Identification of *Plasmodium falciparum* Reticulocyte Binding Protein Homologue 5-Interacting Protein, Pfripr, as a highly conserved Blood-stage Malaria Vaccine Candidate

Ntege Edward Hosea

Submitted in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

March 2017

Faculty of Engineering, Proteo-Science Center

Division of Malaria Research

EHIME UNIVERSITY

Abstract

Genetic variability in *Plasmodium falciparum* malaria parasites hampers current malaria vaccine development efforts. This thesis aimed to address the impact of genetic variability on vaccine efficacy in clinical trials, with a hypothesis that conserved antigen targets can offer robust host immunity across multiple falciparum strains. Therefore, suitable vaccine candidates should be assessed for levels of polymorphism and genetic diversity. Using a total of one hundred and two clinical isolates from a region of high malaria transmission in Uganda, we analyzed extent of polymorphism and genetic diversity in four recently reported novel blood-stage malaria vaccine candidate proteins: Rh5 interacting protein (PfRipr), GPI anchored micronemal antigen (PfGAMA), rhoptry-associated leucine zipper-like protein 1 (PfRALP1) and Duffy binding-like merozoite surface protein 1 (PfMSPDBL1). In addition, utilizing the wheat germ cell-free system, we expressed recombinant proteins for the four candidates based on *P. falciparum* laboratory strain 3D7 sequences, immunized rabbits to obtain specific antibodies (Abs) and performed functional growth inhibition assay (GIA). The GIA activity of the raised Abs was demonstrated using both homologous 3D7 and heterologous FVO strains *in vitro*. Both *pfripr* and *pfralp1* are less polymorphic but the latter is comparatively more diverse, with varied number of regions having insertions and deletions, asparagine and 6-mer repeats in the coding sequences. *Pfgama* and *pfmspdbl1* are polymorphic and genetically diverse among the isolates with antibodies against the 3D7-based recombinant PfGAMA and PfMSPDBL1 inhibiting merozoite invasion only in the 3D7 but not FVO strain. Moreover, although Abs against the 3D7-based recombinant PfRipr and PfRALP1 proteins potently inhibited merozoite invasion of both 3D7 and FVO, the GIA activity of anti-PfRipr was much higher than that of anti-PfRALP1. Thus, PfRipr is regarded as a promising blood-stage vaccine candidate for next-generation vaccines against *P. falciparum*.

Summary

Thesis title: Identification of Plasmodium falciparum reticulocyte binding protein homologue 5-interacting protein, PfRipr, as a highly conserved blood-stage malaria vaccine candidate.

Background

Malaria is a life threatening disease caused by five parasite species within the genus Plasmodia including, *P.falciparum, P.vivax, P.malariae, P. ovale,* and *P. knowlesi*. Of these, *P.falciparum* causes the most severe infections and number of deaths. An infected female Anopheles Mosquito vector transmits malaria. According to the latest World Health Organization (WHO), there were an estimated 212 million new cases of malaria, and 429,000 deaths, 90% of which occurred in sub-Saharan Africa in 2016. Global efforts to control malaria burden rely heavily on the availability and proper use of insecticides to kill the vector by using Insecticide Treated Nets (ITNs) and In-door Residual Spraying (IRS) and the effective antimalarial drug, artemisinin. In the period between 2000 and 2015 increased funding scaled up the use of these effective malaria control intervention especially in the Africa region. This resulted in the reduction of incidence rates by 37% globally, and 42% in Africa, and mortality rates by 60% globally, and 66% in Africa. However, there is a worrisome concern of development and spread of parasite resistance to the artemisinin, and mosquito resistance to the insecticides, that could threaten the progress. There is therefore, a global demand for development of especially effective malaria vaccines that could complement current effective control measures.

The malaria parasite has a complex life cycle that alternates in both the mosquito vector and the human host. In the human host, different stages of the parasite can be found in different organs of the body of during infection. This exposes different sets of parasite proteins (antigens) to the host immune system. The exposure renders the development of an effective malaria vaccine an uphill task. An effective malaria vaccine would require a polyvalent multicomponent vaccine with a combination of candidate antigens from different

stages of the life cycle. Hence the approach of targeting vaccine development by differentiating between stages including, Pre-erythrocytic stage vaccine that targets prevention of sporozoites entry and development in the liver, Asexual blood-stage vaccine that targets disease prevention through blocking of merozoite invasion and intra-erythrocytic parasite development, and Transmission-blocking vaccine that targets sexual and sporogonic stages to prevent parasite development in the mosquito.

Problem Statement

A malaria vaccine of high efficacy is crucial a complementary tool to the current effective control measures against malaria. Its development however, has proved exceptionally challenging. This is because of a number of factors among others, the complicated biology of the malaria parasite as it traverses through the stages of its life cycle expressing different, stage-specific antigens, each stimulating a specific immune response, and extensive antigenic diversity that most times results in allele-specific immune responses leading to selection for non-vaccine serotypes and allow new recombination forms of parasites to emerge in the natural populations. The RTS,S vaccine, a leading pre-erythrocytic subunit vaccine and only vaccine that has completed phase 3 trial, showed moderate level efficacy of modest duration. Therefore, there is need to explore approaches to either boost efficacy of the RTS, S/AS01, and/or other available vaccine candidates of even different stages or discover new antigens in the design of next-generation vaccines with prospects of a highly effective multi-component/multi-stage/multi-antigen formulation.

Targeting vaccines against blood-stage merozoite antigens would improve vaccine efficacy, since the antigens are targets of acquired immunity, and controlling parasite density may reduce generation of the sexual stage parasites and subsequently reduce transmission. Moreover, as analysed, determinants of RTS,S induced immunogenicity in the final results of the phase 3 trial. The analysis revealed anti-CSP antibody titers, a surrogate marker of protection for the magnitude and duration of the vaccine efficacy, waned more rapidly during

participant follow-up at especially higher transmission intensity because of reduced titers levels and lesser blood-stage immunity. The finding is a significant limitation that clearly highlights the importance of blood-stage immunity in preventing malaria. However, the most advanced leading blood-stage vaccine candidates like FMP2.1/AS02A, a subunit vaccine based on P. *falciparum* 3D7 apical membrane antigen 1 (AMA1) sequence, have suffered poor efficacy in human trials mainly due to high genetic polymorphisms of AMA1 that induce not only allele-specific immune responses but also suboptimal concentrations of functional antibodies against malaria parasites.

The Study Rationale

The extensive genetic diversity and polymorphisms in several *P.falciparum* malaria antigen-coding genes arise as a result of selection by the human immune system. Novel, relatively conserved antigens that induce broadly cross-reactive antibody and cell-mediated immune response may provide longer lasting and more efficacious protection. There is therefore, need to prioritize candidate peptides that comprise of conserved epitope targets of immunity in the design of next generation vaccines. The approach of population genetic and structural studies, followed by molecular epidemiological surveys or *in vitro* functional studies has been instrumental in identifying immunologically relevant diversity in pathogens prior to development and testing of vaccines.

In this thesis, a similar approach of population genetics followed by *in vitro* functional studies, growth inhibition assay (GIA) was used to identify immunologically relevant conserved antigens among novel *P. falciparum* malaria blood-stage vaccine candidate antigens; RH5-interacting protein (PfRipr), rhoptry-associated leucin zipper-like protein 1 (PfRALP1), Glycosylphosphatidylinositol (GPI) anchored micronemal antigen (PfGAMA) and Duffy binding-like merozoite surface protein1 (PfDBLMSP1). The antigens are essential to the malaria parasite survival. Wheat germ cell-free system (WGCFS) expressed PfRALP1, PfGAMA and PfMSPDBL1 recombinant proteins were found immunogenic, and antibodies

for each of the recombinant proteins potently inhibited erythrocyte invasion *in vitro.* Additionally, human sera collected from *P. falciparum* malaria endemic regions recognized the WGCFS expressed recombinant proteins. However, investigations into the extent of polymorphism and genetic diversity in PfRipr, PfRALP1, PfGAMA, and PfMSPDBL1 were minimal, especially in the malaria endemic populations in Africa.

Significance And Objectives Of The Thesis

The thesis attempted to contribute to the 2030 WHO Global Technical Strategic goals by developing efficacious next-generation malaria vaccine, through the identification of highly conserved *P.falciparum* antigen targets of robust natural immunity across multiple *P. falciparum* strains.

Specifically the thesis' aims were,

1. To explore extent of polymorphism and genetic diversity in PfRipr, PfGAMA, PfRALP1 and PfMSPDBL1.
2. To evaluate antibodies against WGCFS expressed recombinant PfRipr, PfGAMA, PfRALP1 and PfMSPDBL1 proteins based on *P. falciparum* 3D7 DNA sequence in inhibiting growth of strains 3D7 And FVO.

Materials And Methods

The study utilized a total of 102 *P. falciparum* clinical isolates from a region of high malaria transmission in Uganda, and *P. falciparum* laboratory strains 3D7 and FVO. We also searched and selected Single Nucleotide Polymorphisms (SNPs) among 164 to 203 *P. falciparum* isolates from the online PlasmoDB. We used tools of population genetic analysis to assess extent of polymorphism and genetic diversity in the four above mentioned merozoite proteins; PfRipr, PfGAMA、PfRALP1, and PfMSPDBL1. These proteins were recently reported as well characterized potential blood-stage vaccine candidates that are immunogenic with minimal genetic variability in a few field isolates and laboratory strains. *P.falciparum* AMA1 and the housekeeping protein, adenylosuccinate lyase (ADSL) were positive and

negative controls respectively. Furthermore, we employed the principle of reverse vaccinology by utilizing WGCFS to express recombinant proteins for the four vaccine candidates based on *P. falciparum* strain 3D7 sequences, immunized rabbits to obtain specific antibodies and performed growth inhibition assays (GIA). The GIA activity of the raised antibodies was demonstrated using both homologous 3D7 and heterologous FVO strains *in vitro*.

Results

We demonstrated that approximately 50% of the selected PlasmoDB SNPs were unique to the Uganda isolates, suggesting a finding of new variants in this population. Genetic analyses showed that *pfgama* and *pfmspdbl*1 are polymorphic and genetically diverse, but both *pfripr* and *pfralp*1 are less polymorphic. *Pfralp1* is however, comparatively more diverse than *pfripr*, due to existence of insertion-deletion (INDELs), asparagine and 6-mer repeat regions in the sequences.

In addition, with the WGCFS, we successfully expressed a large fragment of amino acids 717 residues recombinant PfRipr protein. The WGCFS expressed recombinant PfRipr was immunogenic in rabbit, and generated quality specific polyclonal antibodies (IgG). Antibodies against 3D7 recombinant proteins; PfGAMA and PfMSPDBL1 inhibited merozoite invasion of the homologous strain 3D7 but not the strain FVO. The antibodies against strain 3D7 recombinant proteins; PfRipr and PfRALP1, potently inhibited merozoite invasion of homologous 3D7 and heterologous strain FVO. However, the GIA of anti-PfRipr IgG was much higher than that of anti-PfRALP1.

Conclusion

The results suggest that PfRipr is a promising conserved blood-stage antigen target of immunity and suitable for further development as an efficacious second-generation vaccine against *P. falciparum* malaria

Summary (Japanese)

論文名

抗原多型の少ないマラリアワクチン候補抗原 PfRipr の同定

審査結果の要旨

マラリアは、マラリア原虫によって引き起こされる感染症であり、ハマダラカによって媒介され、熱帯・亜熱帯地域に分布している。2016 年現在で毎年２億人以上が罹患し、約 43 万人が死亡しており、その 9 割はサハラ砂漠以南のアフリカ諸国の子ども達である。現在、薬剤耐性マラリア原虫や、殺虫剤耐性ハマダラカが拡散したためマラリア対策は困難に直面している。そこで、新たな対策としてマラリアワクチンの開発が国際的に進められてきたが未完成である。その理由として、これまでマラリアワクチン候補として研究されてきた原虫タンパク質の数が僅かであった事、さらにマラリア原虫はヒトの免疫系から逃れるため、これまで研究開発が進められてきた主要なワクチン候補抗原に遺伝子多型が存在したことがあげられる。そこで、2002 年に熱帯熱マラリア原虫 3D7 株を用いたマラリアゲノム情報が公開され、新たなワクチン候補の同定が期待された。しかし、大腸菌等の従前の技術ではマラリア原虫の組換えタンパク質合成が困難であったため、研究は進んでいなかった。近年、愛媛大学で開発されたコムギ胚芽無細胞タンパク質合成法を用いることにより、マラリア組換えタンパク質合成の効率ならびにその品質が飛躍的に向上することが判明した。そこで申請者の所属する研究部門では、コムギ胚芽無細胞タンパク質合成法を用いて、赤血球への侵入型である熱帯熱マラリア原虫メロゾイトのタンパク質を網羅的に合成し、作製した抗体の培養熱帯熱マラリア原虫株 3D7 に対する増殖阻害活性を指標に、新規マラリアワクチン候補抗原を探索してきた。これまでの当部門の研究により、PfRipr、PfRALP1、PfGAMA および PfMSPDBL1 の４

種類のメロゾイトタンパク質を新規マラリアワクチン候補抗原として同定してきた。そこで、新規マラリアワクチンの開発をさらに推進するためには、これら４種類の抗原遺伝子のフィールド分離株原虫における多型の有無、ならびに、熱帯熱マラリア原虫 3D7 株タイプの抗原に対する抗体が、抗原多型のある熱帯熱マラリア原虫 FVO 株に対しても増殖阻害活性を有するか、を検証することが重要な研究課題と考えられた。

そこで申請者は、熱帯熱マラリア高度流行地であるウガンダから得られた熱帯熱マラリア原虫フィールド分離株 102 人分を用いて、PfRipr、PfRALP1、PfGAMA および PfMSPDBL1 の４種類の遺伝子多型を解析した。その際、細胞質内の酵素タンパク質 PfADSL 遺伝子を多型の少ない陰性対照、遺伝子多型が多いため既にワクチン開発が断念された PfAMA1 遺伝子を陽性対照とした。その結果、PfGAMA および PfMSPDBL1 遺伝子は PfADSL 遺伝子と比較して多型が多く、中でも PfMSPDBL1 は PfAMA1 と同程度に遺伝子多型が多かった。一方、PfRipr と PfRALP1 遺伝子は PfADSL 遺伝子同様多型が少なかった。中でも PfRipr 遺伝子は、PfRALP1 遺伝子に存在する繰り返し配列もなく、今回検討した４種類の新規ワクチン候補遺伝子の中で最も多型が少ないことが判明した。次に申請者は、熱帯熱マラリア原虫 3D7 株タイプの抗原に対する抗体が、遺伝子多型のある熱帯熱マラリア原虫 FVO 株に対しても原虫増殖阻害活性を有するか、を検証するため、3D7 株に対して原虫増殖阻害活性を示すことが判明している PfRipr、PfRALP1、PfGAMA および PfMSPDBL1 に対する抗体を、熱帯熱マラリア原虫 FVO 株の培養液中に添加し、増殖阻害活性を測定した。その結果、PfRipr および PfRALP1 に対する抗体は、遺伝子多型の存在する FVO 株に対しても増殖阻害活性を示すことが明らかになった。中でも PfRipr に対する抗体は PfRALP1 に対する抗体よりも強い増殖阻害活性を示した。一方、比較的遺

伝子多型の多かった PfGAMA および PfMSPDBL1 に対する抗体は、FVO 株に対して増殖阻害活性を認めなかった。したがって、申請者は PfRipr が遺伝子多型の少ない新規マラリアワクチン候補抗原であることを初めて明らかとした。有望な新規マラリアワクチン候補タンパク質 PfRipr を同定しえた本研究は、マラリア制圧に向けワクチンが切望されている現在、時宜を得たものと言える。

Declaration

This is to certify that this thesis comprises of only my original work towards the degree of Doctor of Philosophy except where indicated, and that due acknowledgement has been made in the text to all materials otherwise used.

Name: Ntege Edward Hosea

March 2017

Acknowledgements

I am greatly indebted to the kind support from several people at the Division of Malaria Research, Proteo-Science Center, Ehime University, Ehime Japan and Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Osaka Japan, who in different ways contributed to the success of this PhD program. I am especially deeply grateful to my academic adviser Professor Takafumi Tsuboi, and supervisor Dr. Nobuko Arisue for their unwavering mentorship through out the program.

Professor Tsuboi, I can't thank you enough for the trust and faith entrusted in me, when you kindly accepted my admission into a rare and great opportunity to advance my career at your laboratory. You indeed are an amazing mentor and parental figure. Notably, I am very grateful for your relentless efforts in ensuring a conducive academic environment, and support throughout the training. It's difficult to express my gratitude in words, but sincerely from the bottom of my heart, I say; thank you very much.

Dr. Arisue, I would like to thank you very much, for your constant guidance and training on the different perspectives on my research, and particularly for the mind blowing and challenging email exchanges that greatly improved my communication skills. Thank you for inspiring and motivating me as a scientist.

I would like in a very especial way, to sincerely acknowledge Professor Toshihiro Horii for his generosity. This training was made successful through Professor Toshihiro's kind financial support, as part of his Japan Science and Technology Agency (JST) grant. I am also greatly honored for the opportunity to have worked with him over the years, particularly on the clinical development of BK-SE36 malaria vaccine candidate. Moreover, Professor Horii, yet gladly served on the advisory committee that provided support and wise counsel through out the training. Professor Horii, *hontoni domo aligato ganzaimashita!*

Dr. Nirianne Marie Q. Palacpac, you are such a very special friend and a great mentor. I am very lucky to have met you and for being part of my career life story. I sincerely cherish your friendship and the overwhelming support that seen me grow to this level of training. Thank you very much for being such a great friend and unwavering mentor over the years.

Along this journey, I encountered many wonderful people that in one way or another offered support in either the conduct of experiments or smooth existence as a team in the laboratory. I am extremely grateful for their kind support in this aspect. My gratitude extends to the PROS administration staff past and present, and the International Office where I met really incredible people, many of whom were very instrumental in my day to day living in Matsuyama. I am particularly indebted to many, but mostly Professor Ruth Vergin. Professor Vergin is a God given gift to the Ehime University International Community. Thank you very much for being such a wonderful friend, a colleague and a mentor to the international community.

Last but not least, I acknowledge my dear wife Grace, and our two lovely daughters; Elizabeth and Emeline. I highly appreciate the great love, and consistent support. Your genuine love turned hard days great. Thank you very much.

Table of Contents

List of figures

List of Tables

1.0 CHAPTER 1 – INTRODUCTION

1.1 Overview Of Malaria

1.1.1 The Burden of Malaria

Malaria is a potentially life threatening disease caused by protozoan parasites of the genus Plasmodia that belong to the phylum *Apicomplexa*. There are more than 200 plasmodia species that have been identified to-date (Rich SM et al *Genetic and Evolutionary Aspects. Springer US 2006*), and atleast five are well known to cause human malaria including, *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*. The disease is transmitted to human through an infected pregnant female Anopheles Mosquito vector.

The most severe and lethal forms of malaria are due to *Plasmodium falciparum (P. falciparum),* which remains a major global health problem especially in the world's poorest countries. . *Plasmodium vivax* is the second most important species and is prevalent in Southeast Asia and Latin America. *Plasmodium vivax* and *Plasmodium ovale* have the added complication of a dormant liver stage – the hypnozoites, which can be reactivated later and cause disease. *Plasmodium ovale and Plasmodium malariae* represent only a small percentage of infections worldwide. *Plasmodium knowlesi* species infects both macaque monkeys and human with unclear mode of transmission (WHO *World Malaria report 2016*; Sutherland et al, *Journal of Infectious Diseases. 2010*; Singh et al, *The Lancet. 2004*).

In 2016, the World Health Organization (WHO) estimated 3.2 billion people were at risk of malaria (Figure 1), 212 million new cases of malaria with a death toll of at least 429, 000 people worldwide, 90% of which were in the Africa region, affecting mainly children under five years of age (WHO: *World Malaria report* 2016, Bhatt, S. et al. *Nature*. 2015).

