like to thank The Almighty for giving me strength and motivation to complete my studies successfully.

I would like to express my deep gratitude to Dr. Athar Chishti, my research supervisor, for showing his immense trust in me. He made sure that I remain positive and keep trying, never to give up even during stressful periods. I am also grateful to Dr. Toshihiko Hanada for his invaluable guidance for my experiments and revision for my thesis. I would like to thank Rachel Krueger, the research technician in our lab, for her incomparable guidance, help and support during the entire tenure of my research. I would also like to thank every Chishti lab member (including past members) for providing me with a friendly and collaborative work environment during my studies at Tufts.

I would also like to express my gratitude towards Dr. Amy Yee and Dr. K. Eric Paulson for welcoming me with a smile into his lab for rotation and teaching and guiding me a lot with mouse models and cell culture techniques. Spending a few months in their lab under the expert guidance of his Research Technician and himself helped me achieve some important hands-on experience. I would also like to take this opportunity to thank Dr. Emmanuel Pothos as the Program Director for letting me become a part of the Pharmacology program and his support through the entire 2 years of my stay with his valuable input during the faculty and journal club meetings. I also would like to thank my fellow colleagues in the PDD program of 2021 batch for making this journey fun and memorable.

At last, but not least, I also would love to express appreciation to my parents for providing me with emotional and financial support and without whom this journey would have been impossible and incomplete.

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List of Abbreviations

BLAST: Basic Local Alignment Search Tool
BSA: Bovine Serum Albumin
cDNA: Complementary DNA
E. coli: *Escherichia coli*
EcoRI: EcoR one
Enzyme-linked immunosorbent assay abbreviated as ELISA
IPTG: Isopropyl-beta-D-thiogalactoside
iRBC: Infected Red Blood Cell
kDa: kilo Dalton (unit of measurement)
LB: Luria Broth
mAb:Monoclonal Antibody
mRNA: Messenger RNA
PBS: Phosphate-buffered Saline
PCR: Polymerase Chain Reaction
PEXEL: Plasmodium export element
Pfu: Plaque Forming Unit
RBC: Red Blood Cell
rcf: Relative Centrifugal Force
rpm: Revolutions per minute
WHO: World Health Organization

Chapter 1: Introduction

1.1 Malaria, Epidemiology, and Parasite Strains.

Malaria is a disease transmitted by mosquitoes that is caused by five protozoan species: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale,* and the recently implicated Knowlesi [1]. *P. falciparum* is responsible for over 90% of malaria-related deaths, making it a significant global public health threat. The most common early symptoms of malaria include fever, tiredness, headaches, and vomiting.

According to the WHO World Malaria Report 2022, there were an estimated 241 million malaria cases in 2020 across 85 endemic countries (including French Guiana), up from 227 million in 2019. Most of this increase occurred in countries within the WHO African Region [2][3]. Anyone living in or traveling to Sub-Saharan Africa, Southeast Asia, Eastern Mediterranean, Western Pacific, and South America is at risk of contracting malaria, which represents nearly half of the world's population [4][5]. There are also many imported malaria cases and local transmission occurring in non-malarial countries, including North America and Europe. Sub-Saharan Africa is the country of origin for 75.0% of imported cases where the country of origin is unknown.

Approximately 250 species of malaria parasites have been discovered, but only six have been known to infect humans. These include *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale wallickeri, Plasmodium ovale curtisi, Plasmodium malariae, and Plasmodium knowlesi*. Tertian malaria caused by both *P. falciparum* and *P. vivax* is the largest threat globally. However, *P. falciparum* is the deadliest of all *Plasmodium* species, causing the most severe disease and death [6].

The life cycle of *P. falciparum* is complex and involves specific interactions between parasite and host proteins. It consists of three main stages: the exoerythrocytic stage, erythrocytic stage, and sporogonic stage. *P. falciparum* is primarily transmitted by infected female Anopheles mosquitoes, which introduce sporozoites into the bloodstream when they feed on human blood.

Once in the bloodstream, the sporozoites migrate to human liver hepatocytes, where they undergo the exo-erythrocytic stage. During this stage, the sporozoites proliferate and generate thousands of merozoites, which are then released into the bloodstream. The merozoites infect red blood cells (RBCs) and begin the erythrocytic stage of maturation, which is characterized by three stages of development: ring, trophozoite, and schizont.

The infected RBCs (iRBCs) eventually rupture, releasing approximately 10-30 merozoites (daughter cells). The mechanism of gametogenesis is not well understood, but it is believed that a host-derived lipid, Lys phosphatidylcholine, is involved in the sexual differentiation of parasites into male and female gametocytes [7]. The gametocytes are taken up by the mosquito during a blood meal and undergo sexual reproduction inside the mosquito midgut to form new sporozoites. Thus, the liver cycle of human infection starts again with a subsequent blood meal by an infected mosquito [8].

Moreover, by infecting *Plasmodium falciparum* within the next 24 to 48 hours, parasitemia rises, and immune responses increase accordingly. This is usually associated with an increase in TNF-alpha and other inflammatory markers in the cascade, including interleukin-10 (IL-10) and interferon-gamma (IFN-gamma) [6]. Higher parasitemia is generally associated with a more severe clinical picture, but the relationship is highly variable [9]. Above all, the key features that render the disease fatal are the sequestration

of *P. falciparum* in tissues, in conjunction with the upregulation of cytokines and other toxic substances, and the absence or untimely provision of effective antimalarial therapy [10].

1.2 Treatments and preventions for *Plasmodium falciparum*.

During the erythrocytic stage, some of the merozoites differentiate into male and female gametocytes. These gametocytes are taken up by mosquitoes during a blood meal and undergo sexual reproduction in the mosquito midgut, forming new sporozoites. The sporozoites then migrate to the mosquito's salivary glands, ready to infect another human host during the next blood meal. This complex life cycle is what makes malaria such a difficult disease to control, as it involves both human and mosquito hosts.

The CDC recommends that treatment for malaria be initiated immediately after confirmation of infection. Treatment should be guided by three main factors: the infecting Plasmodium species, the clinical status of the patient, and drug susceptibility of the infecting Plasmodium, determined by the geographic region where the infection was acquired [6]. For general antimalarial treatment, chloroquine is a desirable choice. For *P. falciparum* infections acquired in areas without chloroquine-resistant strains, such as Central America west of the Panama Canal, Haiti, and the Dominican Republic, patients can be treated with oral chloroquine or hydroxychloroquine at recommended doses. However, for *P. falciparum* infections acquired in areas with chloroquine resistance, four treatment options are available, including artemether-lumefantrine (Coartem®), which is the preferred option if readily available, and atovaquone-proguanil (Malarone™). These are fixed-dose combination therapies that can be used for pediatric patients weighing ≥5

kg. Quinine sulfate plus doxycycline, tetracycline, or clindamycin is also a treatment option.

Due to the spread of drug resistance and side effects of antimalarial drugs, there is a pressing need for novel drugs and effective vaccination therapeutics. Vaccines are one of the most efficient prophylactic measures for infectious diseases, ranging from smallpox to SARS-CoV-2. With the developed knowledge of immunity, vaccine strategies have focused on identifying target antigens and inducing a protective immune response with minimal side effects.

Over the years, researchers have used various antigens for vaccine development to target the various stages of the life cycle of *Plasmodium falciparum* (sporozoite, merozoite, and sexual stages). However, it has been challenging to identify the ideal target antigens among the 5,000 proteins encoded in the *P. falciparum* genome [11]. The different protein targets are exploited in distinct ways and can be categorized as three vaccines: pre-erythrocytic vaccines, erythrocytic vaccines, and transmission-blocking vaccines.

Currently, there are several vaccine candidates that have been tested in preclinical studies, and two of them have already been evaluated in Phase IIb trials or beyond, namely Lyophilized PEBS synthetic protein (PfPEBS) and AMA1-C1.

1.3 Phage Display Screen to Identify *Plasmodium falciparum* Antigens.

The function of hundreds of undefined *Plasmodium falciparum* ligands and proteins and their corresponding host receptors is relatively unknown. Identifying and characterizing the physiological role of these proteins is urgent for developing a novel

and effective vaccine to curb malaria. Phage display technique was created by G. Smith in 1985 [12][13] as a method of presenting polypeptides on the surface of lysogenic filamentous bacteriophages. It is now one of the most powerful and effective molecular selection techniques. Phage display can be a useful tool for studying protein-ligand interactions, and the characterization of these ligands involves four steps (biopanning process, plaque assays, DNA purification, and DNA sequencing). Among these four steps, biopanning—the procedure for selecting specific binders—is essential for enriching the desired molecule level and amplifying it in bacterial culture [15]. To clarify binders, many approaches and baits can be used, such as enzyme-linked immunosorbent assay (ELISA) and erythrocytes. This technique can be used to probe the direct linkage between phage phenotype and its encapsulated genotype, which is one of the most effective ways to identify potential vaccine candidates (Figure 1.1).