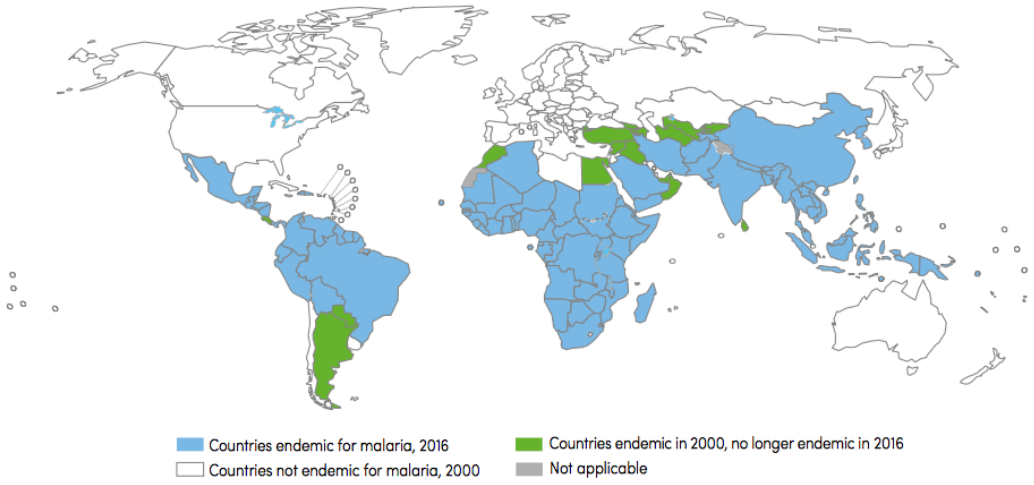


Figure 1. World map showing malaria endemic countries in 2000 and 2016

Source: Adapted from (WHO: *World malaria report 2016*)

In Uganda, malaria is highly endemic in most parts (over 95%), and the country ranks fourth globally in the estimated number of annual cases (Okello PE et al *Am J Trop Med Hyg. 2006*; Yeka A et al *Acta Trop. 2012*). The WHO 2016 estimates were amidst a registered tremendous progress in reduction of incidence rates by 37% globally, and 42% in Africa, and mortality rates by 60% globally, and 66% in Africa, due to scaled up control and elimination strategies in the period between 2000 and 2015. The scaled-up effective strategic interventions include, long-lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS) and intermittent preventive therapy in pregnancy (IPTp), better diagnostics for case ascertainment, and effective treatments using artemisinin-based combination therapies (ACTs) (WHO: *World Malaria report 2016;* Bhatt S, et al. *Nature. 2015;* Irene N. Nkumama, et al. *Trends in Parasitology* 2016) However, critical to the global agenda against malaria, are several threats to current effective control interventions including, stagnation in international funding over the last several years, the emergence and spread of resistance to ACTs, increasing mosquito resistance to pyrethroid insecticides, and evidence of rebound increases

of malaria in some regions where it was previously eliminated (Dondorp AM, Nosten F, Yi P, et al. *New England Journal of Medicine 2009*; Phyo AP, Nkhoma S, Stepniewska K, et al. *The Lancet. 2012*; Ranson, Hilary et al. *Trends in parasite. 2016*). The resistance of malaria parasites to antimalarial drugs and the resistance of mosquitoes to insecticides have in part led to resurgence of malaria in previously declared malaria free areas. This calls for renewed efforts in developing effective malaria vaccines, new antimalarial agents and other novel control interventions (SachJD *Science 2002*; Greenwood B *Acta tropica 2005*; WHO: *World Malaria report 2016*; White NJ. Et al Expert *Opin Pharmacotherapy, 2016*). The effective interventions are desired to initially reduce malaria transmission, and its associated burden, with an ultimate target of eradication.

1.1.2 Life Cycle of the malaria parasite

In the thesis, I focused on *P. falciparum,* the deadliest and most widespread species of human malaria. The life cycle of *P. falciparum* parasite is quite complex. The cycle alternates between extracellular and intracellular forms in both the mosquito and human (Figure-2). This adaptation enables the parasite to successfully exploit, and move through a number of different cellular environments, to ensure propagation and survival of its progeny. In human, the cycle begins with a bite of an infected female Anopheles mosquito during a blood meal. Through the dermis layer of the skin, 25 to 100 sporozoites are injected and quickly migrate through the blood stream to initiate a liver stage infection (Vanderberg JP *Experimental parasitology 1977*; Ponnudurai et al *Transactions of the Royal Society of Tropical Medicine and Hygiene 1991*; Vanderberg & Frevert *International journal for parasitology 2004*). During the liver stage infection, sporozoites traverse the cytosol of several cells prior to establishing themselves within a single hepatocyte (Vanderberg JP *Experimental parasitology 1977*). The triggers and the signals associated with traversal are generally unknown, but the process is thought to be helpful in priming sporozoites for subsequent infection of hepatocytes

(Mota et al *Science 2001*). In the hepatocyte, each sporozoite develops within parasitophorous vacuole into trophozoites that subsequently divides into about 30000 – 40000 liver merozoites. Following unknown triggers, merosomes release the liver merozoites into the bloodstream, which invade circulating Red Blood Cells (RBCs) to start another stage of the cycle, the asexual blood stage.

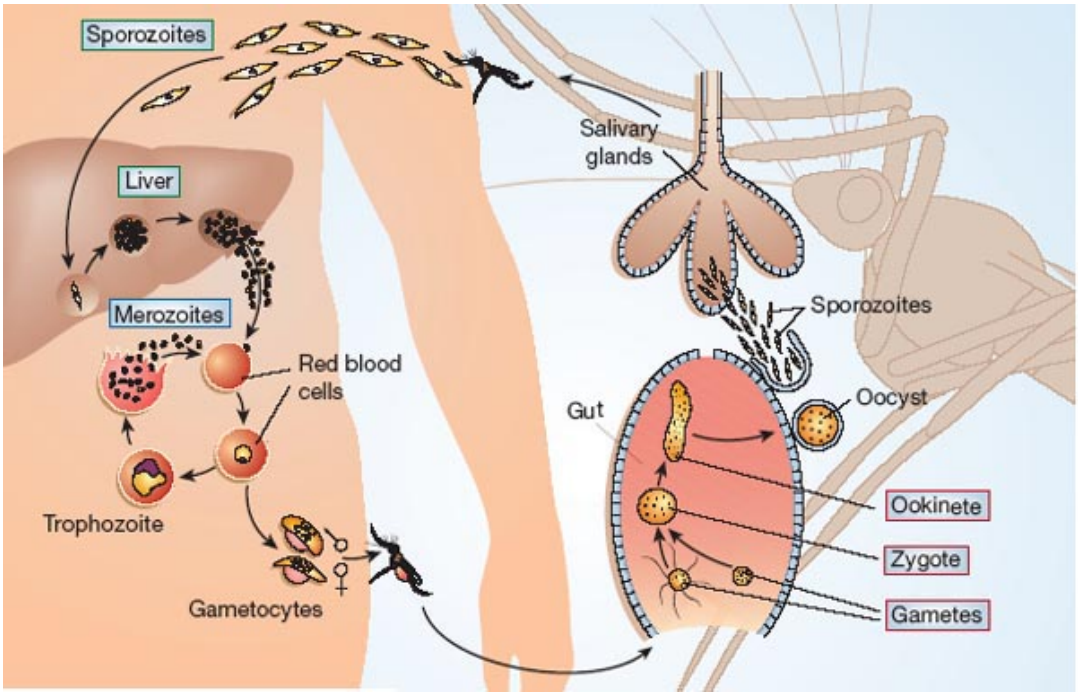


Figure 2. The life cycle of *Plasmodium falciparum*

Source: Adapted from (Ménard et al, *Nature 2005*)

Malaria infection begins when an infected female Anopheles mosquito bites a person, injecting Plasmodium parasites, in the form of sporozoites, into the bloodstream. The sporozoites pass quickly into the human live r. The sporozoites multiply asexually in the liver cells over the next 7 to 10 days, causing no symptoms. In an animal model, the parasites, in the form of merozoites, are released from the liver cells in vesicles (Merosomes), which

eventually disintegrate, freeing the merozoites to enter the blood phase of their development. In the bloodstream, the merozoites invade RBCs and multiply again until the cells burst. Then they invade more RBCs. The cycle is repeated, causing fever each time parasites break free and invade blood cells. Some of the infected blood cells leave the cycle of asexual multiplication and develop into sexual forms of the parasite, called gametocytes that circulate in the blood stream. When a mosquito bites an infected human, it ingests the gametocytes, which develop further into mature sex cells called gametes. The fertilized female gametes develop into actively moving ookinetes that burrow through the mosquito's midgut wall and form oocysts on the exterior surface. Inside the oocysts, thousands of active sporozoites develop. The oocysts eventually burst, releasing sporozoites into the body cavity that travel to the mosquito's salivary glands. The cycle of human infection begins again when the mosquito bites another person.

At the liver stage, infected individuals remain malaria symptom free, with difficulty in early detection of the disease. The asexual blood stage of the parasite begins when liver merozoites invade host RBCs. After successful RBC invasion, merozoites remain within parasitophorous vacuole (PV) that forms around the parasite during the invasion process. Once inside, the parasite then starts an extensive process of RBCs modification that is important to enable access to nutrients and evasion of host immune responses. The infected RBCs become rigid and poorly deformable, resulting in an increased propensity to cytoadhere to endothelial cells. A typical asexual blood stage cycle of *P. falciparum*, lasts for 48-hours, where the parasites develop within the PV, from ring stage through trophozoite and finally schizont stages. At schizont stage the parasite divides asexually (schizogony), to form between 16-32 daughter merozoites that egress from the infected cells to invade other RBCs, continuing the asexual blood stage (Cowman & Crabb *Cell 2006*). The continuous cyclical

merozoite egress, invasion, and sequestration of infected RBCs, leads to the clinical symptoms associated with malaria (Miller et al, *Nature 2002*). The clinical manifestation of malaria varies significantly, from febrile illness accompanied with fever, nausea and headache, to severe anemia and cerebral malaria that can eventually result in coma and death (Heddini A, *International journal for parasitology 2002*; Mackintosh et al, *Trends in parasitology 2004*). A small proportion of merozoites initiate another stage in the life cycle of the parasite called the sexual stage by forming gametocytes, which can be ingested by the mosquito during a blood meal. The sexual stage of the parasite begins in the mosquito. Within the lumen of the mosquito gut, haploid female and male gametocytes develop into gametes and fuse to form zygotes. Through differentiation and maturation, in about 24 hours the zygotes undergo substantial morphological changes as they fuse to form ookinetes. The ookinetes traverse the midgut epithelium to the outer wall of the midgut (Angrisano et al *International journal for parasitology 2012*). In the outer wall, sporogony ensues, when the ookinetes develop into oocytes and undergo multiple cycles of division and maturation to form thousands of sporozoites (Sinden & Strong *Transactions of the Royal Society of Tropical Medicine and Hygiene1978*). The sporozoites subsequently migrate to the mosquito salivary glands, ready to be injected into a human host upon the mosquito's next blood meal, thereby completing the parasite life cycle (Vlachou D et al *Current opinion in genetics & development 2006*; Pradel G *Parasitology 2007*).

1.1.3 The Merozoite

The invasion of *P. falciparum* during the asexual blood stage of is essential for the parasite survival. A successful invasion process is highly dependent on sequentially released proteins from merozoites, the invasive form of the parasite. A merozoite is a small ovoid structure about 1.5-µm in length and 1.0-µm in width (Dvorak JA et al, *Science 1975*; Bannister et al *Cell and tissue research 1986*; Preiser P et al *Microbes and Infection 2000*). It

contains the overall basic eukaryotic cell machinery including among others, Mitochondrion, Nucleus, Golgi network, Endoplasmic reticulum, Ribosomes and plastids (Figure 3) (McFadden et al *In Origins of algae and their plastids 1996*; Preiser P et al *Microbes and Infection 2000*). The highly invasive parasite is enclosed within a pellicle, which has a double membrane architecture consisting of an outer plasma membrane as well as an inner membrane complex. The cytoskeleton sits underneath the pellicle and includes microtubules and dyneins that are involved in motility during invasion. Defining morphologies of the merozoite include, the apical complex and a thick fibril coat on the surface, comprised of large numbers of surface proteins. The apical complex located at the anterior end of the merozoite houses important specialized organelles such as micronemes and rhoptries and is specifically adapted for RBCs invasion (Aikawa M *Experimental parasitology 1971*). The apical organelles contain proteins that are sequentially released and are important in governing motility, PV development as well as cell adhesion and invasion of RBCs. (Aikawa M et al *The Journal of cell biology 1978*; Sam-Yellowe et al. *Journal of Eukaryotic Microbiology 1995*; Preiser P et al. *Microbes and Infection 2000*; Blackman & Bannister MJ et al. *Molecular and biochemical parasitology 2001*; Healer et al. *Infection and immunity 2002*). The surface coat of merozoites is composed of closely spaced fibril clusters of two separate types; one of which forms 5-10 parallel filaments that are around 18-22 nm long and are 2-3 nm thick and a second are much thinner filaments that are at least 40 nm long which bend parallel to the surface (Langreth et al. *The Journal of protozoology 1978*; Bannister et al. *Cell and tissue research 1986*; Galinski & Barnwell *Parasitology today 1996*).

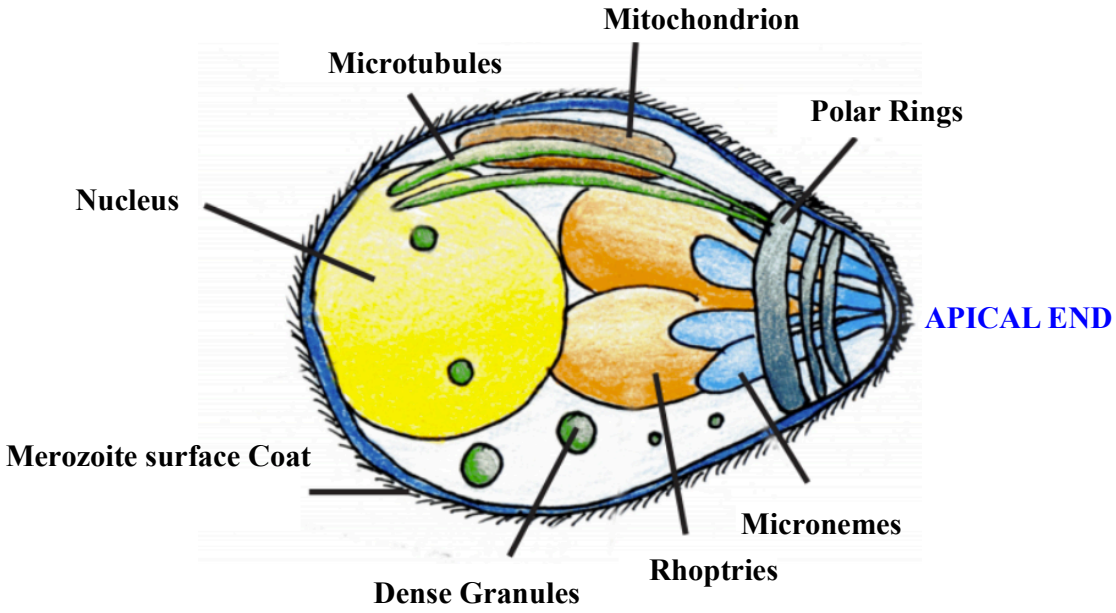


Figure 3. Schematic of a merozoite with labeled important structures

Between these two types of clusters exists an amorphous proteinaceous coat, thought to consist mainly of Merozoite Surface Proteins (MSPs). Many of the MSPs are thought to interact with each other to form macromolecular complexes on the surface, which appear to play important roles in the early stages of merozoite invasion (Bannister et al. *Cell and tissue research 1986*).

1.1.4 The Process of Merozoites Invasion of Erythrocytes

The process of merozoites invasion of erythrocytes is dynamic, and involves a complex series of events (Johnson et al. *Parasitology 1980*; Gilson & Crabb, *International journal for parasitology 2009*). It is a tightly controlled multi-step process, involving multiple specific ligand-receptor interactions between the RBC and parasite. Upon egress, merozoites attach to neighboring erythrocytes and start a process that is very quick and efficient. Invasion takes place within 30-60 seconds and by 20 minutes later, the newly invaded merozoite has transformed into a ring-stage parasite (Dvorak et al. *Science 1975*; Mitchell & Bannister, *Critical reviews in oncology/hematology 1988*).

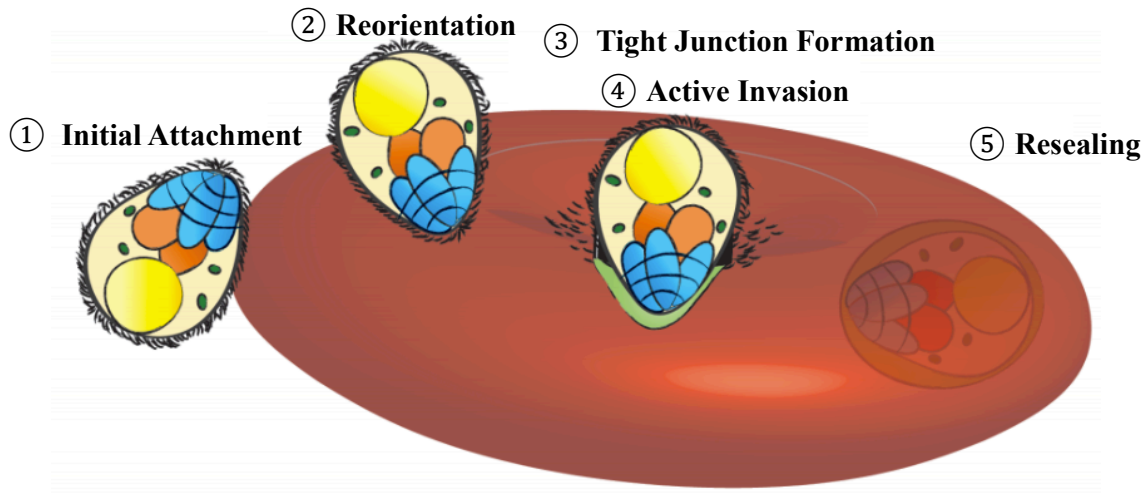


Figure 4. Invasion of merozoites into host RBCs. *Invasion of merozoites into RBCs involves 5 main steps: (1) Initial attachment, a low-affinity interaction that involves the recognition of RBC receptors. (2) Reorientation allows the apical end of the merozoite to interact with the RBC surface. (3) Commitment to invasion occurs when tight junctions are formed between the merozoite and the RBC. (4) As the merozoite is actively propelled into the RBC by an actin-myosin motor, the surface coat is shed off by proteases. (5) Resealing of the RBC surface happens when the entire merozoite has successfully entered the RBC and is enclosed within the newly formed parasitophorous vacuole.*

The invasion process can be described in four steps; initial attachment, reorientation, tight junction formation and active invasion followed by resealing of the erythrocyte (Figure 4). The initial recognition and attachment of the merozoites to erythrocytes is a reversible passive step, which is thought to mediate via low affinity interactions between merozoite surface proteins and erythrocyte receptors (Cowman & Crabb *Cell 2006*; Weiss et al. *PLoS Pathog 2015*). The initial contact may occur on any part of the merozoite surface, and causes very weak deformations on the erythrocyte. Through unknown signaling mechanisms, the

parasite then begins to re-orientate, so that the apical end of the parasite juxtaposes the erythrocyte (Dvorak et al. *Science 1975*). This is particularly an important step, because the ligands mediating downstream steps are found in the apical organelles. The next step in invasion process is the formation of a tight junction. This is an active step that is dependent on the sequential release of proteins (Ligands) from the merozoite apical complex organelles. The main invasion ligands at this step are the Erythrocyte Binding Ligand (EBL) and Reticulocyte binding like Homologue (Rh) family of proteins as well as Apical Membrane Antigen 1 (AMA1) and the Rhoptry Neck Protein (RON) complex, which are released from the rhoptries and micronemes (Dvorak et al. *Science 1975*; Aikawa et al. *The Journal of cell biology 1978*; Adams et al. *Proceedings of the National Academy of Sciences 1992*; Sam-Yellowe et al. *Journal of Eukaryotic Microbiology 1995*). The release of these proteins also coincides with an intracellular calcium release, which is thought to be a critical checkpoint within the invasion process (Gazarini ML et al. *The Journal of cell biology 2003*; Doerig C et al. *The MALSIG consortium 2009*; Alves E et al. *Journal of Biological Chemistry 2011*; Gao et al. *Nature communications 2013*; Yap et al. *Cellular microbiology 2014*; Weiss et al. *PLoS Pathog 2015*). These ligands interact with erythrocyte receptors to form irreversible interactions, committing the parasite to invading the erythrocyte. At the site of tight junction formation, the erythrocyte membrane thickens and is observed as an electron dense region (Aikawa et al. *The Journal of cell biology 1978*; Miller et al *Journal of experimental medicine 1979*). The tight junction is thought to provide an anchor for which the invading merozoite utilizes together with its actomyosin motor to actively pull itself into the erythrocyte (Tyler JS & Boothroyd JC, *PLoS Pathog 2011*; Bargieri D et al. *Trends in parasitology 2012*). As the tight junction moves from the apical to posterior end, the parasite burrows into the host erythrocyte. A parasitophorous vacuole forms around the invading parasite, progressively expanding to accommodate the merozoite as it gets pushed deeper into the erythrocyte. As the

tight junction slides along the merozoite during this active invasion process, a subtilisin-like 2 (PfSub2) protease cleaves off surface antigens and the membrane coat is released into the blood stream (Harris et al, *PLoS Pathog 2005*; O'Donnell et al, *Current opinion in microbiology 2005*). Once the merozoite completes invasion, the erythrocyte surface is resealed and invasion is completed with the parasite replicating in a contained, niche environment within the parasitophorous vacuole (Dvorak et al. *Science 1975*; Aikawa et al. *The Journal of cell biology 1978*; Cooke et al, *In Seminars in hematology 2004*; Marti et al, *The Journal of cell biology 2005*).

1.2 Malaria Vaccines

1.2.1 An Efficacious Vaccine for Malaria

The burden of malaria has decreased substantially over the past decade (Bhatt S et al *Nature. 2015*; Noor AM et al *Lancet. 2014*; O'Meara WP et al *Lancet Infect Dis. 2010*). However, an effective malaria vaccine is considered crucial to the reduction of malaria related morbidity and mortality, particularly in children, and the ultimate disease eradication. Studies on naturally acquired immunity in malaria endemic regions have shown that antibodies (IgG) passively transferred from immune adults to children or non-immune adults offer partial protection against malaria (Fowkes et al, *PLoS Med. 2010*). Moreover, human volunteers immunized with attenuated parasites, either from the sporozoite stage or the blood stage, conferred strong protection in an infection challenge study (McCarthy & Good, *Human vaccines. 2010*; Roestenberg et al. *The Lancet. 2011*). An effective vaccine is envisaged to induce a level of clinical immunity that would at least protect from malaria, as would naturally acquired immunity from natural exposure. This vaccine would be multivalent and also incorporates antigens from multiple stages of the *P. falciparum* life cycle with the idea of inducing more than one type of immune response. The three stages that are currently explored for vaccine strategies are the pre-erythrocytic stage, asexual blood stage and sexual stage

within the parasite lifecycle. The pre-erythrocytic stage vaccine would target invasive sporozoites that infect hepatocytes. Although this stage does not cause disease and is usually asymptomatic, blocking sporozoites would prevent blood stage infections. In contrast, the erythrocytic stage causes all symptoms (disease) associated with malaria due to the egress and invasion of merozoites into red blood cells. Targeting this stage would directly control parasitaemia, and therefore control malaria disease. The third stage is the sexual stage, where targeting gametocytes would render them noninfectious, therefore reducing transmission associated with malaria.

1.2.2 Pre-erythrocytic vaccines

A strong argument for a malaria vaccine targeting the pre-erythrocytic phase of malaria infection are studies showing that inoculation with irradiated *P. falciparum* sporozoites induce durable and strain-transcending protection in healthy volunteers (Clyde et al The *American journal of the medical sciences 1973;* Hoffman et al *Journal of Infectious Diseases 2002*). RTS,S/AS01 is a leading malaria vaccine candidate that in 2015 adopted a positive scientific opinion for its use outside the European Union (EU), and could be the first vaccine designed for young infants to be licensed (Hoffman SL et al. *Vaccine. 2015*). The RTS.S/AS01 vaccine incorporates a pre-erythrocytic stage protein, CSP (Circumsporozoite Protein) from *P.falciparum* co-expressed with the hepatitis B surface antigen as a virus-like particle in the ASO1 adjuvant (Agnandji et al, *PLoS One. 2011*; RTS, *The Lancet. 2015*). Follow up after a phase 3 trial, which involved 15,460 children across 7 African countries showed that RTS.S/ASO1 protects between 20 – 50 % from infection and disease (Agnandji et al, *PLoS One. 2011*; Schwartz et al, *Malaria journal. 2012*; RTS, *PLoS medicine 2014*). More specifically, reduction in severe malaria incidences in children was shown to be 36 % among children and 28 % among infants (RTS, *The New England journal of medicine 2012*; RTS, *The Lancet. 2015*). However, the response is short-lived. Without a booster vaccine

dose, the efficacy of RTS.S/ASO1 against clinical and severe malaria decreased over time after vaccination, dropping from 36 % to 28 % in children and from 28 % to 18 % in young infants. No significant protection from severe malaria was observed 18 months post vaccination (RTS, *The Lancet. 2015*). It is clear that the RTS.S/ASO1 vaccine confers only partial efficacy. In comparison to other pediatric vaccines such as the hepatitis B and measles vaccine, the RTS.S/ASO1 shows very modest efficacy figures. Whilst it is disappointing that the only available vaccine is not able to deliver a more substantial ability to clear and protect against malaria, RTS.S/ASO1 vaccine could serve as an additional malaria control measure, as a more effective second generation malaria vaccine is being developed.

1.2.3 Asexual blood stage vaccines

Pathology from malaria infection develops during the asexual blood stage (Miller et al, *Nature 2002*), making this ground for a strong argument for the development of a vaccine. Vaccine development at this stage targets antigens involved in either invasion or adhesion of infected RBCs. Such a vaccine would prevent symptoms but not infection, similar to the situation where immunity is developed naturally. Antibodies are thought to play a key role in controlling parasite densities and pathology during blood stage infection in humans (Cohen et al. *Nature 1961;* Sabchareon et al. *The American journal of tropical medicine and hygiene1991*, Fowkes et al. *PLoS Med. 2010*), further supporting the development of blood-stage vaccines. A number of merozoite antigens are under current investigation as vaccine candidates, and several vaccines have undergone phase II trials. Antigens being investigated are of importance to the proliferation of the parasite, and naturally acquired antibodies correlate well with protection in many immuno- epidemiological studies (Polley et al. *Genetics. 2003*; Fowkes et al. *PLoS Med. 2010*).

Blood stage vaccine candidate antigens comprise proteins expressed on the surface of merozoites, like MSP1 (Ogutu BR, et al. *PLoS ONE 2009*), MSP2 (Genton B , et al *J. Infect.*

Dis. 2002), MSP3 (Druilhe P, et al. *PLoS Med 2005*; Sirima SB et al. *PLoS ONE 2009*; Audran R, et al. *PLoS ONE 2009*) and glutamate-rich protein (GLURP) [Hermsen CC et al. *Vaccine 2007*; Esen M, et al. *Vaccine 2009*) or proteins released from secretory organelles upon invasion, like AMA1 (Sagara I, et al. *Vaccine 2009*) and EBA-175 (El Sahly HM et al. *Clin. Vaccine Immunol 2010*). Focus has mainly been on MSP1, AMA1, MSP2, MSP3 and EBA-175 (Ogutu et al. *PLoS One. 2009*; Otsyula et al. *Malaria journal 2013*; Malkin et al. *Vaccine 2008*). To date, none of these antigens have alone shown to confer protection in phase II trials (reviewed in Goodman and Draper. *Annals of Tropical Medicine & Parasitology 2010*). The most advanced field trial of a blood-stage vaccine, FMP2.1/AS02A, based on AMA1 from the 3D7 strain of P. falciparum, reported no significant efficacy against clinical malaria but only to parasites with identical AMA1 sequence to 3D7-allele-specific immune response (Thera MA et al. *N Engl J Med. 2011*). Several studies provide evidence that antigens expressed on the surface of the infected red blood cell are involved in pathophysiology through adhesion mechanisms. Special focus has been given to the PfEMP-1 family of proteins, where certain variants of the proteins have been associated with severe malaria and mechanisms such as rosetting and adhesion of infected red blood cells to vascular endothelium (Treutiger CJ et al *Nature medicine 1997*; Heddini A et al *Infection and Immunity. 2001*; reviewed in Craig and Scherf *Molecular and biochemical parasitology 2001*). These antigens are targets for naturally acquired immunity to malaria (Bull et al *Nature medicine 1998*). Certain PfEMP1 variants are associated with specific clinical presentations; for example, conserved epitopes of PfEMP1 encoded by *var2csa* are currently developed as pregnancy associated malaria vaccine (Avril M, et al. *Infect. Immun 2010*)

1.2.4 Transmission blocking vaccines

Antibodies against sexual stages are elicited during natural *P. falciparum* infection (Ouedraogo et al. *Infection and immunity 2011*), and this together with the opportunity to

reduce transmission on a population level provides the strongest argument for the development of a vaccine against gametocytes. A transmission blocking vaccine raises ethical considerations since it does not provide any protection from disease for the individual vaccinated, and this type of vaccine will most likely be co-formulated with antigens that induce protection also against other stages of the malaria parasite's life cycle.

2.0 CHAPTER 2. IDENTIFICATION OF CONSERVED ANTIGENS

2.1 Problem Statement

A malaria vaccine of high efficacy is viewed by many as crucial complementary tool to the current effective control measures against malaria. Its development however, has proved exceptionally challenging. This is because of a number of factors among others, the complicated biology of the malaria parasite as it traverses through the stages of its life cycle expressing different, stage-specific antigens, each stimulating a specific immune response (Michael F Good and Denise L Doolan *Current opinion in immunology 1999*; Hoffman SL, et al. *Vaccine 2015*), and extensive antigenic diversity that most times results in allele-specific immune responses leading to selection for non-vaccine serotypes and allow new recombination forms of parasites to emerge in the natural populations (Takala & Plowe *Parasite immunology 2009*; Dzikowski and Deitsch, *Current genetics 2009;* S.J Draper et al. *Vaccine 2015*). The RTS,S vaccine, a leading pre-erythrocytic subunit vaccine, showed moderate level efficacy of modest duration in Phase II/III clinical trials (Birkett, A. J., Moorthy, V. S., et al. *Vaccine 2013*; Casares S, et al. *Vaccine 2010*; Rts SCTP. *The Lancet 2015*), it is proof that an effective vaccine against malaria is possible to develop and could be useful in expediting the evaluation of next generation vaccines in clinical trials. However, its efficacy is sub-optimal to the global agenda of malaria elimination and ultimate eradication (Moorthy, V.S., et al. *The Lancet. 2013*). There is therefore, need to explore approaches to either boost efficacy of the RTS, S/AS01 (Heppner DG Jr, et al. *Vaccine 2005*) and/or other available vaccine candidates of even different stages or discover new antigens in the design of next-generation vaccines with prospects of a highly effective multi-component/multi-stage/multi-antigen formulation (Hoffman SL, et al. *Vaccine 2015*).

Targeting vaccines against blood-stage merozoite antigens would improve vaccine efficacy (Tsuboi. T., et al *The Lancet Infectious Diseases 2015*), since they are targets of acquired immunity, and controlling parasite density may reduce generation of the sexual stage parasites and subsequently reduce transmission (Richards, J. S., Beeson, J. G., *Immunology and cell biology 2009*; Crompton, P.D., et al *Proceedings of the National Academy of Sciences 2010*; malERA Consultative Group on Vaccines. *PLoS Med. 2011)*. Moreover, reviewed by Tsuboi et al, analysed determinants of RTS,S/AS01 induced immunogenicity in the final results of the trial. The analysis revealed anti-CSP antibody titers, a surrogate marker of protection for the magnitude and duration of the vaccine efficacy, waned more rapidly during participant follow-up at especially higher transmission intensity because of reduced titers levels and lesser blood-stage immunity. The finding is a significant limitation that clearly highlights the importance of blood-stage immunity in preventing malaria (Tsuboi. T, et al, *The Lancet Infectious Diseases 2015*). However, disappointingly, the most advanced leading blood-stage vaccine candidates like FMP2.1/AS02A, a subunit vaccine based on *P. falciparum* strain 3D7 AMA1 sequence, have suffered poor efficacy in human trials mainly due to high genetic polymorphisms of AMA1 that induce not only allele-specific immune responses but also suboptimal concentrations of functional antibodies against malaria parasites (Thera MA, Doumbo OK, et al *N Engl J Med 2011*; Bailey JA, Pablo J, et al, *The American journal of tropical medicine and hygiene.2015*; Halbroth and Draper, *Advances in parasitology. 2015*).

2.2 The Thesis Rationale

The extensive genetic diversity and polymorphisms in several *P.falciparum* malaria antigen-coding genes arise as a result of selection by the human immune system. Novel, relatively conserved antigens that induce broadly cross-reactive antibody and cell-mediated immune response may provide longer lasting and more efficacious protection (Pandey AK et

al *Infect Immun 2013*, Reddy KS et al *Infect Immun 2014*, Douglas AD et al. *Cell Host Microbe 2015*, Hill DL et al *Infect Immun 2016*). There is therefore, need to prioritize candidate peptides that comprise of conserved epitope targets of immunity in the design of next generation vaccines. The approach of population genetic and structural studies, followed by molecular epidemiological surveys or *in vitro* functional studies has been instrumental in identifying immunologically relevant diversity in pathogens prior to development and testing of vaccines (Takala SL, Plowe CV. *Parasite immunology 2009*). In this thesis, a similar approach of population genetics followed by *in vitro* functional studies, growth inhibition assay (GIA) was used to identify immunologically relevant conserved antigens.

Acquired human immunity predominantly targets the blood stage of infection, and Antigens expressed by the merozoite, the extracellular form of *Plasmodium* that infects RBCs, are especially important immune targets and vaccine candidates (Richards, J. S., and J. G. Beeson. *Immunology and cell biology 2009*). RBCs invasion occurs over several steps, with multiple interactions involving proteins on the merozoite surface and proteins contained within dedicated invasion organelles, the micronemes and rhoptries (Cowman, A. F., and B. S. Crabb. *Cell. 2006*). These proteins are thought to represent the major protective antibody targets and most attractive merozoite vaccine candidates because of their exposure to host immune responses and their important roles in invasion. Members of the reticulocyte binding-like homologue (PfRh) protein family involved in binding to and initiating entry of the invasive merozoite into erythrocytes are promising vaccine candidates, (Lin Chen, et al, *PLoS Pathog.2011*) and there is also increasing evidence suggesting that antibodies against merozoite surface proteins (MSPs) play an important role in clinical immunity to malaria (Chris Y. H. Chiu, et al *International journal for parasitology 2015*). We therefore, considered further evaluation of some well-characterized members of these protein families as immune targets and potential candidates for inclusion in the next generation vaccines. These

included novel *P. falciparum* malaria blood-stage vaccine candidate antigens; RH5-interacting protein (PfRipr), rhoptry-associated leucin zipper-like protein 1 (PfRALP1), Glycosylphosphatidylinositol (GPI) anchored micronemal antigen (PfGAMA) and Duffy binding-like merozoite surface protein1 (PfDBLMSP1) (Boyle, M. J., et al. *International journal for parasitology.2013*; Lin Chen, et al, *PLoS Pathog.2011*; Haase S, et al. *Infection and immunity 2008*; Ito D, et al. *Infection and immunity 2013*; Louise Hinds et al *Eukaryotic cell 2009*, Arumugam et al *Infection and immunity 2011*; Richards J.C. et al *The Journal of Immunology 2013;* Wickramarachchi T, et al *International journal for parasitology 2008*; Sakamoto H et al *Vaccine.2012*)

2.3 The Study Merozoite Antigens

2.3.1 Ripr

Emerging evidence suggests that proteins involved in the intricate and essential invasion complex formed by the interaction of *P. falciparum* reticulocyte binding protein homologue 5 (PfRh5) with cysteine-rich Rh5 interacting protein (PfRipr) and tethered to the merozoite surface via a glycosylphosphatidylinositol (GPI)-anchored cysteine-rich protective antigen (PfCyRPA) are likely blood-stage candidates (Drew DR et al *Trends Parasitol 2015*, Chen L et al. *PLoS Pathog 2011*, Reddy KS et al *Proc Natl Acad Sci 2015*, Favuzza P et al *Malar J 2016*). PfRh5 (encoding gene, PF3D7_0424100) and PfCyRPA (encoding gene, PF3D7_0423800) have limited sequence polymorphism in at least five *P. falciparum* strains and are immunogenic (Dreyer AM, et al. *The Journal of Immunology 2012;* Douglas AD et al. *Cell Host Microbe 2015*, Reddy KS et al *Proc Natl Acad Sci 2015*, Bustamante LY et al. *Vaccine 2013*). The genes encoding PfRh5 and PfRipr (PF3D7_0323400) are refractory to gene-targeted deletion, suggesting that the two proteins play an essential role in parasite survival (Douglas AD et al. *Cell Host Microbe 2015*, Chen L et al. *PLoS Pathog 2011*, Baum J et al *Nucleic Acids Res 2009*).

PfRipr is a novel cysteine-rich merozoite protein. It is a secreted protein that localizes in the micronemes and released during erythrocyte invasion to form the membrane-associated interaction with the PfRH5 (Damien R. Drew1, James G. Beeson *Trends in parasitology 2015*). Full-length PfRipr consists of 1,086 amino acids with a molecular weight of 126 kDa. It has a signal sequence at the N-terminus and 87 cysteine-residues, many of which clustered in epidermal growth factor (EGF)-like domains distributed along the entire length of the protein. The EGF-like domains are ten; two in the N-terminal region and eight clustered towards the C-terminus. The protein has neither transmembrane domain nor GPI anchor sequence (Chen L et al. *PLoS Pathog 2011*).

Antibodies against recombinant PfRipr expressed in *Escherichia coli* potently inhibited parasite growth *in vitro* in multiple parasite strains (Chen L et al. *PLoS Pathog 2011*). However, it remains difficult to produce adequately functional proteins for further studies of naturally acquired immunity in PfRipr.

2.3.2 RALP1

PfRALP1 is novel tight-junction protein localized in the merozoite rhoptry neck and translocate to moving junction during invasion of erythrocytes. It is conserved in *Plasmodium* spp. and is refractory to gene knockout attempts [Haase S, et al *Infection and immunity 2008*], suggesting that PfRALP1 might play an important role in invasion. It weighs 87.9 kDa with a full-length amino acid sequence of 749 residues. The protein has a N-terminus signal peptide sequence, a leucine zipper-like domain, and coiled-coil domain at its C-terminal with neither transmembrane domain nor GPI anchor sequence. PfRALP1 is reported to possess an erythrocyte-binding epitope in the C-terminal region which could be useful in bridging the protein between the erythrocyte and merozoite surfaces (Ito D et al *Infection Immunity 2013*) Although further studies are required, it is suggested that lack of a transmembrane region in RALP1 might be associated with an unknown membrane protein that anchors it to the

merozoite surface and hence plays a role as an invasion ligand, like the PfRh proteins (Gunalan K, et al *Nature communications 2013*, Chen L et al. *PLoS Pathog 2011*).

2.3.3 GAMA

PfGAMA is a microneme protein of the merozoite that is conserved throughout Plasmodia spp, is refractory to gene knockout attempts and is essential to parasite invasion. The full-length protein has a molecular mass of 85.2 kDa and consists of; 738 amino acids long, has N-terminal signal peptide, asparagine-rich regions and a C-terminal GPI anchor sequence. GAMA possesses an erythrocyte binding epitope in the C-terminal region and binds to a non-sialylated protein receptor.

2.3.4 MSPDBL1

PfMSPDBL1 belongs to merozoite surface protein 3 family of proteins that play an important role in the successful invasion of merozoites into host erythrocytes (Boyle, M. J et al. *International journal for parasitology 2013*). Full length PfMSPDBL1 is 80.2 kDa in weight with amino acid sequence of 697 residues. The protein contains; a five amino acid (NLRNA/G) conserved motif at its N-terminal common to other MSP3 members, (Singh, Set al *PloS one 2009*, Hodder, A. N et al *Journal of Biological Chemistry 2012*) a cysteine-rich Duffy binding-like (DBL) domain and a glutamate rich C-terminal secreted polymorphic antigen associated with merozoite (SPAM) domain and a terminating LLZ motif (Kauth, C. W et al *Journal of Biological Chemistry 2006*; Burgess, B.R et al *Journal of Biological Chemistry 2005*). PfMSPDBL1 localizes to the surface of the merozoite, possess neither transmembrane domains nor GPI anchors and is presumed to associate extrinsically through other merozoite surface proteins (Hodder, A. N et al *Journal of Biological Chemistry 2012*; Sakamoto, H et al *Vaccine 2012*). It binds to unknown receptors on the erythrocyte surface through its DBL domain, (Hodder, A. N et al *Journal of Biological Chemistry 2012*; Wickramarachchi T, et al. *International journal for parasitology 2009*) and anti-PfMSPDBL1

antibodies partially inhibit parasite growth in vitro (Lin CS, et al. *Journal of Biological Chemistry 2014*; Sakamoto, H et al *Vaccine 2012*). Recent studies report PfMSPDBL1 and a closely related MSP3 member, PfMSPDBL2 which is an important target of immunity albeit its potential resistance to halofantrine, (Amambua-Ngwa, A et al *PLoS Genet 2012*; Van Tyne, D et al *Antimicrobial agents and chemotherapy 2013*; Tetteh, K. K et al *Infection and immunity 2013*) to have a common functionality and probably form a complex on the merozoite surface where MSP1 acts as a platform to display the 2 proteins for binding to their erythrocyte receptors (Lin CS, et al. *Journal of Biological Chemistry 2014*; Chris Y. H et al *Journal of Infectious Diseases 2015*) Studies have also reported that the DBL domains of both PfMSPDBL1 and PfMSPDBL2 are very polymorphic and under selective pressure and the SPAM region is conserved (Amambua-Ngwa, A et al *PLoS Genet 2012*; Ochola LI et al. *Molecular biology and evolution 2010*; Tetteh KK et al *PloS one 2009*),

PfRipr, PfRALP1, PfGAMA and PfMSPDBL1 are essential to parasite survival [Ito D et al Infect Immun 2013Arumugam TU et al *Infect Immun 2011*, Wickramarachchi T et al *Int J Parasitol 2009*, Sakamoto H et al *Vaccine 2012*]. Wheat germ cell-free system (WGCFS) expressed PfRALP1, PfGAMA and PfMSPDBL1 recombinant proteins are immunogenic and the antibodies for each of the recombinant proteins potently inhibited erythrocyte invasion *in vitro* [Arumugam TU et al *Expert Rev Vaccines 2014*]. Human sera collected from *P. falciparum* malaria endemic regions recognized the WGCFS expressed recombinant proteins. However, investigations into the extent of polymorphism and genetic diversity in PfRipr, PfRALP1, PfGAMA, and PfMSPDBL1 are minimal, especially in malaria endemic populations in Africa (Ito D et al *Infect Immun 2013*, Arumugam TU et al *Infect Immun 2011*, Sakamoto H et al *Vaccine 2012*).