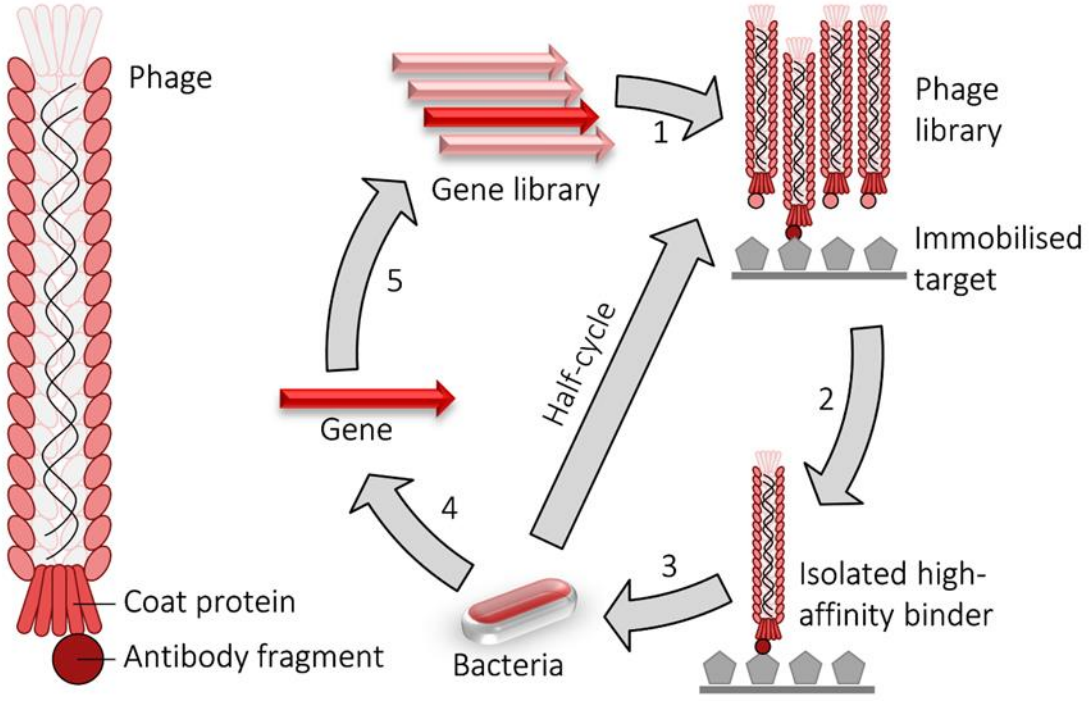


Figure1.1 The complete process of phage display technology.

1.4 Characteristics of *Plasmodium falciparum* PHIST protein.

A major hurdle in developing an effective vaccine against malaria is the antigenic heterogenicity of *P. falciparum*. Some antigens that have been of interest extensively are PHIST proteins. The PHIST protein family, constituting one *Plasmodium falciparum* group, contains around 74 members and is called the Plasmodium (or **P**oly-) **H**elical **I**nterspersed **S**ub **T**elomeric (PHIST) protein family. The PHIST family, originally identified as PEXEL (Plasmodium export element) motif-containing proteins that are exported and required for maintaining parasite growth in the infected erythrocyte, can be classified as three sub-groups, including PHISTa, PHISTb and PHISTc. The PHISTa proteins are relatively small and are composed of a PHIST domain following a signal peptide sequence and a PEXEL motif. PHISTb proteins have a PHIST domain plus a variable carboxy-terminal portion, while some such as RESA infected erythrocyte surface antigen have an additional DnaJ domain. The PHISTc group is the most diverse of the PHIST proteins and consists of 18 proteins that are shared between the *P. falciparum* and *P. vivax* lineages [15]. Some PHIST domains are also identified to bind either the erythrocyte cytoskeletal proteins and change the shape of *Plasmodium falciparum* infected red blood cells whereas others are involved in alteration of RBC membrane mechanical properties or in knob formation [16][17]. Moreover, it has also been observed that PHIST members can interact with PfEMP1 directly and comigrates with PfEMP1 during export and localized in knobs [18]. Several members of the PHIST family are essential for parasite survival, which highlights their significance in malaria biology [19].

Developing an effective vaccine to combat malaria has been an ongoing challenge due to the considerable antigenic heterogenicity of *Plasmodium falciparum*. This

organism is known for its ability to rapidly mutate and adapt to changing conditions, making it difficult to develop a vaccine that can provide broad and long-lasting protection against the disease. Recent studies have shown that the PHIST protein family plays a critical role in malaria pathogenesis and is a promising target for developing new therapeutic interventions [14]. These proteins are involved in a range of processes essential for the parasite's survival, including immune evasion, cytoadherence, and host cell modification. This significant diversity in PHIST protein family functions, structures, and their ability to interact with different proteins highlights their importance in malaria biology and their potential as vaccine targets. By targeting the PHIST family of proteins, researchers hope to develop new treatments that can effectively block the pathogenesis of the disease and prevent its spread.

Several members of the PHIST family are reportedly essential for parasite survival, indicating their importance in malaria biology [19]. The essentiality of PHIST proteins for parasite growth and development makes them attractive candidates for vaccine development. The identification and characterization of PHIST proteins can provide insights into the physiological role of these proteins and their corresponding host receptors, which is essential for developing a novel and effective vaccine to suppress malaria infection.

The identification of specific PHIST proteins can be facilitated by phage display technology, a molecular selection technique that is one of the most powerful and effective tools. It can be used to probe the direct linkage between phage phenotype and its encapsulated genotype, which is one of the most effective ways to identify potential vaccine candidates. The technique involves presenting polypeptides on the surface of

lysogenic filamentous bacteriophages. The characterization of these ligands involves four steps: biopanning process, plaque assays, DNA purification, and DNA sequencing [14]. Among these four steps, biopanning—the procedure for selecting specific binders—is essential for enriching the desired molecule level and amplifying it in bacterial culture [14]. To clarify binders, many tests and baits can be used, such as antibodies-immobilized enzyme-linked immunosorbent assay (ELISA) and erythrocytes.

1.5 Contributions

All contents were assembled by me with minor edits from the advisor.

Chapter 2: Materials and Methods

2.1 Preparation of Red Blood Cells

A 25 mL sample of packed human red blood cells (RBCs) was collected from the Dvorin lab (Blood Bank) at Harvard Medical School. The RBCs were washed three times by RPMI, each time for 10 minutes at 400 rcf, using a Beckman CoulterTM Allegra X-30R refrigerated centrifuge with slow acceleration (9) and deceleration (3) at 4°C. After the first wash, the thin top layer of blood containing white blood cells (WBCs) was removed. The washed RBCs were stored in RPMI at 4°C for up to a maximum of one month.

2.2 Phage Display Using Erythrocytes as Baits

Phage display screening method was employed to identify human RBC binding proteins of *P. falciparum* [22]. The phage cDNA library was constructed from *P. falciparum* 3D7 mRNA. The number of independent clones (phages) in 3D7 cDNA library was 2×10^{10} pfu (plaque forming unit). Phage clones were selected through four rounds of biopanning using human RBCs as bait to capture phages. The first round was performed by incubating 100 µL of 50% hematocrit of human RBCs with 2×10^{10} pfu of *P. falciparum* phage lysate. Thus, each independent phage was screened more than 3,000 times. The binding buffer was autoclaved PBS (Phosphate Buffered Saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 3% BSA (Bovine Serum Albumin, Gold Biotechnology, A-420-100). The volume of the reaction was 700 µL and the incubation of the binding assay was performed for two hours while rotating at room temperature on a rotator (Tube Revolver / Rotator, Thermo Fisher Scientific™ 88881001, 11-676-341). The mixture was centrifuged through 500 µL

Silicone oil- to separate RBCs pellet from supernatant at 12,000 revolutions per minute (rpm) or 13,400 rcf (relative centrifugal force) using Eppendorf® Microcentrifuge 5415D. Phage bound RBCs were washed three times with PBS at 1,800 rpm. Bound phages were eluted by incubating the RBCs with 3.0 M NaCl (sodium chloride) for 20 minutes and centrifuged at 12,000 revolutions per minute (rpm) or 13,400 rcf (relative centrifugal force) using Eppendorf® Microcentrifuge 5415D. Then the supernatant containing the eluted peptides is amplified in BLT5403 *E. coli* (Escherichia coli) bacterial culture by shaking for 3 hours at 37°C. Subsequent rounds of biopanning were performed by using 100 µL of the amplified phage stock from previous round. Plaque assays were performed as described (Novagen's T7Select® System Manual). Phage inserts were amplified from independent plaques by polymerase chain reaction (PCR) and further purified using E.Z.N.A.® Cycle-Pure Kit (VWR, 101318-904), and DNA sequencing was done at Tufts University Core Facility. Positive hits were used for additional experiments.

2.3 Phage Display Using Antibody-immobilized ELISA Plates as Baits

To perform enzyme-linked immunosorbent assays (ELISA), the ELISA plates were first coated with the target antiserum (antibodies) diluted with 1x PBS to between 7 µg/ml and 10 µg/ml. After incubating the coated plates overnight at 4°C covered with parafilm® or plastic wrap to prevent evaporation, the wells were washed three times with 300 µL of 1x PBS to remove unbound target protein. Then, 200 µL of blocking buffer (1% BSA in 1x DPBS) was applied to each well and incubated for 60 minutes at room temperature or overnight at 4°C. Finally, the plates were washed five times with deionized water.

The phage cDNA library was constructed from *P. falciparum* 3D7 mRNA. The 3D7 cDNA library contained 2×10^{10} plaque forming units (Pfu). For the first round, 20 µL of the phage library was diluted with PBST to 200 µL. The diluted phage library was applied to the antibodies-coated ELISA plate wells and incubated for 30 minutes at room temperature. The bound phages were eluted by applying 100 µL of elution buffer (1% SDS in DI water) to each well and incubating at room temperature for 20 minutes. The eluted phages were amplified in BLT5403 *E. coli* (Escherichia coli) bacterial culture by shaking for 1-3 hours at 37°C. Subsequent rounds were performed using 20 µL of the amplified phage stock from the previous round, for a total of four rounds.

2.4 DNA sequencing and Construction of the Expression Plasmids of PHISTb and PHISTc

Two clones, PHISTb and PHISTc, were isolated and purified. To create fusion proteins, the coding sequence for PHISTb and PHISTc were amplified by PCR from the phage and cloned in PGEX-2T vector with a GST fusion tag. We digested the PCR products with EcoRI (New England Biolabs: NEB) and then electrophoresed them in a 1% TAE agarose gel. After excising the corresponding DNA bands from the gel with a new razor blade, we purified the bands using the E.Z.N.A.® Gel Extraction Kit. The amplified and purified PCR products were sequenced using a designed T7 forward primer 5'-GGAGCTGTCGTATTCCAGTC-3' and a T7 reverse primer 5'-AACCCCTCAAGACCCGTTTA-3'. The PHISTb and PHISTc constructs were synthesized by GenScript USA Inc. as codon-optimized DNA cloned into the PGEX-2T vector.

2.5 Recombinant Protein Expression

The PGEX-2T plasmids containing the recombinant proteins of interest, PHISTc and PHISTb, were transformed into *E. coli* BL21 competent cells (DE3). The transformation mix was plated onto LB-agar containing ampicillin (Fisher Scientific, ICN19014805) and incubated overnight at 37°C. Overnight liquid cultures were prepared by inoculating a single colony of recombinant *E. coli* BL21 cells from a freshly streaked plate into 5 mL of LB media (Luria Broth, Affymetrix, VWR, 101170-308) containing 100 µg/mL ampicillin. The cultures were grown at 30°C and 220 rpm for 16 hours. The next day, 50 mL of fresh LB media containing 100 µg/mL ampicillin were inoculated with 1.0 mL of the overnight culture. The cultures were then grown at 30°C and 220 rpm until the OD at 600 nm reached 0.6. At this point, 0.1 mM IPTG (Isopropyl-beta-D-thioacetamide, Gold Biotechnology, I2481C25) was added to the cultures to induce protein expression. The cultures were then grown for an additional 3 hours before harvesting the cells by centrifugation at 4,000 rpm for 30 minutes at 4°C using a Sorvall RC5C Plus Centrifuge with SS-34 rotor (Thermo Electron Corporation). The cell pellets were then resuspended in lysis buffer to release the recombinant protein. The lysate was then centrifuged at 13,000 rpm for 30 minutes at 4°C to remove cell debris, and the supernatant was collected for analysis by SDS-PAGE, Western blotting, and for further characterization.

2.6 SDS-PAGE Analysis of Recombinant PGEX-2T PHISTc and PHISTb

Sample lysates were clarified at 3,000 rcf for 5 minutes. 2X SDS sample buffer with reducing agent was added to each sample and the protein lysates were heated at 90°C for 5 minutes to denature the proteins. The denatured proteins were then separated by SDS-

PAGE on a 12% acrylamide gel. A Coomassie Blue stain was used to visualize the separated proteins.

2.7 Western Blotting with anti-GST mAb

Proteins were transferred onto nitrocellulose membranes, stained with Ponceau S to visualize proteins, washed, and blocked with 3% BSA in PBST for one hour at room temperature. The Western blot was probed with an anti-GST primary antibody to detect the GST-tagged PHISTb and PHISTc proteins. A mouse secondary antibody conjugated with HRP was used to generate a chemiluminescent signal. The size of the expressed proteins was verified by comparing the bands to the molecular weight marker.

2.8 Batch Purification Using Glutathione Resin

GST-tagged PHISTb and PHISTc proteins were purified using batch purification with glutathione agarose beads. This method was used for both small and large volume purification. First, the expressed proteins were concentrated for 10-20 minutes using centrifugal filters (Amicon®Ultra-4 10K). The beads were washed once with PBS by centrifugation for 5 minutes at 500 rcf. Then, the washed beads were centrifuged for 5 minutes at 500 rcf to create a 50% slurry with PBS. The mixture of beads and concentrated proteins were incubated at 4°C overnight for binding. The mixture was then eluted with an elution buffer (20mM reduced glutathione in 50mM Tris HCL PH 8.0) for 10 to 20 minutes on ice and centrifuged for 5 minutes at 500 rcf. The supernatant was collected to harvest purified proteins.

2.9 Contributions

2.1 2.2 2.3 2.4 were finished solely by me, 2.5 2.6 2.7 and 2.7 were finished by me and Rachel Kruger collaboratively.

Chapter 3: Results

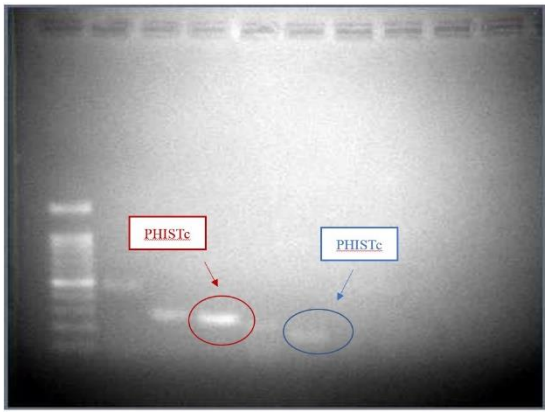
3.1 Phage Display Screening for Novel Malaria Binding Proteins by using RBC as baits.

Two phage display cDNA libraries of *P. falciparum* 3D7 were used. One was generated in our laboratory and another one was kindly shared by Dr. Theresa Coetzer (University of the Witwatersrand, Johannesburg, South Africa) using the T7 phage display system (Novagen). The first library, generated in our lab, was screened to isolate positive cDNA clones recognizing RBC surface-binding proteins. In this screen I used human RBCs as bait. After four rounds of biopanning followed by the plaque assay, several phage clones were detected. These clones were amplified and sequenced based on promising phage inserts that showed clear bands on agarose gels. We decided to sequence 5 out of 10 clones. The clones were sent for sequencing to the Tufts Core Facility. Nucleotide and protein BLAST sequences were obtained using NCBI (the National Center for Biotechnology Information) and PlasmoDB sources [20]. For amino acid translation of nucleotides, we used ExPASy translate tool [20

]. Among these five clones, one phage cDNA clone was identified, *Plasmodium falciparum* 3D7 strain Plasmodium exported protein, PHISTc (PF3D7_0936800) And Sanjana, the former MS student also screened for another member of PHISTb family proteins, *Plasmodium falciparum* 3D7 strain Plasmodium exported protein, PHISTb(PF3D7_1476300), even though there are no alignments between these two sequences, they all belong to PHIST family proteins(Figure 3.1) .For the second library obtained from the Coetzer's lab was used as described above, and three phage cDNA clones were identified, one was for *Plasmodium falciparum* 3D7 strain erythrocyte

membrane protein 1, PfEMP1 (PF3D7_0412700) (Figure 3.2) and the other two were both for *Plasmodium falciparum* 3D7 strain conserved Plasmodium protein of unknown function (PF3D7_0724100) (Figure 3.3).