2.4 Aims Of The Thesis

The overall aim of this thesis was to contribute to the logical design of next generation malaria vaccine discovery efforts for the improvement of vaccine efficacy in the field. We hypothesized that highly conserved merozoite antigen targets of protective immunity could improve efficacy across diverse natural parasite populations. We underscored the importance of post-genomic approaches of understanding evolution of the parasites and the reverse vaccinology (Rappuoli R, *Vaccine 2001*; Donati and Rappuoli, *Annals of the New York Academy of Sciences 2013*).

The specific aims were:

I. To explore extent of polymorphism and genetic diversity in blood-stage merozoite proteins: PfRipr, PfGAMA, PfRALP1 and PfMSPDBL1.

II. To evaluate growth inhibition assay (GIA) activity of rabbit IgG antibody against *P. falciparum* 3D7 recombinant PfRipr, PfGAMA, PfRALP1 and PfMSPDBL1 expressed in wheat germ cell-free system on both 3D7 and heterologous FVO strains.

2.5 Materials And Methods

2.5.1 Parasite isolates and DNA extraction

P.falciparum field isolates were obtained from participants in a BK-SE36 malaria vaccine study from April 2010 to November 2011 (Palacpac NM, Ntege E et al *PLoS One 2013*). The participants were residents (age range 6 – 40 years) of various villages in Lira and the neighboring districts in Northern Uganda. Ethical approvals for blood samples to be taken and stored for use in future studies were obtained from Med Biotech Laboratories (MBL-IRC: 29 Jun 2011) and Uganda National Council for Science and Technology (UNCST HS 635). Informed consent was obtained in writing from participants or parents/legal representatives

(in the case of children) prior to enrollment. *P.falciparum* laboratory strains 3D7 and FVO were kind gifts from the National Institute of Allergy and Infectious Diseases, NIH, and maintained in continuous blood culture in the Division of Malaria Research, Proteo-Science Center, Ehime University, Japan. Finger-prick blood samples from *P. falciparum*-infected participants were collected on Whatman® 31ETCHR filter paper (Whatman, Piscataway, NJ), and air-dried. Genomic DNA (gDNA) was extracted from the filter paper spots using EZ1 DNA Investigator kit on EZ1 BioRobot™ (Qiagen, Hilden, Germany). The 3D7 and FVO gDNA were extracted from late trophozoite-schizont culture using QiaAmp DNA mini kit (Qiagen) according to the manufacturer's instructions. All extracted gDNA was stored at –20 °C until use.

2.5.2 PCR amplification and sequencing of target genes

Nested PCR was used to obtain full-length nucleotide sequences of *pfripr, pfralp1, pfgama* and *pfmspdbl1* from one hundred and two *P. falciparum* clinical isolates and FVO gDNA. Similarly, full-length nucleotide sequences for *P. falciparum* AMA1 gene (*pfama1*) (PF3D7_1133400, 1869bp), as positive control; and the housekeeping gene, adenylosuccinate lyase (*pfadsl*) (PF3D7_0206700, 1416bp) for negative control were obtained. Nested PCR primers for *pfripr, pfralp1, pfgama* and *pfmspdbl1* (Supplementary Table 1); and *ama1* and *adsl* primers [Tanabe K et al *Vaccine 2013*, Tanabe K et al *Curr Biol 2010*] were designed based on the 3D7 gene sequence as reference (http://plasmodb.org). The procedure for nested PCR is described elsewhere (Snounou G et al *Methods Protoc 2002*). Briefly, primary amplification was carried out in a 25 µl reaction mixture containing 1 µl each of 10 pmol/mL forward and reverse primers, 5 µl each of 2.0 mM dNTP, 4 µl of Milli-Q water (Millipore, Billerica, MA), 0.5 unit of KOD-FX-Neo (Toyobo, Osaka, Japan), 12.5 µl of 2×PCR buffer, and 1 µl of gDNA. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, and amplification for 34 cycles at 95 °C for 15 sec and 59 °C for 30 sec, followed by a final

extension at 68 °C for 1 min 30 sec. The primary PCR product was diluted 10-fold, and a 2µl aliquot was used as template for a nested PCR amplification of 29 cycles under similar conditions. All reactions were carried out in a C1000™ Thermal Cycler (Applied Biosystems, Foster City, CA). Nested PCR products were analyzed on a 1 % (w/v) agarose gel electrophoresis, stained in ethidium bromide, visualized on UV transilluminator (BioRad Gel Doc 1000, Bio-Rad, Hercules, CA) and purified using QIAquick® PCR Purification Kit (QIAGEN). Using standard protocols at facilities in Eurofinsgenomics (Tokyo, Japan), the purified nested PCR products were bi-directionally sequenced with both amplification primers and several internal sequencing primers (Supplementary Table 1). Obtained nucleotide sequences were assembled and edited using SeqMan software (Lasergene 7; DNASTAR, Madison, WI). Samples with low quality electropherogram and suspected mixed infections were excluded from analysis after two independent confirmatory rounds of PCR and DNA sequencing.

2.5.3 Polymorphism analyses

Nucleotide sequences were aligned using CLUSTAL W (Thompson JD et al *Nucleic acids research 1994*) implemented in MEGA version 7 (Kumar S et al *Mol Biol Evol 2016*), with manual corrections. Measures of polymorphic sites including, number of segregating sites (*S*), number of synonymous changes (Syn), number of non-synonymous changes (Nonsyn), and DNA polymorphism [nucleotide diversity (*Pi*), and average number of pairwise nucleotide differences (*K*)], were determined using DnaSP version 5.10.01 and MEGA 7 respectively (Kumar S et al *Mol Biol Evol 2016*, Librado P et al *Bioinformatics 2009*). Haplotype number (H) was analysed using an online tool (http://www.gen-info.osaka-u.ac.jp/~uhmin/study/population/index.html) and Haplotype diversity (Hd) and standard deviation (SD) was calculated in Excel using the formulas $Hd = \{n/(n-1)\}(1-\Sigma X^2)$, $Vsh = \{2/n(n-1)\}[2(n-2)\{\Sigma X^3-(\Sigma X^2)^2\} +\Sigma X^2-(\Sigma X^2)^2]$ and $SD=(Vsh)^{1/2}$, where Vsh is variance of

single haplotype, n is the number of sequences and X is the frequency of the haplotype. An Hd value of zero signifies absence of allele diversity and values approaching 1 indicating large number of equally frequent alleles. Natural selection (immune pressure) was determined by obtaining differences between the numbers of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) using Nei and Gojobori method (Nei M et al *Mol Biol Evol 1986*) with Jukes and Cantor correction as implemented in MEGA version 7 (Kumar S et al *Mol Biol Evol 2016*). Statistical significance between dN and dS was measured by MEGA codon based Z-test. Tests for departures from neutrality were based on allele frequency distribution test, (Tajima's D) in DnaSP (Librado P et al *Bioinformatics 2009*). Also, searched and selected single nucleotide polymorphism (SNPs) in *pfripr, pfralp1, pfgama* and *pfmspdbl1* using the query system on PlasmoDB (http://www.plasmodb.org). In brief, using gene IDs, SNPs were selected with a read frequency threshold of 80%, minor allele frequency > 0 and percent isolates with base call > 80% from 164 to 203 *P. falciparum* isolates (Supplementary table 2). The total number of SNPs in gene regions with no insertion, deletion, peptide repeats and recombinant regions were downloaded and considered.

Nucleotide sequence data are available in the GenBank™, EMBL, and DDBJ databases under the accession numbers: LC157434 - LC157845.

2.5.4 Production of recombinant PfRipr protein, and antiserum

For the functional assays (GIA), we used previously generated rabbit polyclonal IgGs against the WGCFS expressed recombinant PfRALP1, PfGAMA and PfMSPDBL proteins (Ito D et al *Infect Immun 2013*, Arumugam TU et al *Infect Immun 2011*, Sakamoto H et al *Vaccine 2012*). The anti-PfRipr IgG against WGCFS expressed recombinant PfRipr was obtained as described (Tsuboi T et al *Infect Immun 2008*). Briefly, the nucleotide sequence of the *pfripr* (PF3D7_0323400) of strain 3D7 was obtained from the malaria genome database

PlasmoDB (http://www.plasmodb.org). In order to generate specific antibodies, a truncated region of the *pfripr* was amplified and expressed as recombinant proteins with the wheat germ cell-free translation system (CellFree Sciences, Matsuyama, Japan) as described previously (Tsuboi T, et al. *Infection and Immunity 2008*). Briefly, the PF3D7_0323400 fragment encoding PfRipr (encompassing 717 amino acids [aa; K279 to D995] was amplified from *P. falciparum* 3D7 gDNA by PCR by using sense primer with XhoI site and antisense primer with NotI restriction site (in lowercase letters in the primer sequences below). Primers PfRipr-sense (5'-ctcgagAAAAATGTTTGTGAAGAAAATTATAGATGTAC-3') and PfRipr-antisense (5'-gcggccgcCTAGTCATTATATTGGAATGTAAAACTTTCATC-3'), were used to generate the DNA fragment encoding the PfRipr protein. The amplified fragment was then restricted and ligated into the wheat germ cell-free expression vector pEU-E01-GST-TEV-N2 (CellFree Sciences). The cloned insert was sequenced with an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA). The recombinant protein with a glutathione S-transferase (GST) tag was expressed with the wheat germ cell-free system and purified with a glutathione-Sepharose 4B column (GE Healthcare). GST-tagged PfRipr protein was purified as GST-fusion protein by glutathione elution. All of the detailed methods used for wheat germ cell-free protein synthesis and affinity purification were described previously (Tsuboi T, et al., *Methods Mol Biol, 2010*).

To generate antisera against the recombinant PfRipr protein, a Japanese white rabbit was immunized subcutaneously with 250 μg of the protein with Freund's complete adjuvant, followed by 250 μg of the protein with Freund's incomplete adjuvant. All immunizations were done three times at 3-week intervals, and the antisera were collected 14 days after the last immunization. All protocols on the animal experiments were approved by the Institutional Animal Care and Use Committee of Ehime University and the experiments were

conducted according to Ethical Guidelines for Animal Experiments of Ehime University, Japan.

2.5.5 Enzyme-linked Immunosorbent assay (ELISA)

The anti-PfRipr, anti-PfRALP1, anti-PfGAMA and anti-PfMSPDBL1 IgG titers in the rabbit antisera were determined by enzyme-linked Immunosorbent assay (ELISA) as previously described (Tachibana M et al *Clin Vaccine Immunol 2011*). ELISA plates were coated with recombinant protein at 50 ng/well. The sera were tested at serial dilutions starting from 1:100 to 1: 1,000,000. A 1:2,000 dilution of goat anti-rabbit IgG HRP conjugate (Biosource, Camarillo, CA) was used as the secondary antibody. Reciprocal serum dilutions that gave a mean absorbance value of 1.0 at 415 nm were determined as the endpoint titers of IgG.

2.5.6 Indirect Immunofluorescence assay (IFA)

Thin smears of schizont-enriched *P. falciparum* 3D7-infected erythrocytes were prepared on glass slides and stored at -80°C. The smears were thawed, fixed with ice-cold acetone for 3 min, and blocked with PBS containing 5% nonfat milk (blocking solution) at 37°C for 30 min. The slides were stained with primary antibodies diluted at the following concentrations in blocking solution at 37°C for 1 h: rabbit anti-PfRipr antibody, 1:500; mouse anti-PfAMA1 antibody, 1:100 (Tsuboi T et al *Infect Immun 2008*). Secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen), were used at a 1:500 dilution in blocking solution at 37°C for 30 min. Slides were mounted in ProLong Gold Anti-fade reagent (Invitrogen) and viewed under a 63x oil immersion lens. High-resolution image capture and processing were performed with a confocal scanning laser microscope (LSM710; Carl Zeiss Micro Imaging, Thornwood, NY). Images were processed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

2.5.7 In-vitro Growth inhibition assay (GIA)

P.falciparum asexual stages were maintained *in vitro* with 1x RPMI 1640 (Trager W, Jensen JB. 1976) supplemented with 5 % heat-inactivated human plasma, 7.5% sodium bicarbonate solution, 10mg/ml gentamycin solution and 0.5 % Albumax I (Thermo Scientific, Waltham, MA). Human erythrocytes are from healthy donors (blood group O^+) obtained from the Japanese Red Cross Society. To harvest synchronized schizonts for GIA, mature schizont/late trophozoite were purified by differential centrifugation on a 70%/40% Percoll-sorbitol gradient, washed with RPMI1640 GlutaMax (Thermo Scientific) and cultured at 37 ˚C for about 12~18 hours to ring stage. Then, the ring stage rich parasites were treated with 5 % D-Sorbitol (Mochida Pharmaceutical, Tokyo, Japan), washed with RPMI 1640 GlutaMax and cultured at 37˚C for about 25~30 hours before initiation of assays. Total rabbit IgGs for GIA were purified from rabbit antisera with HiTrap protein G-Sepharose columns (GE Healthcare) according to the manufacturer's protocol. Purified IgGs were further buffer exchanged into complete culture medium, concentrated with Amicon Ultra-15 centrifugal filter units (Millipore), filter sterilized with an Ultrafree-MC GV 0.22-μm centrifugal filter (Millipore), and pre-absorbed to remove nonspecific anti-erythrocyte surface antibodies using 25μl of packed human O^+ erythrocytes per purified IgG (derived from 1 ml of antiserum) at room temperature for 1 hour. Finally, the concentrations of all rabbit IgG samples were adjusted to 40 mg/ml in incomplete culture medium.

The inhibitory activity of rabbit IgGs on merozoite invasion was tested after one cycle of parasite replication, and parasitemia was determined by flow cytometry as described previously (Arumugam et al *Infection and immunity 2011*). Briefly, the parasite cultures were synchronized a day before starting GIA, so that the majority of parasites were at late trophozoite-to-schizont stage at the beginning of the assay. Twenty microliters of parasite-infected erythrocyte (pRBC) suspension (0.3 ± 0.1 % parasitemia and 2 % hematocrit) and 20

µl of IgGs were added (final concentration of the IgG: 20 mg/ml) per well of half-area flat-bottom 96-well cell culture micro plates (Corning, NY) and gently mixed. For a control, 20 µl of culture medium was added to the pRBC. Twenty µl of culture medium was also added to the pRBC with anti-EBA175 (III-V) IgG for positive control, or with anti-HisGST IgG for negative control as described (Arumugam TU et al *Infect Immun 2011*). Cultures were incubated at 37°C in humidified, gassed (90% N_2, 5% O_2, and 5% CO_2), airtight boxes. After 20 hours of incubation, when most of the invaded parasites had developed to early trophozoite stage, the pRBC were pelleted by brief centrifugation (1,300 × *g* for 5 min) and washed once in 100µl PBS. The cells were then incubated with 50µl of diluted (1:1,000 in PBS) SYBR green I (Invitrogen) for 10 min at RT and washed once in PBS. Parasitemia was measured by flow cytometry with FACSCanto II (BD Biosciences, San Jose, CA) through acquisition of 50,000 events per sample. Data were analyzed by FlowJo 9.1 software (Tree Star, Ashley, OR) through first gating for intact erythrocytes by side scatter and forward scatter parameters and subsequently determining the proportion of SYBR green I-positive cells. Three independent experiments were performed with samples tested in triplicate. Growth inhibition was expressed as a percentage relative to the maximal growth achieved in control wells. For analysis, percentage (%) inhibition was calculated using the following formula: % inhibition = 100−[(parasitemia (%) of infected RBCs with tested IgG – parasitemia (%) of normal RBCs only)/(parasitemia (%) of infected RBCs without any IgG – parasitemia (%) of normal RBCs only) ×100]. Kruskal-Wallis test followed by Dunn's multiple-comparison test was used for the analyses, and two-tailed P values were considered significant if they are < 0.05. The analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

2.6 Results

2.6.1 Polymorphism and genetic diversity in *pfripr, pfralp1, pfgama,* and *pfmspdbl1*

Full-length sequences of *pfripr* and the control genes were successfully obtained. Near full-length *pfralp1, pfgama,* and *pfmspdbl1* sequences were obtained, varying in length especially among the clinical isolates. For the analysis, 80 nucleotide sequences for *pfripr*, 63 for *pfralp1*, 63 for *pfgama*, 58 for *pfmspdbl1*, 61 for *pfama1* and 95 for *pfadsl* were employed. The analyses showed *Pfripr* and *pfralp1* are comparatively less polymorphic than *pfgama*, *pfmspdbl1* and the positive control *pfama1* [Tables 1 and 2]. Relative to the negative control *pfadsl*, *pfripr* is the most conserved among the four vaccine candidates, with 16 non-synonymous mutations distributed along the entire nucleotide sequence, low average number of pairwise nucleotide differences (k=1.054) and a Pi of 0.00032 [Table 1, Figure 5B, 5C]. The antigen has 15 haplotypes among Ugandan isolates (majority of which is UgH1 (57.5%) identical to the reference 3D7), has no insertion/deletion and/or peptide repeat regions and with a low haplotype diversity (Hd) of 0.647 [Table 2, Figure 6A]. *Pfralp1* has 11 non-synonymous mutations but has an asparagine repeat region (codons 205 – 214) with varying number of residues per haplotype and a 6-mer repeat region (codons 411 – 481) with varying number of repeats [Figure 6B, 7A]. Average number of pairwise nucleotide differences (k=1.258) and Pi of 0.00062 was higher than *pfadsl* and *pfripr* [Table 1, Figure 5B, 5D]; and more genetically diverse with 39 haplotypes (38 haplotypes from Ugandan isolates) with only 6.3% (UgH4) being similar to 3D7 [Figure 6B]. The antigen's Hd of 0.960 is close to the highly polymorphic positive control, *pfama1* [Table 2]. *Pfgama* has 15 nonsynonymous mutations, distributed more in the C-terminus but outside the asparagine-rich region [Figure 5E]. It is diverse with Pi of 0.00126 close to *pfama1* [Table 1, Figure 5A, 5E]. Among the four vaccine candidates, *Pfgama* is the most genetically diverse with 59 haplotypes, all 57 Ugandan haplotypes different from 3D7 and FVO [Figure 6C], and an Hd of 0.998 (*pfama1*

Hd=0.990) [Table 2]. *Pfgama* has an insertion/deletion region between codons 49-50 and an asparagine-rich variable sequence region at codons 356-485. *Pfmspdbl1* has 35 non-synonymous mutations, high number of pairwise nucleotide differences (k=10.82) and a Pi of 0.00682 [Table 1, Figure5F]. Only a small percentage (8.6%, UgH3) of *pfmspdbl1* haplotype is identical to 3D7 [Figure 6D]. The DBL domain of PfMSPDBL1 among isolates is trimorphic and probably under meiotic recombination [Figure 7B]. The immune selection analysis (test for neutrality) suggested no evidence of positive or purifying selection in the four candidate genes on evaluation of dN and dS, but the Tajima's D analysis is significant ($P < 0.05$) in *Pfripr* [Table 1]. From the PlasmoDB database, we obtained and analyzed a total of 22 (*pfripr*), 12 (*pfralp1*), 22 (*pfgama*) and 38 (*pfmspdbl1*) non-synonymous SNPs. When compared with the observed SNPs among *P. falciparum* field isolates from Uganda, there were similar rates of non-synonymous substitutions in all the four genes. Remarkably there were many shared and unique SNPs for each gene. Approximately half (~50%) of the SNPs were unique to the Uganda isolates, suggesting a finding of new variants in this population. Noted as well, was that most of the unshared SNPs from the database had low allele frequencies in PfRipr (Table 3)

Gene	N	bp	S	Eta	Syn	Nonsyn	k ± SE	Pi ± SD	θ ± SD	Neutrality tests				
										dS ± SE	dN ± SE	*P* (HA: dN >dS)	*P* (HA: dN <dS)	D (Tj)
Pfadsl	95	1413	9	9	4	5	0.750± 0.465	0.00053± 0.00006	0.00124± 0.0000003	0.0022± 0.0017	0.0001± 0.00004	1	0.082	-1.4409 *P* > 0.10
Pfama1	61	1866	81	90	4[b]	76[b]	25.591 ± 2.823	0.01371± 0.00023	0.00928± 0.00263	0.0019± 0.0001	0.0170± 0.0020	0*	1	1.14353 *P* > 0.10
Pfgama	63	1989[a]	24	24	9	15	2.513± 0.854	0.00126± 0.00009	0.00256± 0.00052	0.0014± 0.0005	0.0012± 0.0005	1	0.148	-1.59 *P* > 0.05
Pfmspdbl1	58	1587[a]	51	51	16	35	10.82± 1.819	0.00682± 0.00057	0.00694± 0.00097	0.0090± 0.0030	0.0063± 0.0014	1	0.165	- 0.06 *P* > 0.10
Pfralp1	63	2031[a]	12	13	2	11	1.258± 0.558	0.00062± 0.00008	0.00125± 0.00036	0.0004± 0.0004	0.0007± 0.0003	0.319	1	-1.571 *P* > 0.05
Pfripr	80	3258	18	18	2	16	1.054± 0.392	0.00032± 0.00004	0.00112± 0.00026	0.0002± 0.0002	0.0004± 0.0001	0.172	1	-2.084 *P* < 0.05*

Table 1. Polymorphisms and immune selection in full-length *pfama1*, *pfadsl*, *pfgama*, *pfmspdbl1*, *pfralp1*, and *pfripr*

N: number of nucleotide sequences. bp: Total number of nucleotide sites. S: number of segregating sites; Eta: total number of mutations; Syn: synonymous mutations; Nonsyn: non synonymous mutations; k: Average number of pairwise nucleotide differences; Pi and θ: nucleotide diversity values; dS: the number of synonymous substitutions per synonymous sites; dN: the number of non-synonymous substitutions per non-synonymous sites; HA: assumptions for alternative hypothesis; SD: Standard deviation; SE: Standard error; D (Tj): Tajimas' D value.