A



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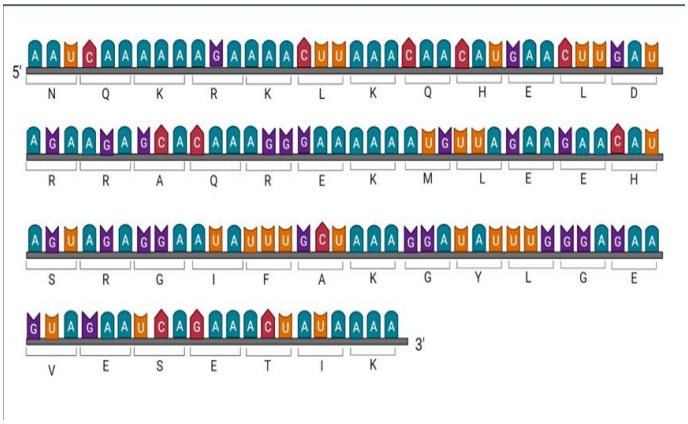

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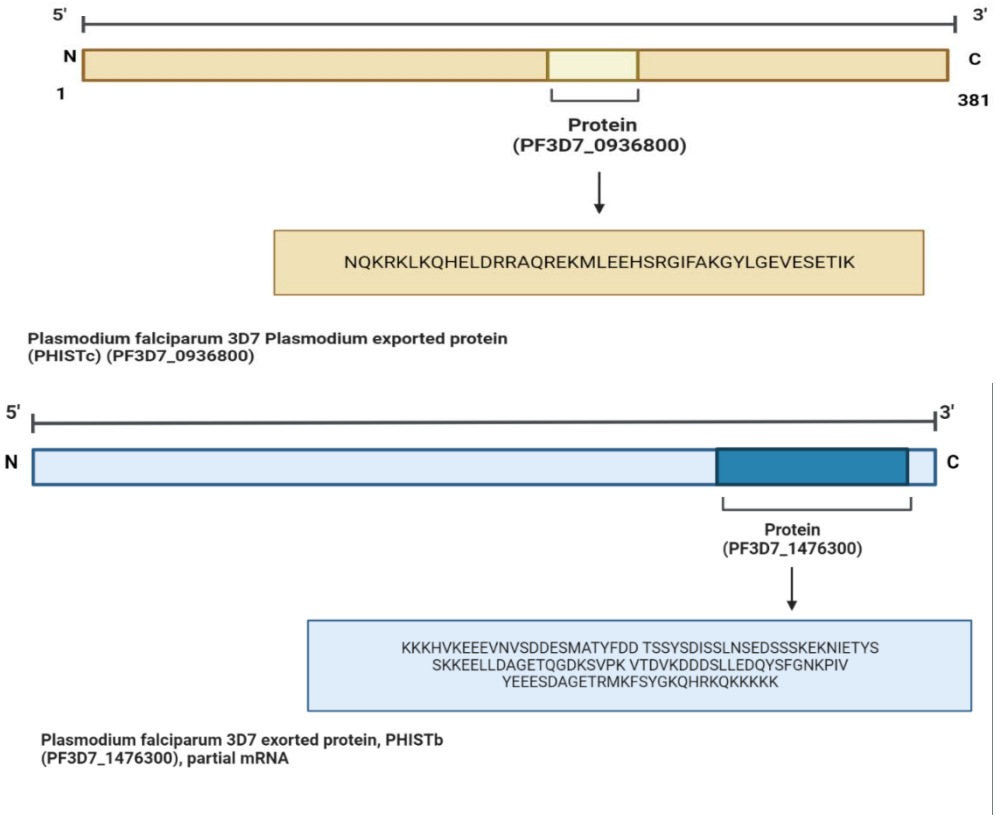

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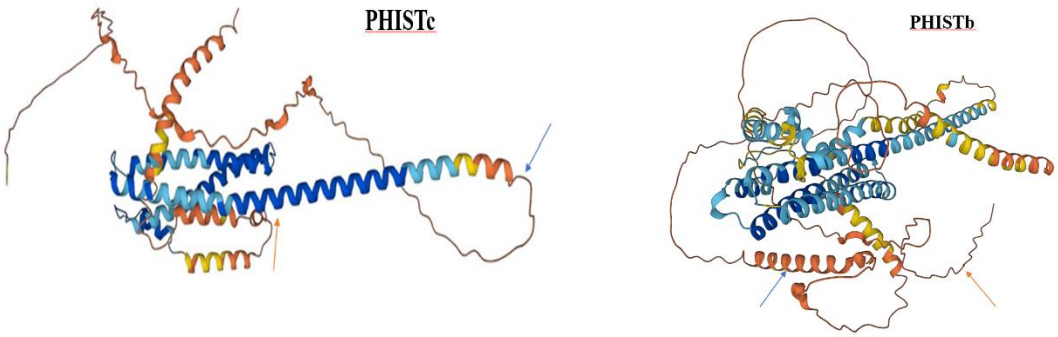
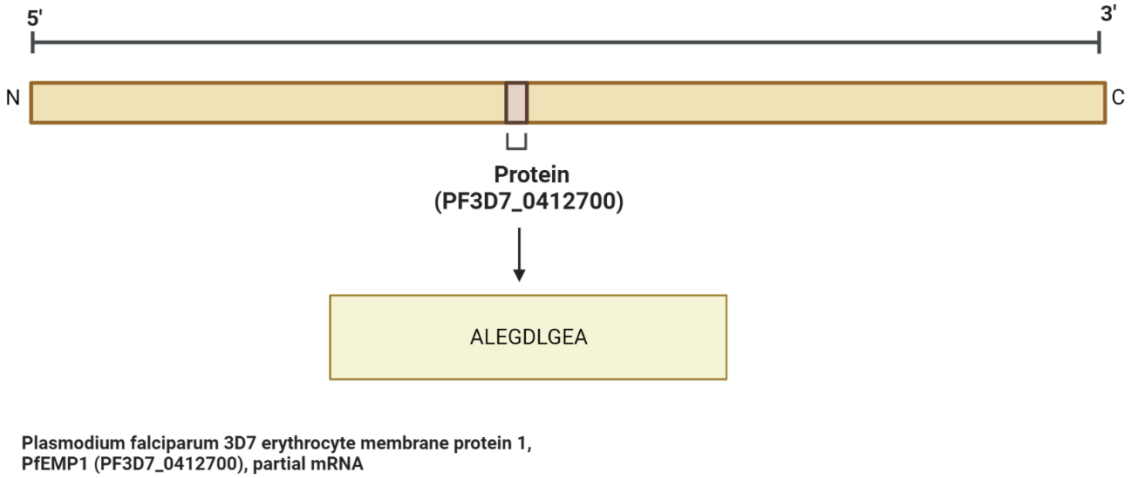


Figure 3.1 Phage display sequence for *Plasmodium falciparum* 3D7 Plasmodium exported protein (PHISTc). A) Isolation of PHISTc from Phage Display cDNA Screening. Phage clones of the fourth round of biopanning using human RBCs as bait. Phage inserts were amplified from independent plaques by PCR and arranged according to their base pair size in 1% agarose gels containing ethidium bromide. Bands in agarose gel show DNA inserts that were sequenced. DNA sequencing of the PCR product identified two of the clones as PHISTc and PfEMP1, marked with the red circle and blue circle. Other bands could not be identified by the nucleotide Blast analysis due to the lack of significant sequence similarities. B) Purified PCR products isolated by phage display were sequenced by TUCF and the translated amino acids were obtained from NCBI databases and ExPasy. C). Selected Protein Sequences of Positive Clones Bound to Human RBCs Using the Phage cDNA P. falciparum 3D7 Library Screen which was generated in Chishti's lab. The sequence of proteins expressed on the surface of phages that bound selectively to human RBCs was located in 237 to 279 of Plasmodium falciparum 3D7 Plasmodium exported protein (PHISTc). D) 3D protein structure predicted by Alphafold. For PHISTc recombinant protein, the start position (237th amino acids) was marked with the blue arrow and the end position (279th amino acids) was marked with the orange arrow whereas for PHISTb recombinant protein, the start position (445th amino acids) was marked with the blue arrow and the end position (565th amino acids) was marked with the orange arrow.