**Significant, [a] Insertion, deletion, amino acid repeat region and recombination region were removed from the analysis. [b] Complex codons were removed from the analysis.*

Table 2. Genetic diversity among full-length sequences of *P. falciparum* field isolates from Uganda and strains 3D7 and FVO.

Gene	N	aa	H	Hd ± SD
Pfadsl	95	471	6	0.103 ± 0.043
Pfama1	61	622	49	0.990 ± 0.006
Pfgama	63	730 - 753	59	0.998 ± 0.003
Pfmspdbl1	58	690 - 698	46	0.989 ± 0.007
Pfralp1	63	733 - 765	39	0.960 ± 0.014
PfRipr	80	1086	16	0.647 ± 0.057

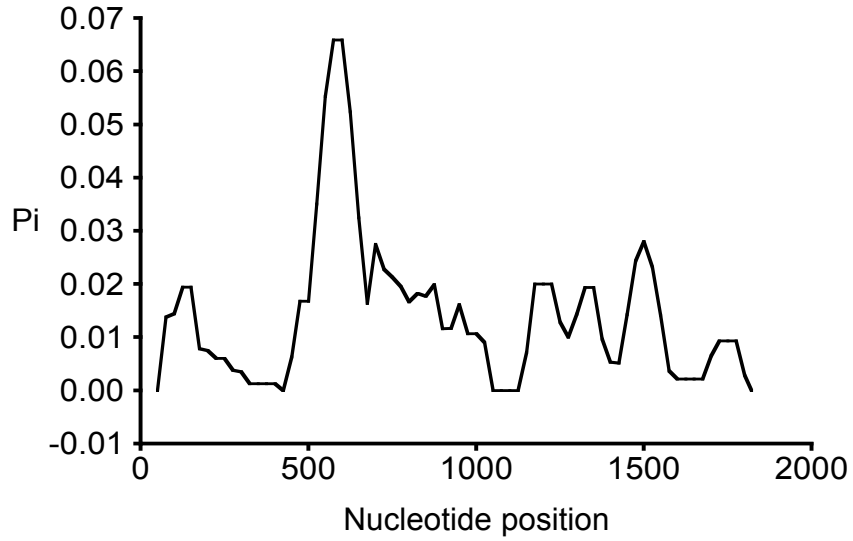
N: number of amino acid sequences; aa, total number of amino acids sequenced; H: number of haplotypes; Hd: haplotype diversity; SD: standard deviation. First and second amino acid position of PfRipr and position 312 of Pfmspdbl1 were removed from the analysis because some sequences were ambiguous at these positions

Gene ID	Annotation	No. of Isolates		Nucleotide length analyzed	No. of mutations				Uganda + PlasmoDB	Uganda
					Uganda		PlasmoDB			
		Uganda	PlasmoDB		Syn	Non-syn	Syn	Non-syn	Shared Non-syn	Unique Non-syn
Pf3D7_0323400	*Pfripr*	80	198 - 203	3258bp	2	16	3	22	8/22 (36%)	8/16 (50%)
Pf3D7_0722200	*Pfralp1*	63	194 - 203	2031bp*	2	11	3	12	5/12 (41%)	6/11(54%)
Pf3D7_0828800	*Pfgama*	63	192 - 203	1989bp*	9	15	9	22	8/22 (36%)	7/15 (46%)
Pf3D7_1035700	*Pfmspdbl1*	58	164 - 201	1587bp*	16	35	10	38	12/38(32%)	23/35 (65%)

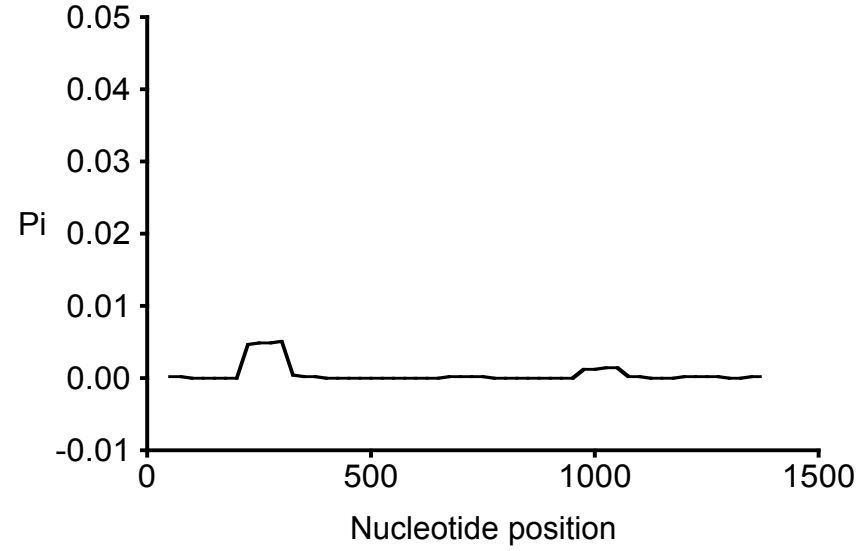
Table 3. Comparison of Single Nucleotide Polymorphism (SNPs) data in PlasmoDB with the polymorphism in Uganda isolates

*bp: base pairs. Syn: synonymous substitutions; Non-syn: non synonymous substitutions; * availed SNPs in regions of Insertion, deletion, amino acid repeat region and recombination region were not considered.*

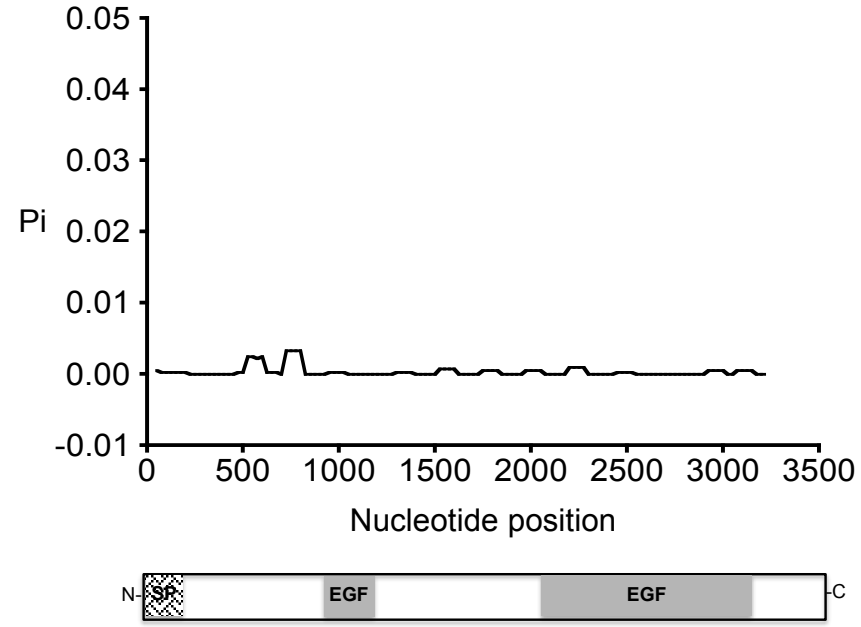
A ***Pfama1***



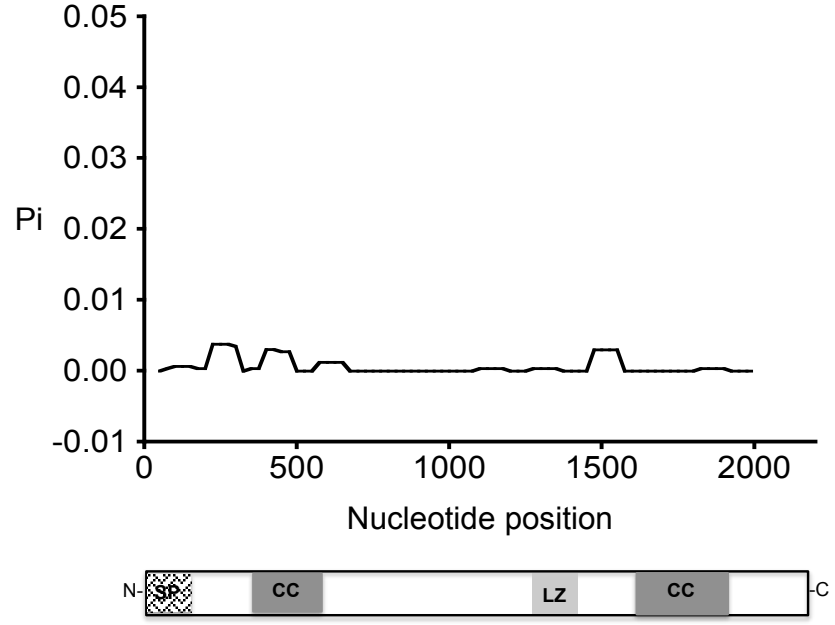
B ***Pfadsl***



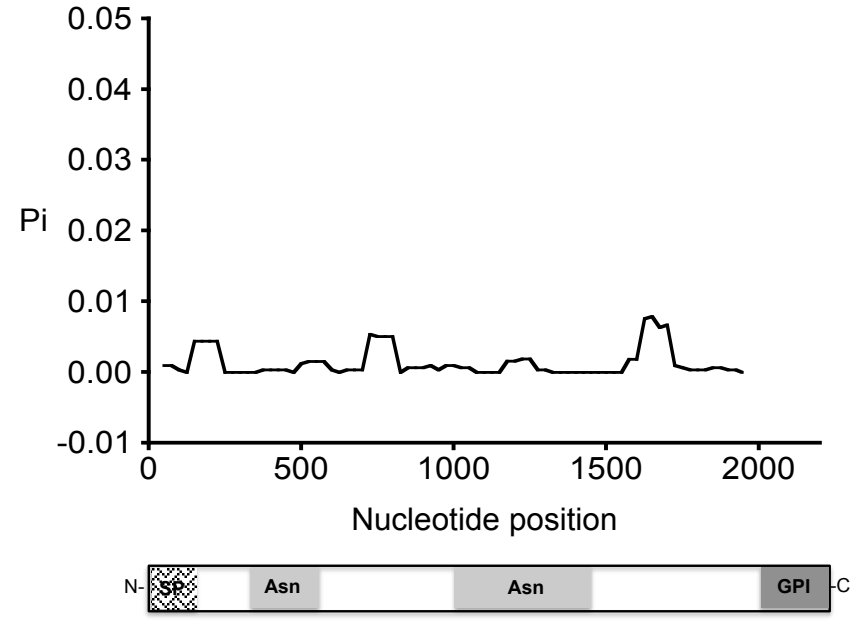
C ***PfRipr***



D ***Pfralp1***



E ***Pfgama***



F ***Pfmspdbl1***

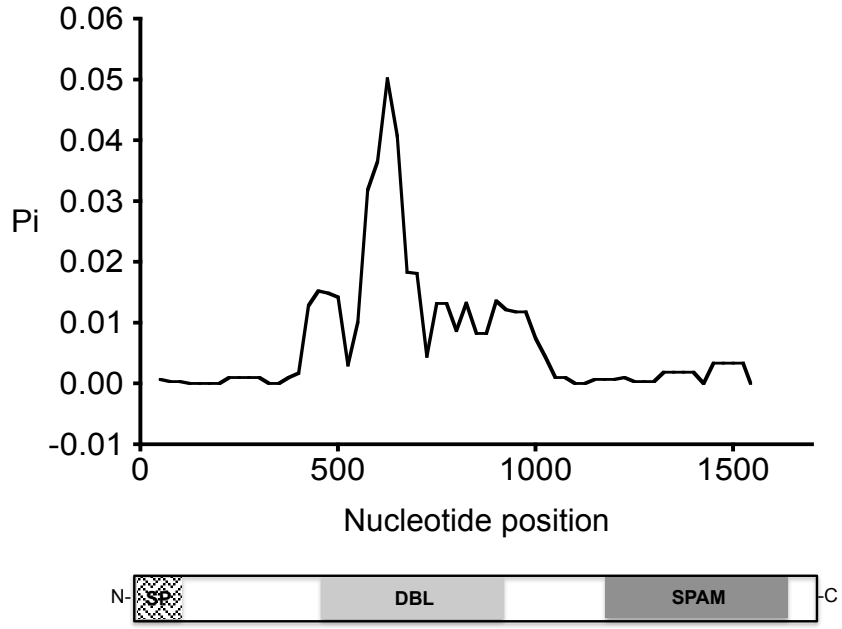


Figure 5. Sliding window analysis of nucleotide diversity (Pi) per site to compare the level of genetic diversity across four candidate genes. *(A) Pfama1, (B) Pfadsl, (C) Pfripr, (D) Pfralp1, (E) Pfgama, and (F) Pfmspdbl1. Pi is nucleotide diversity calculated using DnaSP ver. 5.10.01 with window length of 100 bases and step size of 25 bases. Pi is plotted against midpoint of window length. Schematic representation of the primary structures of full-length protein as described in references (Ito D et al Infect Immun 2013, Arumugam TU et al Infect Immun 2011, Sakamoto H et al Vaccine 2012), are indicated below each graph. PfRipr protein consists of 1,086 amino acids with a molecular weight of 126 kDa, a putative hydrophobic signal sequence (SP) at the N-terminus and Epidermal growth factor (EGF)-like domains in the protein with two at the N-terminal region and eight clustered towards the C-terminus. RALP1 protein consists of 749 aa with a calculated molecular mass of 87.9 kDa. The predicted signal peptide (SP; residues 1 to 17), leucine zipper-like domain (LZ; residues 511 to 532), and coiled-coil domain (CC; residues 86 to 158 and 595 to 624) are shown. GAMA protein consists of 738 amino acids, with a calculated molecular mass of 85.24 kDa. Shown are the predicted signal peptide (SP; residues 1 to 24), asparagine-rich region (Asn, residues 356 to 485), and C-terminal GPI anchor attachment site (GPI; residues 715 to 738). PfMSPDBL1 protein has signal peptide (SP, amino acid (aa) 1–25), Duffy binding-like (DBL, aa 159–419) domain and secreted polymorphic antigen associated with merozoites (SPAM, aa 543–697) domain.*

Figure 6A

Haplotype	Position of amino acid substitution																Frequency
	3	19	56	190	215	255	259	327	444	524	596	598	673	829	985	1039	
Pf3D7/ Ripr_UgH1	**R**	**A**	**N**	**V**	**N**	**M**	**Y**	**T**	**A**	**H**	**D**	**L**	**L**	**E**	**Y**	**I**	**46**
PfFVO	•	•	•	**A**	•	•	•	•	•	•	•	•	**V**	•	**N**	•	1
Ripr_UgH2	•	•	•	•	•	•	H	•	•	•	•	•	•	•	•	•	11
Ripr_UgH3	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	•	7
Ripr_UgH4	•	•	•	•	•	•	•	•	•	L	•	•	•	•	•	•	3
Ripr_UgH5	•	•	•	•	•	•	H	•	•	•	•	•	•	•	•	M	2
Ripr_UgH6	•	•	•	•	•	I	•	•	•	•	•	•	•	•	•	•	1
Ripr_UgH7	T	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	1
Ripr_UgH8	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	1
Ripr_UgH9	•	•	•	•	K	•	•	•	•	•	•	•	•	•	•	•	1
Ripr_UgH10	•	E	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1
Ripr_UgH11	•	•	•	•	•	•	H	•	•	•	•	•	•	Q	•	•	1
Ripr_UgH12	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	1
Ripr_UgH13	•	•	•	•	•	•	•	•	•	•	•	F	•	•	•	•	1
Ripr_UgH14	•	•	•	A	•	•	H	•	•	•	•	•	•	•	•	•	1
Ripr_UgH15	•	•	D	•	•	•	•	•	•	•	•	•	•	•	•	•	1

Figure 6B

Haplotype	Position of Amino acid substitution												Frequency
	41	52	79	87	90	150 Asparagine repeat region (205-214) - No. of Asparagine residues		395 6 mer repeat region (411-481)- No. of 6 mer repeats		512	578	690	
Pf3D7, RALP1_UgH4	**A**	**V**	**V**	**V**	**K**	**S**	**10**	**A**	**12**	**E**	**L**	**H**	**4**
PfFVO	•	•	•	•	•	•	10	•	12	•	I	•	1
RALP1_UgH1	•	•	•	•	•	N	10	•	13	•	V	•	10
RALP1_UgH2	•	•	•	•	•	•	10	•	13	•	•	•	6
RALP1_UgH3	•	•	•	•	E	•	10	•	11	•	•	•	5
RALP1_UgH5	•	•	•	•	•	•	7	•	12	•	•	•	2
RALP1_UgH6	•	•	•	•	•	•	11	•	12	•	•	•	2
RALP1_UgH7	•	•	•	•	E	•	10	•	13	•	•	•	2
RALP1_UgH8	S	•	•	•	•	•	8	•	13	•	•	•	1
RALP1_UgH9	•	•	•	•	•	•	9	•	13	•	•	•	1
RALP1_UgH10	•	•	•	•	•	•	8	•	10	•	•	•	1
RALP1_UgH11	•	•	•	•	•	•	8	•	14	•	•	•	1
RALP1_UgH12	•	•	•	•	•	•	8	•	15	•	•	•	1
RALP1_UgH13	•	•	•	•	•	•	8	•	14	•	•	•	1
RALP1_UgH14	•	•	•	•	•	•	9	•	14	•	•	•	1
RALP1_UgH15	•	•	•	•	•	•	10	•	13	•	•	•	1
RALP1_UgH16	•	•	•	•	•	•	12	•	12	•	•	•	1
RALP1_UgH17	•	•	•	•	•	•	11	•	12	•	•	•	1
RALP1_UgH18	•	•	•	•	•	•	8	•	12	•	•	•	1
RALP1_UgH19	•	•	•	•	E	•	10	•	13	•	•	•	1
RALP1_UgH20	•	•	•	•	•	•	8	•	12	•	•	•	1
RALP1_UgH21	•	•	•	•	•	•	13	•	12	•	•	•	1
RALP1_UgH22	•	•	•	•	•	•	7	•	15	•	•	•	1
RALP1_UgH23	•	•	•	•	•	•	8	•	13	•	•	Y	1
RALP1_UgH24	•	•	•	•	•	•	7	•	13	•	•	•	1
RALP1_UgH25	•	•	•	•	E	•	10	•	12	•	•	•	1
RALP1_UgH26	•	•	•	•	•	•	13	•	14	•	•	•	1
RALP1_UgH27	•	•	•	•	•	•	10	•	14	•	•	•	1
RALP1_UgH28	•	•	•	•	•	•	10	•	15	•	•	•	1
RALP1_UgH29	•	•	I	•	E	•	10	•	14	•	•	•	1
RALP1_UgH30	•	•	•	•	•	•	10	•	13	•	•	•	1
RALP1_UgH31	•	•	•	•	•	•	10	•	15	•	•	•	1
RALP1_UgH32	•	•	•	•	•	•	8	•	10	Q	•	•	1
RALP1_UgH33	•	•	•	•	E	•	12	•	12	•	•	•	1
RALP1_UgH34	•	•	•	•	•	•	13	E	12	•	•	•	1
RALP1_UgH35	•	•	•	•	•	•	8	•	13	•	•	•	1
RALP1_UgH36	•	I	•	I	•	•	10	•	15	•	•	•	1
RALP1_UgH37	•	•	•	•	E	•	15	•	13	•	•	•	1
RALP1_UgH38	•	•	•	•	E	•	8	•	12	•	•	•	1

Figure 6 C

Haplotype	Position of Amino acid substitution																				Frequency
	14	22	between 49 and 50								67	141	185	229	258	340	356 - 485	616	632	637	691	
Pf3D7	A	L	–	–	–	–	–	–	–	–	N	F	D	T	M	K	Asparagine rich region	L	S	K	N	1
PfFVO	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH1	V	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	2
Gama_UgH2	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	2
Gama_UgH3	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	2
Gama_UgH4	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	2
Gama_UgH5	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH6	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH7	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	Q		•	•	•	K	1
Gama_UgH8	•	P	K	N	E	F	N	N	N	E	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH9	•	•	–	–	–	–	–	–	–	–	•	Y	•	A	I	•		•	•	•	•	1
Gama_UgH10	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	1
Gama_UgH11	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH12	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH13	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH14	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH15	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		M	•	•	•	1
Gama_UgH16	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	R	•	•	1
Gama_UgH17	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	1
Gama_UgH18	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH19	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH20	•	•	K	N	E	F	N	N	N	E	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH21	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH22	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		M	•	•	•	1
Gama_UgH23	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	1
Gama_UgH24	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH25	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH26	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	N	•	•	1
Gama_UgH27	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH28	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH29	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH30	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH31	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH32	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH33	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH34	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	1
Gama_UgH35	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH36	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	N	•	•	1
Gama_UgH37	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH38	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH39	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH40	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	Q		•	•	•	•	1
Gama_UgH41	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH42	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH43	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	Q	•	1
Gama_UgH44	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	N	•	•	1
Gama_UgH45	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH46	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		M	•	•	•	1
Gama_UgH47	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH48	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	•	•	•	1
Gama_UgH49	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	•	•	•	1
Gama_UgH50	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH51	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH52	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH53	•	•	–	–	–	–	–	–	–	–	I	•	•	A	I	•		•	N	•	•	1
Gama_UgH54	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	•	•	•	1
Gama_UgH55	•	•	–	–	–	–	–	–	–	–	•	•	•	A	•	•		•	N	•	•	1
Gama_UgH56	•	•	–	–	–	–	–	–	–	–	•	•	•	A	I	•		•	R	•	•	1
Gama_UgH57	•	•	–	–	–	–	–	–	–	–	I	•	E	A	I	•		•	N	•	•	1