A.



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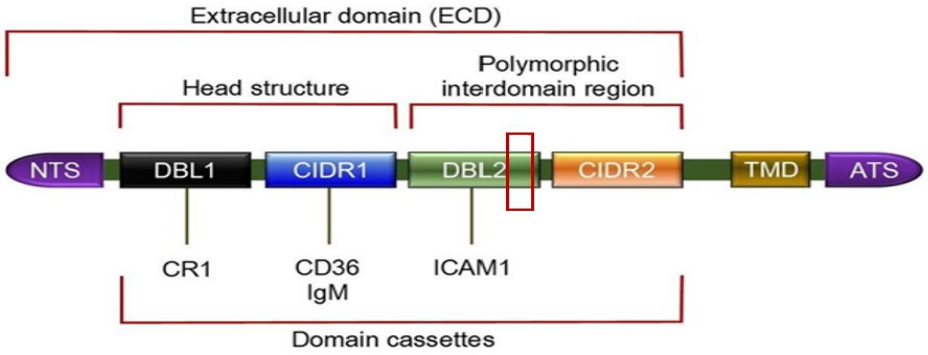
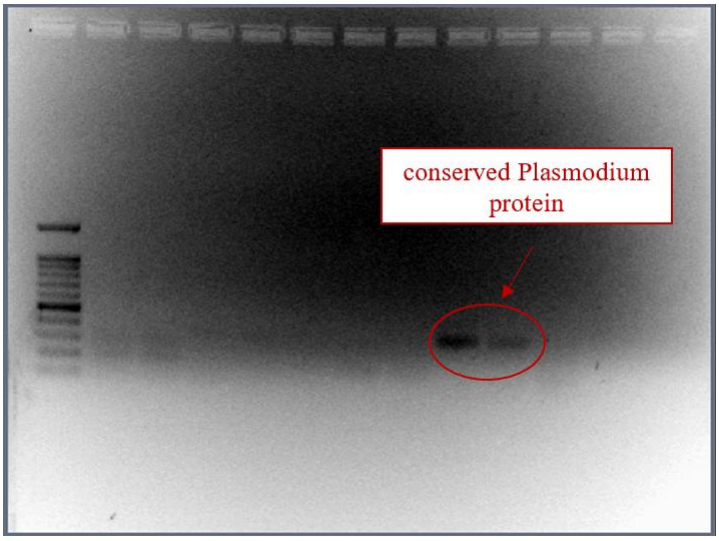


Figure 3.2 Phage display sequence for *Plasmodium falciparum* 3D7 erythrocyte membrane protein 1.A) Selected protein sequences of positive clones bound to human RBCs using the Phage cDNA *P. falciparum* 3D7 Library Screen that was generated in Chishti's lab. The sequence of proteins expressed on the surface of phages that bound selectively to human RBCs was located in 898 to 907 of *Plasmodium falciparum* 3D7 Plasmodium erythrocyte membrane protein 1. B) The protein structure of PfEMP1 is shown. Selected Protein Sequences of positive clones bound to human RBCs was located in extracellular domains in DBL2 module which is labeled as red box.

A



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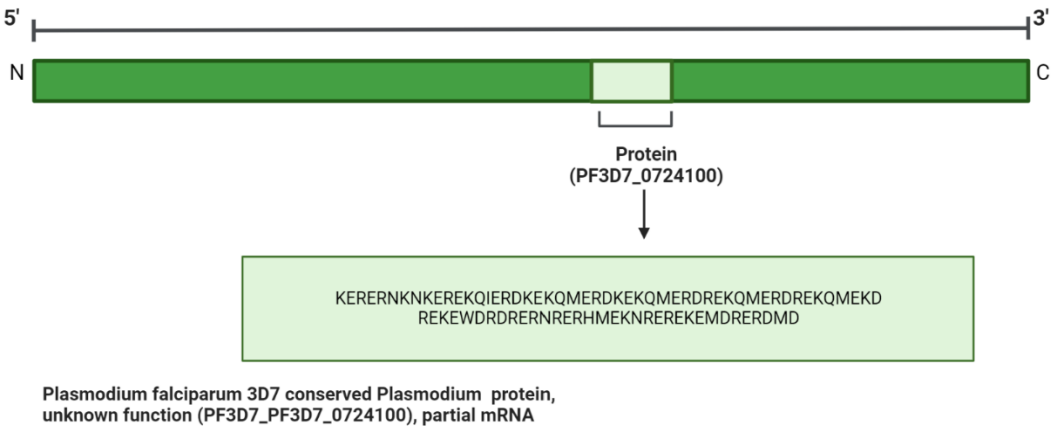


Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function (PF3D7_PF3D7_0724100), partial mRNA

C.

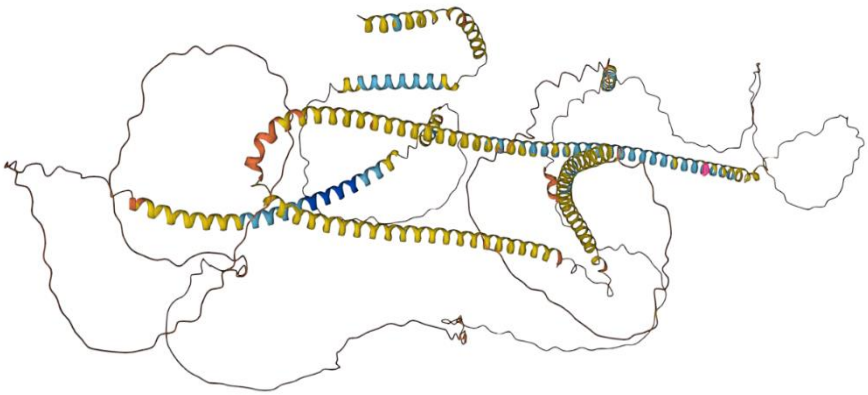


Figure 3.3 Phage display sequence for *Plasmodium falciparum* 3D7 conserved Plasmodium protein of unknown function. A) Amplified PCR products were identified by 1% agarose gel electrophoresis. Bands of DNA inserts were sequenced. DNA sequencing of the PCR products identified as two clones as conserved Plasmodium protein (unknown function), marked with the red circle. Both clones encoded the same protein, plasmodium falciparum 3D7 conserved Plasmodium unknown function protein. Other bands could not be identified by the nucleotide Blast analysis due to the lack of significant sequence similarities. B) Selected Protein Sequences of positive clones bound to human RBCs using the Phage cDNA P. falciparum 3D7 Library Screen that was generated in Coetzer's lab. Two identical sequences of proteins expressed on the surface of phages that bound selectively to human RBCs was located in 595th to 678th of amino acids in Plasmodium falciparum 3D7 conserved Plasmodium protein, unknown function (PF3D7_0724100).

Protein name	PlasmoDB Gene ID	Protein FASTA sequence	Phage library
PHISTc	PF3D7_0724100	NQKRKLKQHELDRRAQREKMLEEHSRGIFAKGYLGEVESETIK	PF3D7 generated in Chishti's lab
PfEMP1	PF3D7_0412700	ALEGDLGEA	PF3D7 generated in Chishti's lab
Conserved Plasmodium protein (unknown function)	PF3D7_0724100	KERERNKNKEREKQIERDKEKQMERDKEKQMERDREKQMERDREKQMEKDREKEWDRDRERNRERHMEKNREREKEMDRERDMD	PF3D7 generated in Coetzer's lab

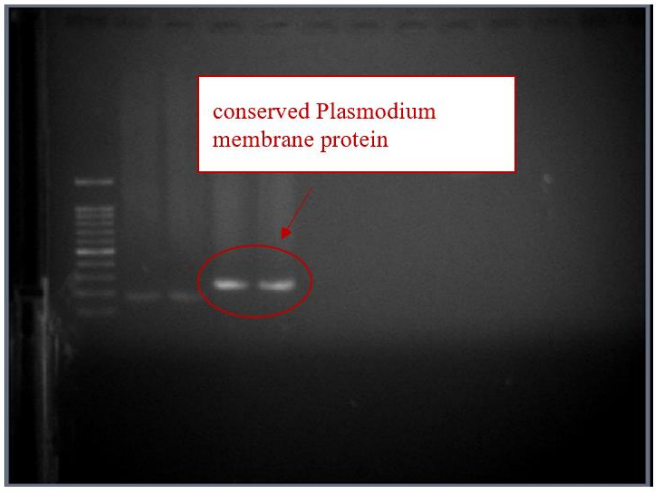
Table3.1: Selected Protein Sequences of Positive Clones Bound to Human RBCs Using the Phage cDNA P. falciparum 3D7 Library Screen. Sequence of proteins expressed on the surface of phages that bound selectively to human RBCs. Bound phages were eluted after several washes and amplified through four rounds of biopanning. FASTA sequences were obtained using NCBI database. Three positive hits were shown here.