Figure 6D

Haplotype	Position of amino acid substitution																																									Frequency
	91	139	140	147	151	154	156	157	158	161	164	166	169	172-323	348	351	355	356	358	362	371	372	377	413	417	441	466	471	472	478	479	500	546	609	610	611	612	613	614	615	616	622	
Pf3D7/UgH3	**Y**	**G**	**N**	**E**	**K**	**K**	**V**	**L**	**S**	**P**	**S**	**G**	**G**	**DBL domain (recombination occurence)**	**D**	**K**	**I**	**Q**	**F**	**D**	**K**	**K**	**E**	**I**	**K**	**K**	**S**	**S**	**V**	**L**	**D**	**V**	**L**	**D**	**D**	**T**	**E**	**D**	**T**	**E**	**D**	**K**	**3**
PfFVO	D	•	•	•	•	•	•	•	•	•	•	•	R		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH1	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	5
MSPDBL1-UgH2	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH4	D	•	•	•	•	•	A	•	•	•	•	•	•		•	•	V	R	I	N	•	•	G	R	I	•	•	•	•	•	N	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH5	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	T	•	•	•	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH6	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH7	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH8	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	2
MSPDBL1-UgH9	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH10	D	•	S	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	•	•	•	•	•	•	•	T	•	G	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH11	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	•	•	•	•	•	•	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH12	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH13	D	•	•	•	•	•	A	•	•	•	•	•	•		•	•	•	•	•	N	•	E	G	R	I	•	•	•	•	•	•	A	V	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH14	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH15	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	I	•	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH16	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	•	•	•	•	•	•	A	•	–	–	–	–	–	–	•	•	•	1
MSPDBL1-UgH17	D	•	•	•	•	•	•	•	•	•	•	R	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH18	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	•	I	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH19	D	R	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH20	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH21	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH22	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH23	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	•	•	•	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH24	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	N	•	•	•	•	•	A	V	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH25	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	•	•	•	•	•	•	A	•	•	•	–	–	–	–	–	–	•	1
MSPDBL1-UgH26	D	•	•	•	•	•	•	•	•	•	•	•	R		N	E	V	R	I	N	•	•	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH27	D	•	•	K	N	Q	•	P	N	L	F	•	•		•	•	•	•	•	•	•	•	•	•	•	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH28	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH29	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH30	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	•	•	•	•	•	•	•	•	A	•	–	–	–	–	–	–	•	•	•	1
MSPDBL1-UgH31	D	•	•	K	N	Q	•	P	N	L	F	•	•		•	•	•	•	•	•	•	•	•	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH32	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH33	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH34	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	T	•	G	•	N	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH35	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH36	D	•	•	•	•	•	A	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH37	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH38	D	•	S	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	I	•	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH39	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	•	•	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH40	D	•	•	•	•	•	A	•	•	•	•	•	•		N	E	V	R	I	N	E	•	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH41	D	•	•	•	•	•	•	•	•	•	•	•	•		N	E	V	R	I	N	•	E	G	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH42	D	•	•	•	•	•	A	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	R	I	N	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH43	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	E	1
MSPDBL1-UgH44	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1
MSPDBL1-UgH45	D	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	E	G	R	I	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	1

Figure 6. Amino acid sequence polymorphism alignment for PfRipr, PfRALP1, PfGAMA, and PfMSPDBL1 among field isolates and strains 3D7 and FVO; *(A) PfRipr, (B) PfRALP1, (C) PfGAMA, (D) PfMSPDBL1. Polymorphic amino acid residues are listed for each haplotype. Amino acid residues identical to the 3D7 reference sequence are marked by dots. Insertion and deletions and repeat regions are in shaded in black. The total number of sequences for each haplotype is detailed in the "Frequency" column.*

A Amino acid alignment of 6-mer repeat region in RALP1

```
                                          30                                  60                                  90
                                          |                                   |                                   |
3D7,H4  DQNDDG DDTKDG DDTKDE DDHKNE DDHKNE DDHKNE DDHKNE DDHKN GDDNKN GDDHKN GDDNKN GDDDNG
H1      ...... ...... .....G ..T.D. ...... ...... ...... ..... E..H.. ...N.. ...H.. ...NKN GDDDNG
H2      ...... ...... ...... ...... ...... ...... ...... ..... E..H.. ...N.. ...H.. ...NKN GDDDNG
H3      ...... ...... ...... ...... ...... ...... .....G ..N.. ...H.. ...N.. ...DNG
H10     ...... ...... ...... ...... ...... ...... .....G ..N.. ...H.. ...GNG
H11     ...... ...... .....G ..T.D. ...... ...... ...... ..... E..H.. E..... ...... ...HKN GDDNKN GDDDNG
H12     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. ...N.. ...H.. ...NKN GDDNKN GDDNKN GDDDNG
H13     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. E..... ...... ...HKN GDDNKN GDDDNG
H14     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. ...N.. ...H.. ...NKN GDDNKN GDDDNG
H17     ...... .....E ..H.N. ...... ...... ...... ...... ..... ...... ...... ...... ......
H19     ...... ...... ...... ...... ...... ...... ...... ..... ...... ...... ...... ...NKN GDDDNG
H22     ...... ...... ...... ...... ...... ...... ...... ..... ...H.. ...... E..H.. ...HKN EDDHKN GDDNKN GDDDNG
H28     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. E..... E..H.. ...HKN EDDHKN GDDNKN GDDDNG
H29     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. ...N.. ...H.. ...HKN GDDNKN GDDDNG
H30     ...... .....E ..H.N. ...... ...... ...... ...... ..... ...... ...... ...... ...NKN GDDDNG
H31     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. E..... ...... ...NKN GDDDNG
H32     ...... ...... ...... ...... ...... ...... ...... ..... ...... ...DNG
H33     ...... ...... ...... ...... ...... ...... ...... ..... ...H.. E..... ..... ......
H36     ...... ...... ...... ...... ...... ...... ...... ..... E..H.. E.....E ..H.. ...NKN GDDHKN GDDNKN GDDDNG
```

Identical sequences : [3D7, H4 ,FVO, H5, H6, H16, H20, H21, H25, H38]; [H1, H15]; [H2, H7, H8, H9, H23, H24, H35,H37]; [H11, H26]; [H13, H27]; [H17, H18, H34]

B **Amino acid alignment of DBL domain in MSPDBL1**

```
       171                           200                           230
        |                             |                             |
3D7/H3 WISVAVKESSTTNKGVLVPPRRTKLCLR-NINKVWHRIKDEKNFKEEFVKVALG-ESNALMKHYKEKNLNALTAIKYGFS
H1     ....T.....................-.........................-.........................
H4     ......................K.....-...Q.....................-.........................
H5     ......................K.....-...Q.....................-.........................
H17    ......................K.....-.........................-.........................
H26    .............................-...N.........I..........-.........................
H36    .............................-.........................-.........................
FVO    .............................-.........................-.........................
H9     ......................KQM.F.I...NFPELK.T.GK.-.N.IYSSA.S.AKQ.I.L.GNNTEK..Q.M....A
H2     .A.SN.RNFL.V..........KQM.F.I...NFPELK.T.GK.-.N.IYSSA.S.AKQ.I.L.GNNTEK..Q.M....A
H27    .A.SN.RNFL.V..........KQM.F.I...NFPELK.T.GK.-.N.IYSSA.S.AKQ.I.L.GNNTEK.HQ..R.S.A
H22    .A.SN.RNFL.V..........KQM.F.I...NFPELK.TKGK.-.N.IYSSA.S.AKQ.I.L.GNNTEK.HQ..R.S.A

                260                           290                           320
                 |                             |                             |
3D7/H3 DMGDIIKGTDLIDYQITKNINRALDKILRNE--TSNDKIKKRVDWWEANKSAFWDAFMCGYKVHINKPCPEHDNMDRI
H1     ............................G..--.............................................
H4     ............................G..--.............................................
H5     ..............................--.............................................
H17    ............................G..--.............................................
H26    ............................G..--.............................................
H36    ............................GK.--.............................................
FVO    ............................G..--.............................................
H9     .I.N.VQ.N.M..TPTSNKTKTY.EEV.GKQYKNV..-P.DAKT..IQ..HRV...M.....................
H2     .I.N.VQ.N.M..TPTSNKTKTY.EEV.GKQYKNV..-P.DAKT..IQ..HRV...M.....................
H27    .I.N..R.D.MM.TPTS.ETITY.E.V.-KIYNEN...P.DAKK..TE.RHHV.E.M....QSAQDNQ.TGYG.I.D.
H22    .I.N..R.D.MM.TPTS.ETITY.E.V.-KIYNEN...P.DAKK..TE.RHHV.E.M....QSAQDNQ.TGYG.I.D.
```

Type I

Type II

Type III

Identical sequences : [3D7, H3, H6,H29, H32]; [H1, H20, H33]; [H4, H7, H12, H13, H14, H18, H23, H34, H35, H37, H39, H40]; [H5, H8, H25, H28, H42, H44]; [H17, H43];[FVO, H10, H15, H21, H38, H41]; [H2, H11, H16, H19, H24, H30, H45]; [H27, H31]

Figure 7. Amino acid sequence polymorphism alignment for: (A) PfRALP1 6-mer repeat region and (B) PfMSPDBL1-DBL recombination region. *Polymorphic amino acid residues are listed for each haplotype. Amino acid residues identical to the 3D7 reference sequence are marked by dots. Identical sequences are listed at the bottom of the figure.*

2.6.2 Recombinant PfRipr protein expression, IFA and ELISA

Recombinant PfRipr protein was designed [Figure 8A] and expressed it as a soluble protein using the WGCFS. The GST-fused recombinant PfRipr was affinity-purified and visualized as a band around 100 kDa [Figure 8B, arrowhead]. Noted are additional two bands around 50 kDa and 18 kDa, which are probably due to premature stoppage of the translation process and a wheat germ contaminant (Tsuboi T et al *Infect Immun 2008*), respectively. To confirm the specificity of the rabbit anti-PfRipr antibody, IFA was performed with acetone-fixed smears of late schizont stage parasites. Fluorescence signals were seen in the apical region of each merozoite, co-localizing with PfAMA1 indicating the localization of PfRipr in micronemes [Figure 8C]. These results are in agreement with previous reports (Chen L et al *PLoS Pathog 2011*), suggesting the specificity of the anti-PfRipr antibody used in this study. With the standardized ELISA protocols, IgG titers against each recombinant protein were obtained; $10^{3.8}$ (PfRipr) (Figure 9), $10^{5.7}$ (PfRALP1) (Ito D et al *Infect Immun 2013*), $10^{5.3}$ (PfGAMA) (Arumugam TU et al *Infect Immun 2011*), and $10^{4.9}$ (PfMSPDBL1) (Sakamoto H et al *Vaccine 2012*).

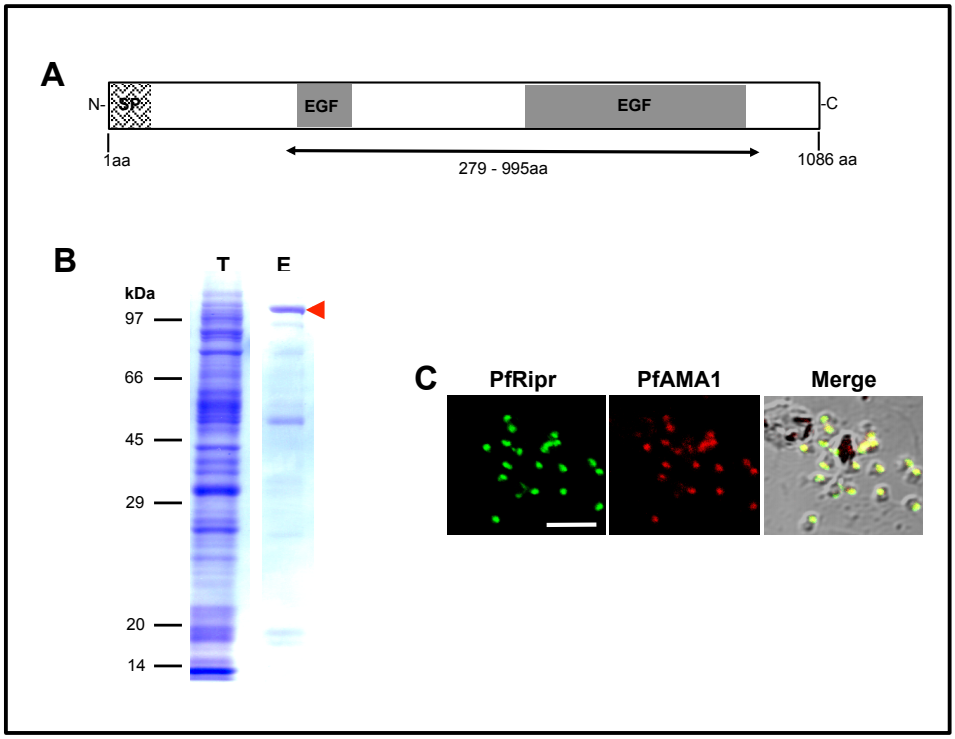


Figure 8. Primary structure, recombinant PfRipr expression, and reactivity of anti-PfRipr antibody against parasite proteins: *(A) Schematic representation of the primary structure of PfRipr showing the region used to express recombinant protein. The double-headed black arrow corresponds to the approximate position of the expressed fragment of 717 amino acids (279 aa – 995 aa), with a calculated molecular mass of 100 kDa. (B) SDS-PAGE of recombinant proteins of P. falciparum 3D7 Ripr expressed in WGCFS. The fraction of affinity-purified recombinant PfRipr proteins is resolved in an SDS-PAGE gel and stained with Coomassie brilliant blue R-250. T represents total lysate. E represents fraction of purified proteins eluted from affinity purification columns, respectively. Red arrowhead indicates the GST-fused recombinant PfRipr as a band around 100 kDa. (C) The localization of PfRipr in asexual blood-stage parasites using indirect immunofluorescence assay (IFA): Acetone-fixed P. falciparum 3D7 mature schizonts were probed with rabbit anti-PfRipr (green) and mouse anti-PfAMA1 (microneme marker) (red). Scale bar: 5 µm.*

Anti PfRipr serum dilution rate	Average	OD values			SD
10^2	**2.611**	2.654	2.492	2.687	0.104
10^3	**2.654**	2.732	2.611	2.619	0.068
10^4	**1.925**	2.012	1.866	1.896	0.077
10^5	**0.520**	0.535	0.528	0.499	0.019
10^6	**0.056**	0.061	0.061	0.047	0.008
None	**0.005**	0.000	0.012	0.003	0.006
None average + 3SD	0.024				

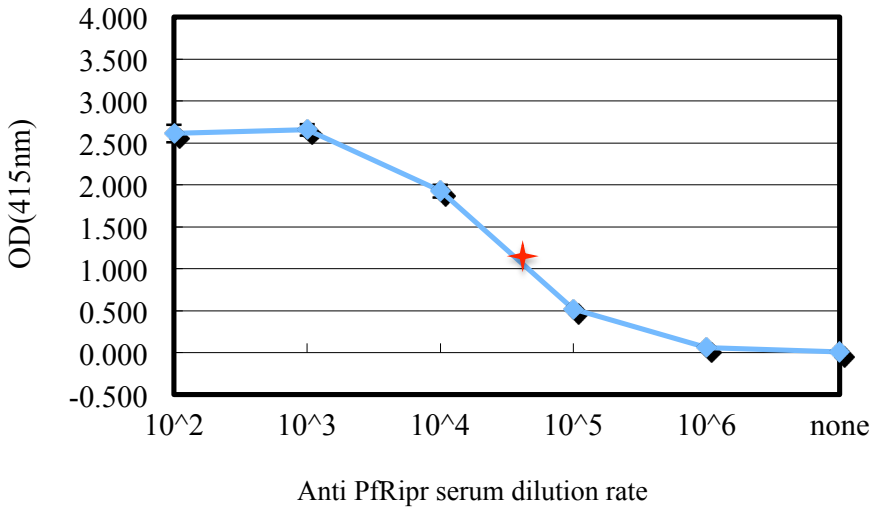


Figure 9. Results of standardized ELISA; *measuring levels of IgG titers against the WGCFS expressed PfRipr recombinant protein.*

2.6.3 GIA activities of anti-3D7 antibodies against PfRipr, PfRALP1, PfGAMA, and PfMSPDBL1 on *P. falciparum* FVO strain

Anti-PfRipr, anti-PfRALP1, anti-PfGAMA, anti-PfMSPDBL1, anti-EBA175 (region III-V) (positive control), and anti-GST (negative control) antibodies were tested at a final concentration of 20 mg/ml (total IgG concentration). The GIA activity of anti-PfRipr IgG against homologous strain 3D7 was 62.8 ± 5.7% (mean ± SD), and 76.6 ± 3.4% for the heterologous FVO. In both strains, the invasion-inhibitory activity of anti-PfRipr antibody was significantly higher than that of the negative-control anti-GST antibody ($P < 0.05$)[Figure 10]. Anti-PfRALP1 antibody inhibited invasion of both 3D7 and FVO by 23.7 ± 5.3% (mean ± SD) and 28.7 ± 8.9%, respectively. Both inhibitory activities were also significantly higher than that of the negative-control ($P < 0.05$) [Figure 10]. Anti-PfGAMA significantly inhibited invasion of 3D7 by 52.8 ± 5.7% (mean ± SD) ($P < 0.05$), but not of FVO strain (GIA activity =14.4 ± 3.4%) [Figure10]. Strain specific inhibition was also observed for anti-PfMSPDBL1 antibody: 26.2 ± 11.8% (mean ± SD) for 3D7 ($P < 0.05$) compared to no inhibition in the FVO strain (1.8 ± 7.3%) [Figure10]. Taken together, anti-PfRipr and anti-PfRALP1 antibodies significantly inhibited invasion of both 3D7 and FVO strains with the observed GIA activity of anti-PfRipr higher than that of anti-PfRALP1.

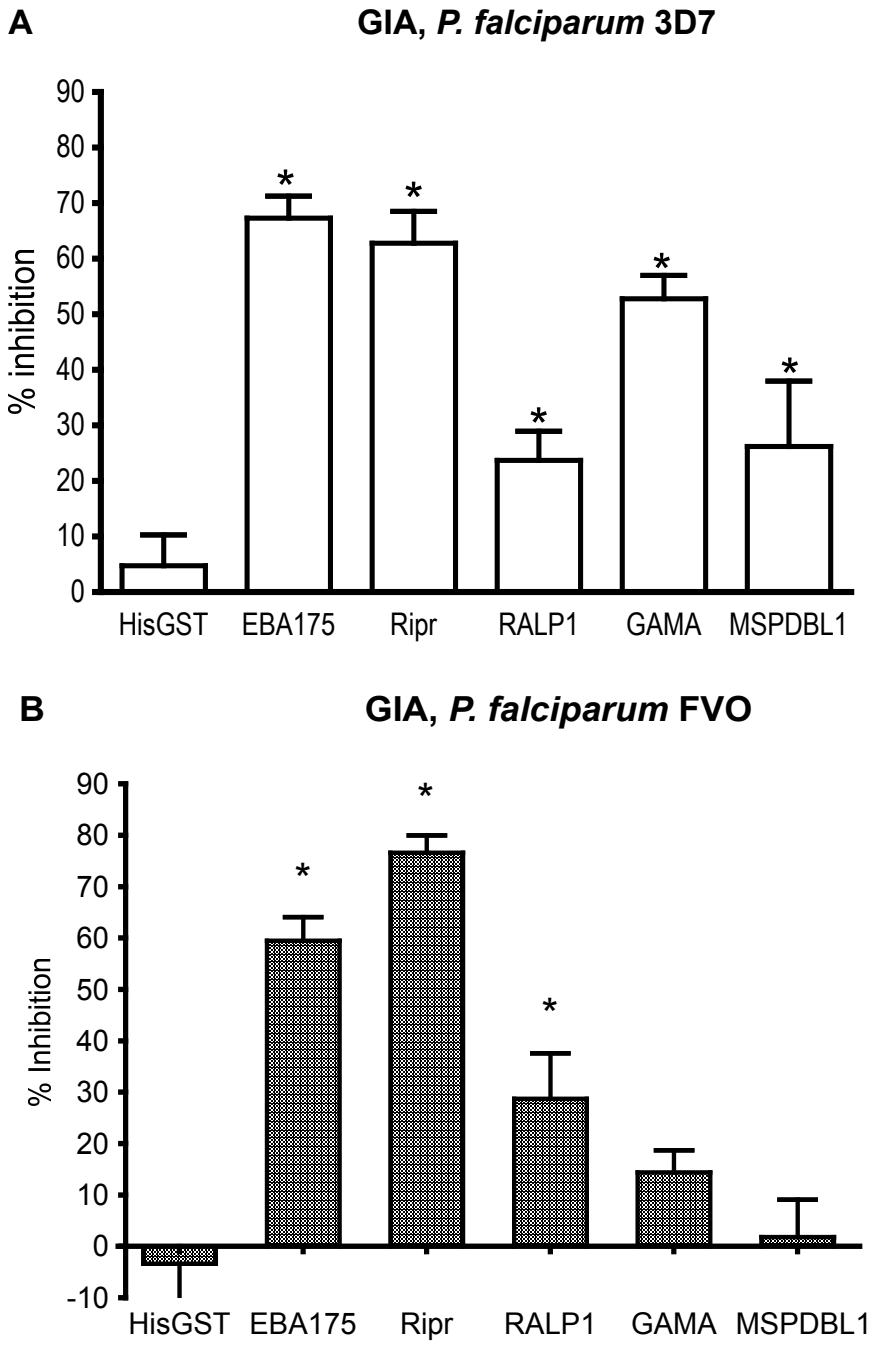


Figure 10. GIA of P. *falciparum* 3D7: anti-PfRipr, anti-PfRALP1, anti-PfGAMA, and anti-PfMSPDBL1 IgG. *(A) Invasion-inhibitory activity of anti-PfRipr, anti-PfRALP1, anti-PfGAMA, and anti-PfMSPDBL1 antibodies against 3D7 strain. (B) Invasion-inhibitory activity of 3D7 type anti-PfRipr, anti-PfRALP1, anti-PfGAMA, and anti-PfMSPDBL1 antibodies against FVO strain. The error bars represent the standard deviation (SD) of three independent experiments. * Statistically significant by Kruskal-Wallis test followed by Dunn's multiple-comparison test ($P < 0.05$)*

2.7 Discussion

High polymorphism levels in *P. falciparum* blood-stage malaria vaccine antigens often result in strain-specific immunity that hampers vaccine efficacy in the clinical trials (Lyon JA et al *PLoS One 2008*, Ogutu BR et al *PLoS One 2009*). Various studies that have attempted to address the effects of allele specific protective efficacy by combining a number of diversity-covering variants were met with a number of challenges (Boes A et al *Malar J 2016*). Moreover, emerging evidence suggests that conserved antigens across multiple strains could be a more straightforward approach to attain high protective efficacy in the field (Pandey AK et al *Infect Immun 2013*, Reddy KS et al *Infect Immun 2014*, Douglas AD et al *Cell Host Microbe 2015*, Hill DL et al *Infect Immun 2016*).