3.2 Phage Display Screening for novel malaria binding proteins using erythrocytes as baits.

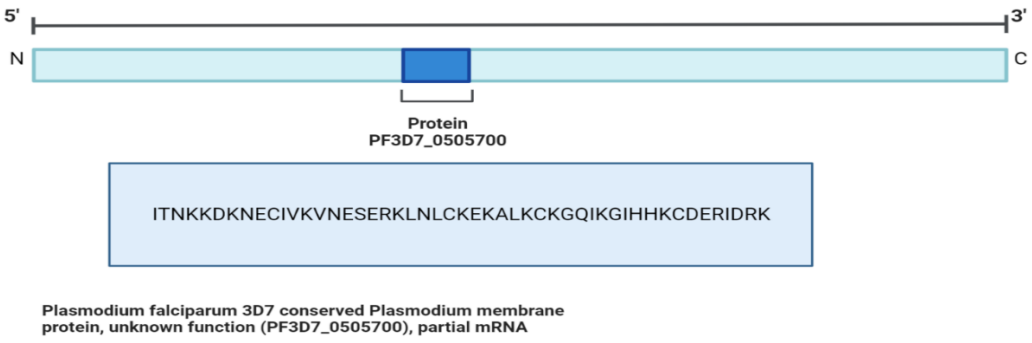
The phage display cDNA library of *P. falciparum* 3D7 strain was generated in Coetzer's lab, using the T7 phage display system (Novagen). The library was screened to isolate positive cDNA clones recognized by mAb7899 that is believed to inhibit malaria parasite growth by inhibiting an unknown protein. By using mAb7899-coated ELISA

plate as bait, several clones were identified after four rounds of biopanning followed by plaque assays. Based on the bands on the 1% agarose gel, 16 clones were sent for sequencing to Tufts Core Facility. Nucleotide and protein BLAST sequences were obtained using NCBI (the National Center for Biotechnology Information) and PlasmoDB sources [20]. For amino acid translation of nucleotides, we used ExPASy translate tool [20]. The BLAST analysis of cDNA clone revealed one sequence that encoded 49 amino acids from the organism of *Plasmodium falciparum* 3D7 strain protein of unknown function (PF3D7_0505700) (Figure 3.4). This protein appears to be a promising target for malaria vaccine.

A.



B.



Plasmodium falciparum 3D7 conserved Plasmodium membrane protein, unknown function (PF3D7_0505700), partial mRNA

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C.

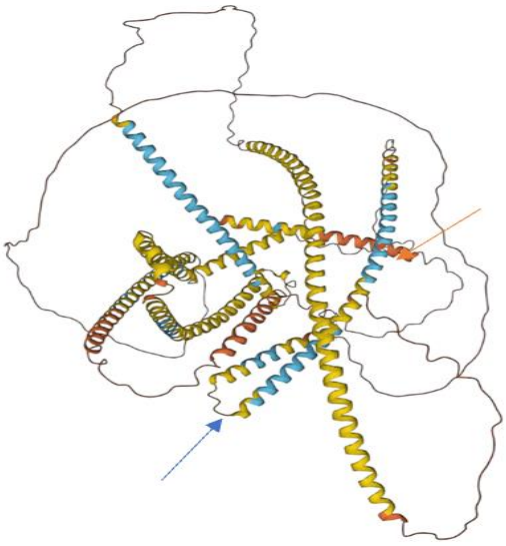


Figure 3.4 Phage display sequence for *Plasmodium falciparum* 3D7 strain conserved Plasmodium membrane protein (unknown function). A) Amplified PCR products were identified by 1% agarose gel electrophoresis. Bands corresponding to DNA inserts were sequenced. DNA sequencing of the PCR product identified a single clone as conserved Plasmodium membrane protein (unknown function), marked with the red circle. Other bands from another sample could not be identified by the nucleotide Blast analysis due to either the lack of significant sequence similarities or too short inserts. B) Selected Protein Sequences of Positive Clones Bound to Human RBCs Using the Phage cDNA P. falciparum 3D7 Library Screen which was generated in Coetzer's lab. The sequences of proteins expressed on the surface of phages that bound selectively to human RBCs was located in 359th to 407th of amino acids that are labeled as orange arrow and blue arrow separately in *Plasmodium falciparum* 3D7 conserved Plasmodium protein, unknown function (PF3D7_0505700).

Protein name	PlasmoDB Gene ID	Protein FASTA sequence	Phage library
Conserved Plasmodium membrane protein (unknown function)	PF3D7_0505700	ITNKKDKNECIVKVNESERKLN LCKEKALKCKGQIKGIHHKCDERIDRK	Coetzer's lab

Table3.2 Selected Protein Sequences of Positive Clones Bound to Human RBCs Using the Phage cDNA P. falciparum 3D7 Library Screen.

3.3 PCR amplification of Codon optimized PHISTb and PHISTc protein.

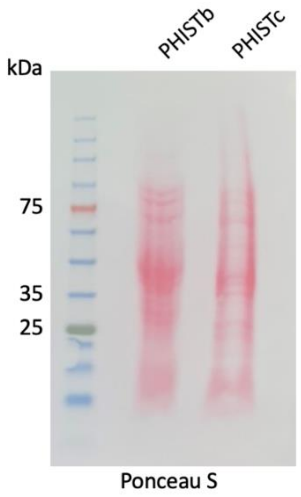
One of the main aims of this project was to determine the functional role of PHIST family protein in malaria infection. Both PHISTb and PHISTc were identified as potential RBC binding protein through phage display screening against human RBCs. In these two proteins, PHISTb was screened and sequenced by Sanjana C. Nair. We planned to codon optimize two clones, PHISTb(121AA) and PHISTc(43AA). Codon Optimization was done by GenScript USA Incorporation. The codon optimized PHISTb and PHISTc sequence were both cloned into pGEX-2T vector, and a GST tag was added as a fusion protein. To confirm the successful cloning of S antigen into the pGEX-2T vector, the isolated plasmids, PHISTb and PHISTc were both sent to TUCF to successfully confirm that these cloned were transformed.

3.4 Expression and Purification of Recombinant PHISTb and PHISTc proteins.

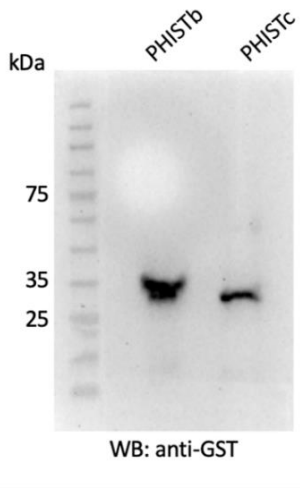
To validate the function of the proteins PHISTb and PHISTc, we carried out an experiment in which we tested their expression and purified them separately. The recombinant protein was expressed in E. coli BL21 with GST as the fusion tag. To visualize the results, we analyzed the protein samples using gel electrophoresis. The first lanes of the gel show the GST-PHISTb protein, which corresponds to 32 kDa, while the second lanes show the GST-PHISTc protein, which corresponds to 30 kDa(Figure 3.5).The purification was detected by loading with glutathione beads with PHISTb and PHISTc recombinant proteins. Purification was detected by loading with glutathione beads with PHISTb and PHISTc recombinant proteins. Purification of PHISTc recombinant protein corresponding to 30 kDa is shown in Figure 3.5 (lane 4), whereas the purification of PHISTb recombinant protein that corresponds to 64 kDa is shown in

Figure 3.5 (lane 5). The size of PHISTb suggests that it may form a dimer in solution. This experiment was conducted with the aim of better understanding the properties and functions of these proteins, which are of particular interest in the field of biochemistry. It is hoped that the results of this study will contribute to the wider scientific community in this area and provide a foundation for further research in the future.