In this study, through use of population genetic analysis tools on *P. falciparum* field isolates obtained from a malaria endemic region in Uganda, the identification and confirmation through GIA of such conserved antigens were attempted. Among the candidate antigens, *pfripr* was found the most conserved. The antigen is less polymorphic, had the most common haplotype identical to 3D7 sequence, and the only candidate we consistently obtained a predicted full-length gene of 3261 bp. Sequence lengths for *pfralp1*, *pfgama* and *pfmspdbl1* differed greatly among the isolates due to the presence of encoded variable regions. Moreover, even with the exclusion of variable regions in the near full-length sequences, extensive polymorphism was seen in *pfgama*, and *pfmspdbl1*. *Pfralp1* was less polymorphic, but unlike *pfripr*, the sequences contained a 6-mer repeat and an asparagine-repeat region. Consistent with previous reports (Wickramarachchi T et al *Int J Parasitol 2009*, Sakamoto H et al *Vaccine 2012*), the DBL domain of *pfmspdbl1* is highly variable, and the SPAM region is conserved among Ugandan isolates. There was no demonstrated strong evidence of immune selection in all the candidate genes. The findings could be explained based on the antigens expression levels, distribution, and possible roles during merozoite

egress and invasion. PfRipr reportedly localizes in the microneme, where it migrates to the merozoite membrane surface and forms a complex with the PfRh5 that is tethered to the surface by a GPI moiety of PfCyRPA (Drew DR et al *Trends Parasitol 2015*, Chen L et al *PLoS Pathog 2011*, Reddy KS et al *Proc Natl Acad Sci 2015*, Favuzza P et al *Malar J 2016*). PfRALP1 is initially stored in the rhoptry neck, where it migrates to the moving junction (Ito D et al *Infect Immun 2013*). The micronemal protein PfGAMA is expectedly more exposed, since it migrates to the surface at an early mediation phase and interacts with unknown erythrocyte protein(s) prior to invasion (Arumugam TU et al *Infect Immun 2011*); while PfMSPDBL1 is dominantly expressed on the entire surface of the merozoite (Wickramarachchi T et al *Int J Parasitol 2009*, Sakamoto H et al *Vaccine 2012*). Therefore, although all are demonstrated to have essential roles to the parasite survival/invasion, and are immunogenic, the expression profiles and level of exposure to the host immunity is different and could influence their sequence diversity (Chen L et al *PLoS Pathog 2011*, Ito D et al *Infect Immun 2013*, Arumugam TU et al *Infect Immun 2011*, Wickramarachchi T et al *Int J Parasitol 2009*, Sakamoto H et al *Vaccine 2012*). A smaller number of *P. falciparum* isolates were sequenced from Uganda, compared to a larger number obtained from the database. However, even with the difference in the sample sizes, similar amino acid substitutions in the four genes and low allele frequency rates in PfRipr were observed. Remarkably, new variants were found that are unique to the *P. falciparum* parasites population in Uganda. This would translate into demand for deeper regional specific genetic variation surveys in the process of developing an effective blood-stage malaria vaccine.

In addition, varying numbers of amino acid substitutions were observed in PfRipr, PfRALP1, PfGAMA, and PfMSPDBL1 between *P. falciparum* 3D7 and FVO strains (Figures 6A-D). The FVO strain has also been reported as heterologous to 3D7 (Kennedy MC et al *Infect Immun 2002*, Polhemus ME et al *Vaccine 2007*). Functional assays such as GIA have

been used to further down select blood-stage vaccine candidates (Duncan CJ et al *Hum Vaccines Immunotherapeutics 2012*). However, impact of the observed amino acid substitutions on GIA is unknown. Chen et al. (Chen L et al *PLoS Pathog 2011*) reported the inhibition of parasite invasion by anti-PfRipr C-terminal (amino acid 791-900 of 3D7) IgG on multiple *P. falciparum* strains (FCR3, W2mef, T994, CSL2, E8B, MCAMP, 7G8, D10, HB3, and 3D7). However, this region had no amino acid substitutions. Using WGCFS, a cell-free eukaryotic system, we synthesized a larger, recombinant PfRipr fragment that includes all the epidermal growth factor (EGF)-like domains and determined whether or not the protein that had observed amino acid substitutions affected GIA. Also, for the first time, as all proteins were synthesized using WGCFS, this allowed an unbiased GIA assessment of anti-PfRipr, anti-PfRALP1, anti-PfGAMA, and anti-PfMSPDBL1 IgG in the heterologous *P. falciparum* FVO. Anti-PfRipr had the highest percentage inhibition in both strains. This was unlikely to be due to difference of IgG titers between the anti-recombinant protein antibodies as confirmed by ELISA. Both anti-PfRipr and anti-PfRALP1 IgG significantly inhibited merozoite invasion in homologous 3D7 and the heterologous FVO strains. The inhibition by anti-PfRipr was higher than that of PfRALP1 for both 3D7 and FVO. In contrast, the GIA activity for anti-PfGAMA and anti-PfMSPDBL1 IgG was only significant for homologous 3D7 strain. These results suggest that antibodies from recombinant PfRipr and PfRALP1 are not allele or strain-specific and the highly conserved antigen targets could elicit protective antibodies able to target heterologous parasites in a malaria endemic area such as Uganda. The utility of PfRipr as a vaccine candidate is continuously being studied. Various *in vitro* and animal experiments demonstrate that PfRipr is part of a multi-protein invasion complex with PfRh5 and PfCyRPA (Douglas AD et al *Cell Host Microbe 2015*, Drew DR et al *Trends Parasitol 2015*, Favuzza P et al *Malar J 2016*). Antibodies to *Escherichia coli*-expressed C-terminal and N-terminal fragments of PfRipr inhibit merozoite invasion *in vitro* but

recombinant PfRipr was not able to recognize antibodies from exposed individuals in a malaria endemic area (Chen L et al *PLoS Pathog 2011*). Recently, Chiu et al. (Chiu CY et al *Front Microbiol 2014*) demonstrated that recombinant C-terminal PfRipr expressed in *E. coli* and baculovirus-infected cells recognized antibodies from exposed individuals, but compared to PfRh5, the association with protection from high-density parasitemia was modest (Chiu CY et al *Front Microbiol 2014*). These differences in immunogenicity could be attributed to the difficulties in the expression and proper folding of cysteine-rich *Plasmodium* antigens in heterologous hosts. The WGCFS recombinant protein successfully elicited antibodies in rabbit that potently inhibited parasite growth in two genetically distinct *P. falciparum* lines. Thus, PfRipr is highly conserved, less polymorphic and has the most common field haplotype identical to the 3D7 haplotype.

Presently, antibody levels in human sera collected from malaria-exposed individuals against the WGCFS expressed PfRipr to infer on any possible association with clinical malaria are yet to be determined. Further analysis of polymorphism and genetic diversity in different field isolates worldwide would also give valuable information on the utility of this vaccine candidate. Nevertheless, our findings identify another component of the PfRh5 invasion complex that is highly conserved and could be prioritized for next-generation blood-stage malaria vaccine. Lastly, these data further substantiates the WGCFS as a valuable tool for the identification of novel malaria vaccine candidates.

I) Bibliography

Agnandji ST, Fendel R, Mestré M, Janssens M, Vekemans J, Held J, Gnansounou F, Haertle S, von Glasenapp I, Oyakhirome S, Mewono L. Induction of *Plasmodium falciparum*-specific CD4+ T cells and memory B cells in Gabonese children vaccinated with RTS, S/AS01 E and RTS, S/AS02 D. PLoS One. 2011 Apr 11;6(4):e18559.

Aikawa M, Miller LH, Johnson J, Rabbege J. Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. The Journal of cell biology. 1978 Apr 1;77(1):72-82.

Aikawa M. Plasmodium: the fine structure of malarial parasites. Experimental parasitology. 1971 Oct 31;30(2):284-320.

Amambua-Ngwa A, Tetteh KK, Manske M, Gomez-Escobar N, Stewart LB, Deerhake ME, Cheeseman IH, Newbold CI, Holder AA, Knuepfer E, Janha O. Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. PLoS Genet. 2012 Nov 1;8(11):e1002992.

Angrisano F, Tan YH, Sturm A, McFadden GI, Baum J. Malaria parasite colonisation of the mosquito midgut–placing the Plasmodium ookinete centre stage. International journal for parasitology. 2012 May 15;42(6):519-27.

Arumugam TU, Ito D, Takashima E, Tachibana M, Ishino T, Torii M, Tsuboi T. Application of wheat germ cell-free protein expression system for novel malaria vaccine candidate discovery. Expert review of vaccines. 2014 Jan 1;13(1):75-85.

Arumugam TU, Takeo S, Yamasaki T, Thonkukiatkul A, Miura K, Otsuki H, Zhou H, Long CA, Sattabongkot J, Thompson J, Wilson DW. Discovery of GAMA, a *Plasmodium falciparum* merozoite micronemal protein, as a novel blood-stage vaccine candidate antigen. Infection and immunity. 2011 Nov 1;79(11):4523-32.

Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S,

Anderson JM, Mao S, Sam B, Sopha C. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. New England Journal of Medicine. 2014 Jul 31;371(5):411-23.

Bailey JA, Pablo J, Niangaly A, Travassos MA, Ouattara A, Coulibaly D, Laurens MB, Takala-Harrison SL, Lyke KE, Skinner J, Berry AA. Seroreactivity to a large panel of field-derived *Plasmodium falciparum* apical membrane antigen 1 and merozoite surface protein 1 variants reflects seasonal and lifetime acquired responses to malaria. The American journal of tropical medicine and hygiene. 2015 Jan 7;92(1):9-12.

Bannister LH, Mitchell GH, Butcher GA, Dennis ED, Cohen S. Structure and development of the surface coat of erythrocytic merozoites of *Plasmodium knowlesi*. Cell and tissue research. 1986 Aug 1;245(2):281-90.

Baum J, Papenfuss AT, Mair GR, Janse CJ, Vlachou D, Waters AP, Cowman AF, Crabb BS, de Koning-Ward TF. Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. Nucleic acids research. 2009 Jun 1;37(11):3788-98.

Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJ, Richards JS. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. FEMS microbiology reviews. 2016 May 1;40(3):343-72.

Berger SS, Turner L, Wang CW, Petersen JE, Kraft M, Lusingu JP, Mmbando B, Marquard AM, Bengtsson DB, Hviid L, Nielsen MA. *Plasmodium falciparum* expressing domain cassette 5 type PfEMP1 (DC5-PfEMP1) bind PECAM1. PLoS One. 2013 Jul 9;8(7):e69117.

Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature. 2015; 526:207–11.

Birkett AJ, Moorthy VS, Loucq C, Chitnis CE, Kaslow DC. Malaria vaccine R&D in the

Decade of Vaccines: breakthroughs, challenges and opportunities. Vaccine. 2013 Apr 18;31:B233-43.

Blackman MJ, Bannister LH. Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation. Molecular and biochemical parasitology. 2001 Sep 28;117(1):11-25.

Boes A, Spiegel H, Kastilan R, Bethke S, Voepel N, Chudobová I, Bolscher JM, Dechering KJ, Fendel R, Buyel JF, Reimann A. Analysis of the dose-dependent stage-specific in vitro efficacy of a multi-stage malaria vaccine candidate cocktail. Malaria journal. 2016 May 17;15(1):1.

Boyle MJ, Wilson DW, Beeson JG. New approaches to studying *Plasmodium falciparum* merozoite invasion and insights into invasion biology. International journal for parasitology. 2013 Jan 31;43(1):1-0.

Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nature medicine. 1998 Mar;4(3):358.

Burgess BR, Schuck P, Garboczi DN. Dissection of merozoite surface protein 3, a representative of a family of *Plasmodium falciparum* surface proteins, reveals an oligomeric and highly elongated molecule. Journal of Biological Chemistry. 2005 Nov 4;280(44):37236-45.

Bustamante LY, Bartholdson SJ, Crosnier C, Campos MG, Wanaguru M, Nguon C, Kwiatkowski DP, Wright GJ, Rayner JC. A full-length recombinant *Plasmodium falciparum* PfRH5 protein induces inhibitory antibodies that are effective across common PfRH5 genetic variants. Vaccine. 2013 Jan 2;31(2):373-9.

Casares S, Brumeanu TD, Richie TL. The RTS, S malaria vaccine. Vaccine. 2010 Jul 12;28(31):4880-94.

Chen L, Lopaticki S, Riglar DT, Dekiwadia C, Uboldi AD, Tham WH, O'Neill MT, Richard D, Baum J, Ralph SA, Cowman AF. An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. PLoS Pathog. 2011 Sep 1;7(9):e1002199.

Chiu CY, Healer J, Thompson JK, Chen L, Kaul A, Savergave L, Raghuwanshi A, Suen CL, Siba PM, Schofield L, Mueller I. Association of antibodies to *Plasmodium falciparum* reticulocyte binding protein homolog 5 with protection from clinical malaria. Front Microbiol. 2014 Jun 30;5:314.

Chiu CY, Hodder AN, Lin CS, Hill DL, Suen CS, Schofield L, Siba PM, Mueller I, Cowman AF, Hansen DS. Antibodies to the *Plasmodium falciparum* proteins MSPDBL1 and MSPDBL2 opsonize merozoites, inhibit parasite growth, and predict protection from clinical malaria. Journal of Infectious Diseases. 2015 Aug 1;212(3):406-15.

Chotivanich K, Udomsangpetch R, McGready R, Proux S, Newton P, Pukrittayakamee S, Looareesuwan S, White NJ. Central role of the spleen in malaria parasite clearance. Journal of Infectious Diseases. 2002 May 15;185(10):1538-41.

Clyde Df, Mccarthy Vc, Miller Rm, Hornick Rb. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. The American journal of the medical sciences. 1973 Dec 1;266(6):398-404.

Cohen S, McGregor IA, Carrington S. Gamma-globulin and acquired immunity to human malaria. Nature. 1961 Nov 25;192:733.

Conway DJ. Paths to a malaria vaccine illuminated by parasite genomics. Trends in genetics. 2015 Feb 28;31(2):97-107.

Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell. 2006 Feb 24;124(4):755-66.

Craig A, Scherf A. Molecules on the surface of the *Plasmodium falciparum* infected

erythrocyte and their role in malaria pathogenesis and immune evasion. Molecular and biochemical parasitology. 2001 Jul 31;115(2):129-43.

Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, Weiss GE, Molina DM, Burk CR, Waisberg M, Jasinskas A, Tan X. A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. Proceedings of the National Academy of Sciences. 2010 Apr 13;107(15):6958-63.

Donati C, Rappuoli R. Reverse vaccinology in the 21st century: improvements over the original design. Annals of the New York Academy of Sciences. 2013 May 1;1285(1):115-32.

Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P. Artemisinin resistance in *Plasmodium falciparum* malaria. New England Journal of Medicine. 2009 Jul 30;361(5):455-67.

Douglas AD, Baldeviano GC, Lucas CM, Lugo-Roman LA, Crosnier C, Bartholdson SJ, Diouf A, Miura K, Lambert LE, Ventocilla JA, Leiva KP. A PfRH5-based vaccine is efficacious against heterologous strain blood-stage *Plasmodium falciparum* infection in Aotus monkeys. Cell host & microbe. 2015 Jan 14;17(1):130-9.

Draper SJ, Angov E, Horii T, Miller LH, Srinivasan P, Theisen M, Biswas S. Recent advances in recombinant protein-based malaria vaccines. Vaccine. 2015 Dec 22;33(52):7433-43.

Drew DR, Beeson JG. PfRH5 as a candidate vaccine for *Plasmodium falciparum* malaria. Trends in parasitology. 2015 Mar 31;31(3):87-8.

Dreyer AM, Matile H, Papastogiannidis P, Kamber J, Favuzza P, Voss TS, Wittlin S, Pluschke G. Passive immunoprotection of *Plasmodium falciparum*-infected mice designates the CyRPA as candidate malaria vaccine antigen. The Journal of Immunology. 2012 Jun 15;188(12):6225-37.

Duncan CJ, Hill AV, Ellis RD. Can growth inhibition assays (GIA) predict blood-stage

malaria vaccine efficacy? Human vaccines & immunotherapeutics. 2012 Jun 12;8(6):706-14.

Dvorak JA, Miller LH, Whitehouse WC, Shiroishi T. Invasion of erythrocytes by malaria merozoites. Science. 1975 Feb 28;187(4178):748-50.

Dzikowski R, Deitsch KW. Genetics of antigenic variation in *Plasmodium falciparum*. Current genetics. 2009 Apr 1;55(2):103-10.

Favuzza P, Blaser S, Dreyer AM, Riccio G, Tamborrini M, Thoma R, Matile H, Pluschke G. Generation of *Plasmodium falciparum* parasite-inhibitory antibodies by immunization with recombinantly-expressed CyRPA. Malaria journal. 2016 Mar 15;15(1):1.

Fowkes FJ, Richards JS, Simpson JA, Beeson JG. The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: A systematic review and meta-analysis. PLoS Med. 2010 Jan 19;7(1):e1000218.

Fried M, Avril M, Chaturvedi R, Fernandez P, Lograsso J, Narum D, Nielsen MA, Oleinikov AV, Resende M, Salanti A, Saveria T. Multilaboratory approach to preclinical evaluation of vaccine immunogens for placental malaria. Infection and immunity. 2013 Feb 1;81(2):487-95.

Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science. 1996 Jun 7;272(5267):1502.

Galinski MR, Barnwell JW. *Plasmodium vivax*: Merozoites, invasion of reticulocytes and considerations for malaria vaccine development. Parasitology today. 1996 Dec 31;12(1):20-9.

Gao X, Gunalan K, Yap SS, Preiser PR. Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*. Nature communications. 2013 Nov 27;4.

Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. Journal of Infectious Diseases. 2002 Mar

15;185(6):820-7.

Gilson PR, Crabb BS. Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. International journal for parasitology. 2009 Jan 31;39(1):91-6.

Good MF, Doolan DL. Immune effector mechanisms in malaria. Current opinion in immunology. 1999 Aug 1;11(4):412-9.

Goodman AL, Draper SJ. Blood-stage malaria vaccines—recent progress and future challenges. Annals of Tropical Medicine & Parasitology. 2010 Apr 1;104(3):189-211.

Greenwood B, Doumbo OK. Implementation of the malaria candidate vaccine RTS, S/AS01. The Lancet. 2016 Jan 29;387(10016):318-9.

Greenwood B. Malaria vaccines: Evaluation and implementation. Acta tropica. 2005 Sep 30;95(3):298-304.

Guillet P, Alnwick D, Cham MK, Neira M, Zaim M, Heymann D, Mukelabai K. Long-lasting treated mosquito nets: a breakthrough in malaria prevention. Bulletin of the World Health Organization. 2001 Jan;79(10).

Haase S, Cabrera A, Langer C, Treeck M, Struck N, Herrmann S, Jansen PW, Bruchhaus I, Bachmann A, Dias S, Cowman AF. Characterization of a conserved rhoptry-associated leucine zipper-like protein in the malaria parasite *Plasmodium falciparum*. Infection and immunity. 2008 Mar 1;76(3):879-87.