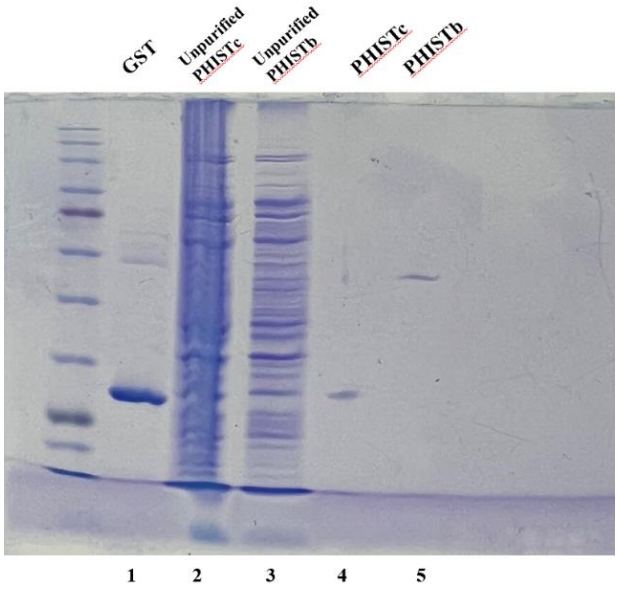
A.



B.



C.



D.

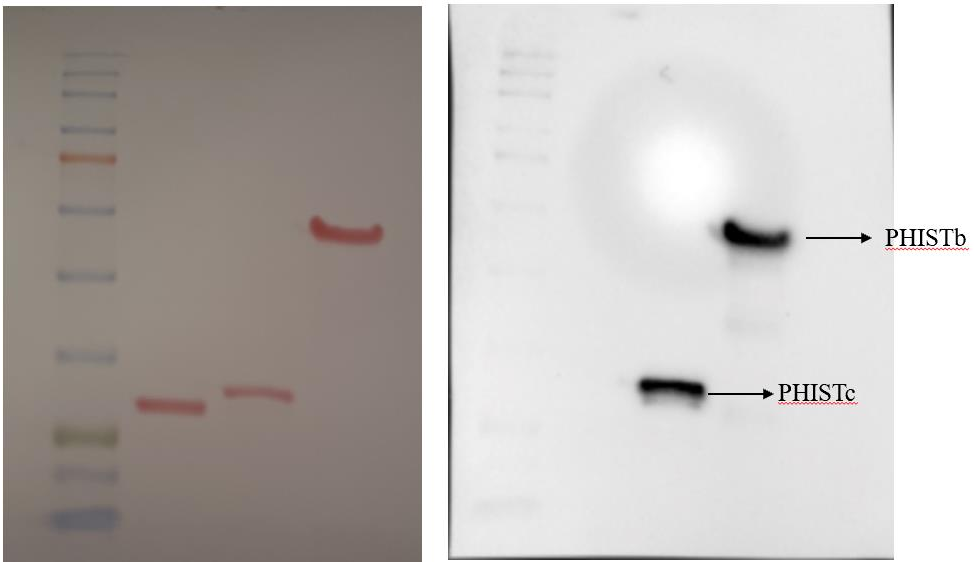


Figure 3.5 (A) Ponceau S stain of the unpurified recombinant transferred PHIST proteins onto the nitrocellulose membrane, visualizing the proteins on the membrane and confirming that the transfer was successful. (B) Proteins were transferred onto nitrocellulose membranes and probed with an anti-GST primary antibody to detect GST-tagged PHISTb and PHISTc proteins. A mouse secondary antibody conjugated with HRP was used to generate a chemiluminescent signal. The size of the expressed proteins was verified by comparing the bands to the molecular weight marker. (C)comassie-stained SDS PAGE showing expression of purified recombinant PHIST proteins in *Escherichia coli*. Proteins were extracted from BL21 E. coli transformed with pGEX-2T plasmids containing the genes encoding GST-fused PHISTc and PHISTb proteins, including PF3D7_0724100 and PF3D7_1476300, and purified using glutathione sepharose beads. The presence of GST-fused PHIST proteins is indicated. The purity of the recombinant proteins was assessed by SDS-PAGE and Coomassie staining, demonstrating the successful expression and purification of the recombinant proteins. (D) Purified Proteins were transferred onto nitrocellulose membranes and probed with an anti-GST primary antibody to detect GST-tagged PHISTb and PHISTc proteins. A mouse secondary antibody conjugated with HRP was used to generate a chemiluminescent signal. The size of the expressed proteins was verified by comparing the bands to the molecular weight marker. PHISTc corresponds to 30kDa whereas PHISTb corresponds to 64kDa since it formed dimer in the solution.

3.5 Contributions

Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4 were completed solely by me. Figure 3.5 and Figure 3.6 were finished by Rachel Kruger and me collaboratively. Additionally, Table 3.1 and Table 3.2 were completed by me solely.

Chapter 4: Discussion

4.1 Functional role of PHISTb and PHISTc proteins in *P. falciparum*-infected human erythrocytes (iRBCs)

Previous studies on the PHIST proteins family have shown that PHIST proteins family bind to the host RBC membrane and play a functional role in the regulation of cellular adhesion by altering the RBC membrane mechanical properties and knob formation [18]. Also, one member termed PHISTc protein, PF3D7_0801000, has been localized in the merozoites, and antibodies against PHISTc modestly inhibit *P. falciparum* parasite growth in in vitro [21]. Phage display cDNA library screening strategy has been employed before to identify human RBC binding proteins of *P. falciparum* [20]. This approach has been successful in identifying novel ligand-receptor interactions [22]. Using this methodology, previous studies have identified novel ligand-receptor interactions and selected RBC positive binders using *P. falciparum* cDNA libraries. Some of these ligand-receptor interactions are critical for parasite life cycle [23]. The Recently, the PHIST protein family has emerged as parasite ligands of interest. It is believed that PHIST protein family facilitates iRBC remodeling process in *P. falciparum* infected host cells [24]. Also, over the past few years, PHIST proteins have attracted more attention due to their localization at various organelles in infected erythrocytes and their interactions with PfEMP1 at knobs [24].

Despite the functional importance of PHIST protein family in malaria pathogenesis, it is not known whether PHISTb and PHISTc directly bind to iRBCs. Several other members of the PHIST protein family have not been characterized. Also, it is not known which core binding site(s) in the PHISTb and PHISTc proteins mediate their binding to

human RBCs. This information will have direct translational implications in the future development of an effective malaria vaccine. Based on this published evidence, the main objective of this project was to isolate and characterize PHIST proteins and investigate their mechanisms of binding to human erythrocytes.

I screened the phage display cDNA libraries to identify novel cDNA clones encoding *P. falciparum* ligands (polypeptides) that directly bind to the surface of human erythrocytes. From this screen, I identified PHISTc as potential ligand for binding human erythrocytes. Sanjana Nair, a previous PDD student, performed a similar screen and has identified PHISTb as putative ligand. Both PHISTc and PHISTb belong to the PHIST family proteins. A unique advantage of the phage display technology is its potential to identify precise target binding domains within proteins. Since the phage-encoded peptides are relatively short, it is necessary to express them as fusion proteins. First, we started to codon-optimize the PHISTb and PHISTc proteins for subsequent expression and purification of recombinant proteins. Several fusion tags including GST, MBP, Trx, and His were considered to facilitate purification of recombinant peptides. These tags serve to stabilize the protein sequence and facilitate protein purification by affinity chromatography. In this study we elected to use a GST tag that binds to glutathione beads suitable for the stability of the polypeptide and purification of the recombinant protein. The size of GST tag is ~ 26 kDa, thus making the detection of PHISTb and PHISTc peptides feasible by Western blotting. We successfully expressed GST-tagged PHISTb and PHISTc fusion proteins in bacteria (Fig 3.6). Currently, we are optimizing the conditions to affinity purify sufficient amount of fusions proteins for direct RBC-binding assays for functional characterization of PHISTb and PHISTc polypeptides.

4.2 Fusion tag-based detection of recombinant proteins.

The sequences identified from the phage display cDNA libraries are reltively short. The PHISTb polypeptide identified consists of 121 amino acids whereas the PHISTc peptide is composed of 43 amino acids. These relatively short sequences will be difficult to identify by immunoblotting. Thus, GST and MBP are two feasible options to express PHISTb and PHISTc peptides. We plan to test the MBP tag if the GST fusion proteins interfere with protein solubility and stability of the recombinant polypepties.

4.3 Phage display can be used as a tool to identify new malaria proteins.

Phage display screening has been proven to be an essential tool in identifying major host-parasite interactions and binding sites [14]. The Chishti lab has been utilizing this technique for quite some time to identify new malaria proteins [21]. I performed several rounds of phage display using human RBCs as well as an immobilized monoclonal antibody as baits and identified at least four different protein sequences as potential target antigens (Figure 3.1 and Figure 3.2). Importantly, these screens have identified the core binding segments of potential antigens of clinical significance.

4.4 Binding sites between PfEMP1 and ICAM1

Rosetting occurs when parasite–derived *P. falciparum* Erythrocyte Membrane Protein One (PfEMP1) on the surface of infected erythrocytes binds to human receptors on uninfected erythrocytes. Each PfEMP1 protein consists of single intracellular and trans-membrane domains, and several extracellular domains (2 to 7 domains, ~30–45 kDa per domain) heavily cross-linked by disulfide bonds [25]. All PfEMP1 domains have been classified into several sub-classes: Duffy binding like (DBL) α, β (previously called βC2, a combination of the β and C2 domains), γ, δ, ζ, ε, and X; and cysteine-rich

interdomain regions (CIDR) α, β, and γ[26]. The sequence I isolated originated from the domain of DBL2, which binds to endothelial receptor, ICAM-1. And it is also observed that naturally acquired antibodies that block a PfEMP1 domain, DBL2β of PF11_0521 allele, from binding to the human ICAM1 receptor, reduce the risk of malaria hospitalization in children. However, the specific binding sites from DBL2 to ICAM1 are still unknown. Therefore, the short sequence identified in this study, ALEGDLGEA, may mediate the binding of iRBCs to endothelial cells via ICAM1 receptor.

4.5 Future plans.

In these studies, we pursued two promising RBC-binding proteins, PHISTb and PHISTc from *P. falciparum*, a highly virulent species causing human malaria. We successfully expressed both PHISTb and PHISTc peptides as recombinant GST fusion proteins (Figure 3.5 and Figure 3.6). In the future, it would be necessary to determine the binding affinity of two PHISTb and PHISTc peptides to human RBCs and evaluate whether these core binding peptides can inhibit malaria parasite invasion in RBCs. Since the PHIST proteins have been implicated in the interaction of PfEMP1 and remodeling of knobs, it would be important to determine whether the PHISTb and PHISTc peptides can block adhesion of iRBCs to endothelial cells. Finally, the functional epitope identified by mAb7899 remains controversial and poorly understood. The *P. falciparum* protein of unknown function identified in this screen using immobilized mAb7899 needs to be further evaluated to determine if it is physiological target of mAb7899 in parasite growth and adhesion assays. Also, the specific domains for binding receptors on erythrocyte membrane surface are still not clarified, but this short sequence from DBL2 may be a potential binders to ICAM-1. In the future, we can use immunoblots to validate the

binding efficacy and interactions between this sequence and ICAM-1. Together with PHISTb and PHISTc proteins and other sequences we screened, findings reported in this Thesis may facilitate the development of novel compounds as potential anti-malarial drugs and vaccine candidates against *P. falciparum* infection in human malaria.

4.6 Contributions

All contents were completed by me solely with minor editing by the advisor.

Chapter 5: Bibliography

[1] Snow RW. Global malaria eradication and the importance of Plasmodium falciparum epidemiology in Africa. BMC Med. 2015;13:23. Published 2015 Feb 3. doi:10.1186/s12916-014-0254-7

[2] WHO. 2022. World Health Organization.World Malaria Report 2022. Accessed March 20, 2022.https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022

[3] Mace KE, Lucchi NW, Tan KR. Malaria Surveillance - United States, 2018. MMWR Surveill Summ. 2022;71(8):1-35. Published 2022 Sep 2. doi:10.15585/mmwr.ss7108a1

[4] Zekar L, Sharman T. Plasmodium Falciparum Malaria. In: *StatPearls*. Treasure Island (FL): StatPearls Publishing; August 8, 2022.

[5] Daily JP, Minuti A, Khan N. Diagnosis, Treatment, and Prevention of Malaria in the US: A Review. *JAMA*. 2022;328(5):460-471. doi:10.1001/jama.2022.12366

[6] Milner DA Jr. Malaria Pathogenesis. Cold Spring Harb Perspect Med. 2018;8(1):a025569. Published 2018 Jan 2. doi:10.1101/cshperspect.a025569

[7] Brancucci NMB, Gerdt JP, Wang C, et al. Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum. *Cell*. 2017;171(7):1532-1544.e15. doi:10.1016/j.cell.2017.10.020

[8] Aly AS, Vaughan AM, Kappe SH. Malaria parasite development in the mosquito and infection of the mammalian host. Annu Rev Microbiol. 2009;63:195-221. doi:10.1146/annurev.micro.091208.073403

[9] White NJ. Anaemia and malaria. *Malar J*. 2018;17(1):371. Published 2018 Oct 19. doi:10.1186/s12936-018-2509-9

[10] Zekar L, Sharman T. Plasmodium Falciparum Malaria. [Updated 2022 Aug 8]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK555962/

[11] Coppel RL. Vaccinating with the genome: a Sisyphean task?. *Trends Parasitol*. 2009;25(5):205-212. doi:10.1016/j.pt.2009.02.006

[12] Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*. 1985;228(4705):1315-1317. doi:10.1126/science.4001944

[13] Bazan J, Całkosiński I, Gamian A. Phage display--a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. Hum Vaccin Immunother. 2012;8(12):1817-1828. doi:10.4161/hv.21703

[14] Bazan J, Całkosiński I, Gamian A. Phage display--a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. Human Vaccine Immunotherapy. 2012 Dec 1;8(12):1817-28. doi: 10.4161/hv.21703.

[15] de Koning-Ward TF, Gilson PR, Boddey JA, et al. A newly discovered protein export machine in malaria parasites. *Nature*. 2009;459(7249):945-949. doi:10.1038/nature08104

[16] Proellocks NI, Herrmann S, Buckingham DW, et al. A lysine-rich membrane-associated PHISTb protein involved in alteration of the cytoadhesive properties of Plasmodium falciparum-infected red blood cells. *FASEB J*. 2014;28(7):3103-3113. doi:10.1096/fj.14-250399

[17] Parish LA, Mai DW, Jones ML, Kitson EL, Rayner JC. A member of the Plasmodium falciparum PHIST family binds to the erythrocyte cytoskeleton component band 4.1. *Malar J*. 2013;12:160. Published 2013 May 11. doi:10.1186/1475-2875-12-160
[18] Oberli A, Slater LM, Cutts E, et al. A Plasmodium falciparum PHIST protein binds the virulence factor PfEMP1 and comigrates to knobs on the host cell surface. *FASEB J*. 2014;28(10):4420-4433. doi:10.1096/fj.14-256057
[19] Zhang M, Wang C, Otto TD, et al. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science*. 2018;360(6388):eaap7847. doi:10.1126/science.aap7847
[20] Lauterbach SB, Lanzillotti R, Coetzer TL. Construction and use of Plasmodium falciparum phage display libraries to identify host parasite interactions. *Malar J*. 2003;2(1):47. Published 2003 Dec 17. doi:10.1186/1475-2875-2-47
[21] Nagaoka H, Kanoi BN, Morita M, et al. Characterization of a Plasmodium falciparum PHISTc protein, PF3D7_0801000, in blood- stage malaria parasites. *Parasitol Int*. 2021;80:102240. doi:10.1016/j.parint.2020.102240
[22] Almukadi H, Schwake C, Kaiser MM, et al. Human erythrocyte band 3 is a host receptor for *Plasmodium falciparum* glutamic acid-rich protein. *Blood*. 2019;133(5):470-480. doi:10.1182/blood-2018-07-865451
[23] Chang HH, Falick AM, Carlton PM, Sedat JW, DeRisi JL, Marletta MA. N-terminal processing of proteins exported by malaria parasites. *Mol Biochem Parasitol*. 2008;160(2):107-115. doi:10.1016/j.molbiopara.2008.04.011
[24] Warncke JD, Vakonakis I, Beck HP. Plasmodium Helical Interspersed Subtelomeric (PHIST) Proteins, at the Center of Host Cell Remodeling. *Microbiol Mol Biol Rev*. 2016;80(4):905-927. Published 2016 Aug 31. doi:10.1128/MMBR.00014-16
[25] Ghumra A, Khunrae P, Ataide R, et al. Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to Plasmodium falciparum. PLoS One. 2011;6(1):e16414. Published 2011 Jan 31. doi:10.1371/journal.pone.0016414
[26] Pasternak ND, Dzikowski R. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum. Int J Biochem Cell Biol. 2009;41(7):1463-1466. doi:10.1016/j.biocel.2008.12.012