Halbroth BR, Draper SJ. Chapter One-Recent Developments in Malaria Vaccinology. Advances in parasitology. 2015 Apr 30;88:1-49.

Healer J, Crawford S, Ralph S, McFadden G, Cowman AF. Independent translocation of two micronemal proteins in developing *Plasmodium falciparum* merozoites. Infection and immunity. 2002 Oct 1;70(10):5751-8.

Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M,

Marsh K. Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors. Infection and Immunity. 2001 Sep 1;69(9):5849-56.

Heddini A. Malaria pathogenesis: a jigsaw with an increasing number of pieces. International journal for parasitology. 2002 Dec 4;32(13):1587-98.

Heppner DG, Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, Stewart VA, Dubois P, Lanar DE, Krzych U, Moris P. Towards an RTS, S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. Vaccine. 2005 Mar 18;23(17):2243-50.

Hill DL, Eriksson EM, Suen CS, Chiu CY, Ryg-Cornejo V, Robinson LJ, Siba PM, Mueller I, Hansen DS, Schofield L. Opsonising antibodies to *P.falciparum* merozoites associated with immunity to clinical malaria. PLoS One. 2013 Sep 9;8(9):e74627.

Hill DL, Wilson DW, Sampaio NG, Eriksson EM, Ryg-Cornejo V, Harrison GA, Uboldi AD, Robinson LJ, Beeson JG, Siba P, Cowman AF. Merozoite antigens of *Plasmodium falciparum* elicit strain-transcending opsonizing immunity. Infection and immunity. 2016 May 16:IAI-00145.

Hinds L, Green JL, Knuepfer E, Grainger M, Holder AA. Novel putative glycosylphosphatidylinositol-anchored micronemal antigen of *Plasmodium falciparum* that binds to erythrocytes. Eukaryotic cell. 2009 Dec 1;8(12):1869-79.

Hodder AN, Czabotar PE, Uboldi AD, Clarke OB, Lin CS, Healer J, Smith BJ, Cowman AF. Insights into Duffy binding-like domains through the crystal structure and function of the merozoite surface protein MSPDBL2 from *Plasmodium falciparum*. Journal of Biological Chemistry. 2012 Sep 21;287(39):32922-39.

Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M, Paul C, Gordon DM. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. Journal of Infectious Diseases.

2002 Apr 15;185(8):1155-64.

Hoffman SL, Vekemans J, Richie TL, et al. The march toward malaria vac- cines. Vaccine. 2015.

Hviid L. The immuno-epidemiology of pregnancy-associated *Plasmodium falciparum* malaria: a variant surface antigen-specific perspective. Parasite immunology. 2004 Nov 1;26(11-12):477-86.

Ito D, Hasegawa T, Miura K, Yamasaki T, Arumugam TU, Thongkukiatkul A, Takeo S, Takashima E, Sattabongkot J, Han ET, Long CA. RALP1 is a rhoptry neck erythrocyte-binding protein of *Plasmodium falciparum* merozoites and a potential blood-stage vaccine candidate antigen. Infection and immunity. 2013 Nov 1;81(11):4290-8.

Johnson JG, Epstein N, Shiroishi T, Miller LH. Factors affecting the ability of isolated *Plasmodium knowlesi* merozoites to attach to and invade erythrocytes. Parasitology. 1980 Jun 1;80(03):539-50.

Kauth CW, Woehlbier U, Kern M, Mekonnen Z, Lutz R, Mücke N, Langowski J, Bujard H. Interactions between merozoite surface proteins 1, 6, and 7 of the malaria parasite *Plasmodium falciparum*. Journal of Biological Chemistry. 2006 Oct 20;281(42):31517-27.

Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, Long CA, Miller LH, Stowers AW. In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infection and immunity. 2002 Dec 1;70(12):6948-60.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular biology and evolution. 2016 Mar 22:msw054.

Langreth SG, Jensen JB, Reese RT, Trager W. Fine structure of human malaria in vitro. The Journal of protozoology. 1978 Nov 1;25(4):443-52.

Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA

polymorphism data. Bioinformatics. 2009 Jun 1;25(11):1451-2.

Lin CS, Uboldi AD, Marapana D, Czabotar PE, Epp C, Bujard H, Taylor NL, Perugini MA, Hodder AN, Cowman AF. The merozoite surface protein 1 complex is a platform for binding to human erythrocytes by *Plasmodium falciparum*. Journal of Biological Chemistry. 2014 Sep 12;289(37):25655-69.

Lyon JA, Angov E, Fay MP, Sullivan JS, Girourd AS, Robinson SJ, Bergmann-Leitner ES, Duncan EH, Darko CA, Collins WE, Long CA. Protection induced by *Plasmodium falciparum* MSP1 42 is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. PloS one. 2008 Jul 30;3(7):e2830.

Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. Trends in parasitology. 2004 Dec 31;20(12):597-603.

malERA Consultative Group on Vaccines. A research agenda for malaria eradication: vaccines. PLoS Med. 2011 Jan 25;8(1):e1000398.

Malkin E, Hu J, Li Z, Chen Z, Bi X, Reed Z, Dubovsky F, Liu J, Wang Q, Pan X, Chen T. A phase 1 trial of PfCP2. 9: an AMA1/MSP1 chimeric recombinant protein vaccine for *Plasmodium falciparum* malaria. Vaccine. 2008 Dec 9;26(52):6864-73.

McCarthy JS, Good MF. Whole parasite blood stage malaria vaccines: a convergence of evidence. Human vaccines. 2010 Jan 1;6(1):114-23.

Mcfadden GI, Waller RF, Reith ME, Lang-Unnasch N. Plastids in apicomplexan parasites. In Origins of algae and their plastids 1997 (pp. 261-287). Springer Vienna.

Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002 Feb 7;415(6872):673-9.

Mitchell GH, Butcher GA, Voller A, Cohen S. The effect of human immune IgG on the in vitro development of *Plasmodium falciparum*. Parasitology. 1976 Apr 1;72(02):149-62.

Moorthy V, Reed Z, Smith PG, WHO Study Group on Measures of Malaria Vaccine

Efficacy. Measurement of malaria vaccine efficacy in phase III trials: report of a WHO consultation. Vaccine. 2007 Jul 9;25(28):5115-23.

Moorthy VS, Newman RD, Okwo-Bele JM. Malaria vaccine technology roadmap. The Lancet. 2013 Nov 23;382(9906):1700-1.

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodríguez A. Migration of Plasmodium sporozoites through cells before infection. Science. 2001 Jan 5;291(5501):141-4.

Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular biology and evolution. 1986 Sep 1;3(5):418-26.

Nkumama IN, O'Meara WP, Osier FH. Changes in Malaria Epidemiology in Africa and New Challenges for Elimination. Trends in Parasitology. 2016 Dec 6.

Noor AM, Kinyoki DK, Mundia CW, Kabaria CW, Mutua JW, Alegana VA, et al. The changing risk of *Plasmodium falciparum* malaria infection in Africa: 2000–10: a spatial and temporal analysis of trans- mission intensity. Lancet. 2014; 383:1739–47.

O'Meara WP, Mangeni JN, Steketee R, Greenwood B. Changes in the burden of malaria in sub-Saharan Africa. Lancet Infect Dis. 2010; 10:545–55.

Ochola LI, Tetteh KK, Stewart LB, Riitho V, Marsh K, Conway DJ. Allele frequency–based and polymorphism-versus-divergence indices of balancing selection in a new filtered set of polymorphic genes in *Plasmodium falciparum*. Molecular biology and evolution. 2010 Oct 1;27(10):2344-51.

Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, Tucker K, Waitumbi JN, Diggs C, Wittes J, Malkin E. MSP-1 Malaria Vaccine Working Group: Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in western Kenya. PLoS One. 2009;4:e4708.

Okello PE, Van Bortel W, Byaruhanga AM, Correwyn A, Roelants P, Talisuna A, et al. Variation in malaria transmission intensity in seven sites throughout Uganda. Am J Trop Med Hyg. 2006;75(2):219–25.

Osier FH, Mackinnon MJ, Crosnier C, Fegan G, Kamuyu G, Wanaguru M, Ogada E, McDade B, Rayner JC, Wright GJ, Marsh K. New antigens for a multicomponent blood-stage malaria vaccine. Science translational medicine. 2014 Jul 30;6(247):247ra102-.

Otsyula N, Angov E, Bergmann-Leitner E, Koech M, Khan F, Bennett J, Otieno L, Cummings J, Andagalu B, Tosh D, Waitumbi J. Results from tandem Phase 1 studies evaluating the safety, reactogenicity and immunogenicity of the vaccine candidate antigen *Plasmodium falciparum* FVO merozoite surface protein-1 (MSP1 42) administered intramuscularly with adjuvant system AS01. Malaria journal. 2013 Jan 23;12(1):1.

Ouédraogo AL, Roeffen W, Luty AJ, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, Cuzin-Ouattara N, Teleen K, Tiono AB, Sirima SB, Verhave JP. Naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission. Infection and immunity. 2011 Dec 1;79(12):4957-64.

Palacpac NM, Ntege E, Yeka A, Balikagala B, Suzuki N, Shirai H, Yagi M, Ito K, Fukushima W, Hirota Y, Nsereko C. Phase 1b randomized trial and follow-up study in Uganda of the blood-stage malaria vaccine candidate BK-SE36. PloS one. 2013 May 28;8(5):e64073. doi:10.1371/journal.pone.0064073.

Pandey AK, Reddy KS, Sahar T, Gupta S, Singh H, Reddy EJ, Asad M, Siddiqui FA, Gupta P, Singh B, More KR. Identification of a potent combination of key *Plasmodium falciparum* merozoite antigens that elicit strain-transcending parasite-neutralizing antibodies. Infection and immunity. 2013 Feb 1;81(2):441-51. doi:10.1128/IAI.01107-12.

Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, ler Moo C, Al-Saai S, Dondorp AM, Lwin KM, Singhasivanon P. Emergence of artemisinin-resistant

malaria on the western border of Thailand: a longitudinal study. The Lancet. 2012 Jun 1;379(9830):1960-6.

Polhemus ME, Magill AJ, Cummings JF, Kester KE, Ockenhouse CF, Lanar DE, Dutta S, Barbosa A, Soisson L, Diggs CL, Robinson SA. Phase I dose escalation safety and immunogenicity trial of *Plasmodium falciparum* apical membrane protein (AMA-1) FMP2. 1, adjuvanted with AS02A, in malaria-naive adults at the Walter Reed Army Institute of Research. Vaccine. 2007 May 22;25(21):4203-12. doi:10.1016/j.vaccine.2007.03.012.

Polley SD, Chokejindachai W, Conway DJ. Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. Genetics. 2003 Oct 1;165(2):555-61.

Ponnudurai T, Lensen AH, Van Gemert GJ, Bolmer MG, Meuwissen JT. Feeding behaviour and sporozoite ejection by infected Anopheles stephensi. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1991 Mar 1;85(2):175-80.

Pradel G. Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. Parasitology. 2007 Dec 1;134(14):1911-29.

Preiser P, Kaviratne M, Khan S, Bannister L, Jarra W. The apical organelles of malaria merozoites: host cell selection, invasion, host immunity and immune evasion. Microbes and Infection. 2000 Oct 31;2(12):1461-77.

Proietti C, Doolan DL. The case for a rational genome-based vaccine against malaria. Breaking the cycle: attacking the malaria parasite in the liver. Front Microbiol 2016 Jan 6:74.

Ranson H, Lissenden N. Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. Trends in parasitology. 2016 Mar 31;32(3):187-96.

Reddy KS, Amlabu E, Pandey AK, Mitra P, Chauhan VS, Gaur D. Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for Plasmodium

falciparum erythrocyte invasion. Proceedings of the National Academy of Sciences. 2015 Jan 27;112(4):1179-84.

Reddy KS, Pandey AK, Singh H, Sahar T, Emmanuel A, Chitnis CE, Chauhan VS, Gaur D. Bacterially expressed full-length recombinant *Plasmodium falciparum* RH5 protein binds erythrocytes and elicits potent strain-transcending parasite-neutralizing antibodies. Infection and immunity. 2014 Jan 1;82(1):152-64.

Rich SM, Ayala FJ. Evolutionary Origins of Human Malaria Parasites. In Krishna R. Dronamraju, Paolo Arese (Ed). Emerging Infectious Diseases of the 21st Century: Malaria – Genetic and Evolutionary Aspects. Springer US 2006. pp.125-146.

Richards JS, Arumugam TU, Reiling L, Healer J, Hodder AN, Fowkes FJ, Cross N, Langer C, Takeo S, Uboldi AD, Thompson JK. Identification and prioritization of merozoite antigens as targets of protective human immunity to *Plasmodium falciparum* malaria for vaccine and biomarker development. The Journal of Immunology. 2013 Jun 17:1300778.

Richards JS, Beeson JG. The future for blood-stage vaccines against malaria. Immunology and cell biology. 2009 Jul 1;87(5):377-90.

Richards JS, Stanisic DI, Fowkes FJ, Tavul L, Dabod E, Thompson JK, Kumar S, Chitnis CE, Narum DL, Michon P, Siba PM. Association between naturally acquired antibodies to erythrocyte-binding antigens of *Plasmodium falciparum* and protection from malaria and high-density parasitemia. Clinical Infectious Diseases. 2010 Oct 15;51(8):e50-60.

Riley EM, Stewart VA. Immune mechanisms in malaria: new insights in vaccine development. Nature medicine. 2013 Feb 1;19(2):168-78.

Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, Arens T, Beckers P, Van Gemert G, van de Vegte-Bolmer M, van der Ven AJ. Long-

term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. The Lancet. 2011 May 27;377(9779):1770-6.May 27;377(9779):1770-6.

Rts SC, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, Adegnika AA, Mordmüller B, Issifou S, Kremsner PG. A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants. The New England journal of medicine. 2012;367(24):2284-95.

Rts SC. Efficacy and safety of RTS, S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. The Lancet. 2015 Jul 10;386(9988):31-45.

Rts SC. Efficacy and safety of the RTS, S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS medicine. 2014;11(7):e1001685.

Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. The American journal of tropical medicine and hygiene. 1991 Sep;45(3):297-308.

Sachs J, Malaney P. The economic and social burden of malaria. Nature. 2002 Feb 7;415(6872):680-5.

Sachs JD. A new global effort to control malaria. Science. 2002 Oct 4;298(5591):122-4.

Sakamoto H, Takeo S, Maier AG, Sattabongkot J, Cowman AF, Tsuboi T. Antibodies against a *Plasmodium falciparum* antigen PfMSPDBL1 inhibit merozoite invasion into human erythrocytes. Vaccine. 2012 Mar 2;30(11):1972-80.

Sam-Yellowe TY, Fujioka H, Aikawa M, Messineo DG. *Plasmodium falciparum* Rhoptry Proteins of 140/130/110 kd (Rhop-H) Are Located in an Electron Lucent Compartment in the Neck of the Rhoptries. Journal of Eukaryotic Microbiology. 1995 May 1;42(3):224-31.

Schwartz L, Brown GV, Genton B, Moorthy VS. A review of malaria vaccine clinical projects based on the WHO rainbow table. Malaria journal. 2012 Jan 9;11(1):1.

Silverman PH, Schooley JC, Mahlmann LJ. Murine malaria decreases hemopoietic stem cells. Blood. 1987 Feb 1;69(2):408-13.

Sinden RE, Strong K. An ultrastructural study of the sporogonic development of *Plasmodium falciparum* in Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1978 Jan 1;72(5):477-91.

Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. The Lancet. 2004 Mar 27;363(9414):1017-24.

Singh S, Soe S, Weisman S, Barnwell JW, Pérignon JL, Druilhe P. A conserved multi-gene family induces cross-reactive antibodies effective in defense against *Plasmodium falciparum*. PloS one. 2009 Apr 30;4(4):e5410.

Snounou G, Singh B. Nested PCR analysis of Plasmodium parasites. Malaria Methods and Protocols: Methods and Protocols. 2002:189-203.

Snow RW, Marsh K. Malaria in Africa: progress and prospects in the decade since the Abuja Declaration. Lancet. 2010 Jul 10;376(9735):137.

Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrittayakamee S, Dolecek C, Hien TT, Do Rosário VE, Arez AP, Pinto J. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. Journal of Infectious Diseases. 2010 May 15;201(10):1544-50.

Tachibana M, Wu Y, Iriko H, Muratova O, MacDonald NJ, Sattabongkot J, Takeo S, Otsuki H, Torii M, Tsuboi T. N-terminal prodomain of Pfs230 synthesized using a cell-free system is sufficient to induce complement-dependent malaria transmission-blocking activity. Clinical and Vaccine Immunology. 2011 Aug 1;18(8):1343-50.

Takala SL, Plowe CV. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'. Parasite immunology. 2009 Sep 1;31(9):560-73.

Tanabe K, Mita T, Jombart T, Eriksson A, Horibe S, Palacpac N, Ranford-Cartwright L, Sawai H, Sakihama N, Ohmae H, Nakamura M. *Plasmodium falciparum* accompanied the human expansion out of Africa. Current Biology. 2010 Jul 27;20(14):1283-9.

Tanabe K, Mita T, Palacpac NM, Arisue N, Tougan T, Kawai S, Jombart T, Kobayashi F, Horii T. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin. Vaccine. 2013 Feb 18;31(9):1334-9.

Tetteh KK, Osier FH, Salanti A, Kamuyu G, Drought L, Failly M, Martin C, Marsh K, Conway DJ. Analysis of antibodies to newly described *Plasmodium falciparum* merozoite antigens supports MSPDBL2 as a predicted target of naturally acquired immunity. Infection and immunity. 2013 Oct 1;81(10):3835-42.

Tetteh KK, Stewart LB, Ochola LI, Amambua-Ngwa A, Thomas AW, Marsh K, Weedall GD, Conway DJ. Prospective identification of malaria parasite genes under balancing selection. PloS one. 2009 May 15;4(5):e5568.

Thera MA, Doumbo OK, Coulibaly D, et al. A field trial to assess a blood-stage malaria vaccine. N Engl J Med. 2011;365 (11):1004–1013.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research. 1994 Nov 11;22(22):4673-80.

Treutiger CJ, Heddini A, Fernandez V, Muller WA, Wahlgren M. PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes. Nature medicine. 1997 Dec 1;3(12):1405-8.

Tsuboi T, Takashima E. Antibody titre as a surrogate of protection of the first malaria subunit vaccine, RTS, S/AS01. The Lancet Infectious Diseases. 2015 Dec 31;15(12):1371-2.

Tsuboi T, Takeo S, Iriko H, Jin L, Tsuchimochi M, Matsuda S, Han ET, Otsuki H, Kaneko O, Sattabongkot J, Udomsangpetch R. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infection and immunity. 2008 Apr 1;76(4):1702-8.

Van Tyne, Daria, et al. "Modulation of PF10_0355 (MSPDBL2) alters *Plasmodium falciparum* response to antimalarial drugs." Antimicrobial agents and chemotherapy 57.7 (2013): 2937-2941.

Vanderberg JP, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. International journal for parasitology. 2004 Aug 31;34(9):991-6.

Vanderberg JP. *Plasmodium berghei*: quantization of sporozoites injected by mosquitoes feeding on a rodent host. Experimental parasitology. 1977 Jun 30;42(1):169-81.

Vlachou D, Schlegelmilch T, Runn E, Mendes A, Kafatos FC. The developmental migration of Plasmodium in mosquitoes. Current opinion in genetics & development. 2006 Aug 31;16(4):384-91.

Weiss GE, Crabb BS, Gilson PR. Overlaying Molecular and Temporal Aspects of Malaria Parasite Invasion. Trends in parasitology. 2016 Jan 7.

White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. The Lancet. 2014 Feb 22;383(9918):723.

White NJ. Can new treatment developments combat resistance in malaria?. Expert opinion on pharmacotherapy. May 2016.

Wickramarachchi T, Cabrera AL, Sinha D, Dhawan S, Chandran T, Devi YS, Kono M, Spielmann T, Gilberger TW, Chauhan VS, Mohmmed A. A novel *Plasmodium*

falciparum erythrocyte binding protein associated with the merozoite surface, PfDBLMSP. International journal for parasitology. 2009 Jun 30;39(7):763-73.

World Health Organization: World Malaria Report 2016. 2016, http://www.who.int/malaria/publications/world-malaria-report-2016/report/en/.

Yeka A, Gasasira A, Mpimbaza A, Achan J, Nankabirwa J, Nsobya S, et al. Malaria in Uganda: challenges to control on the long road to elimination: I. Epidemiology and current control efforts. Acta Trop. 2012;121:184–95.

II) Supplementary Data

Nested PCR and DNA sequencing primers

Pfripr		Primer sequence (5' to 3')
PCR primer	Ripr-F1	CTACCATGCTTCGTTAGTAAATAGAAAGGGGAC
	Ripr-F2	GGTTCCTCATCAATGCTAATTATGTACTTACAT
	Ripr-R1	GTTGCAACATATATACGTTTAAGGATATTCC
	Ripr-R2	AGGTATATATTAAAATGAAGAAATATGCATTGG
Sequencing primer	Ripr-FA	CAAGAAAACATCGGCAATTGATTT
	Ripr-R1	GTTGCAACATATATACGTTTAAGGATATTCC
	Ripr-F1	CTACCATGCTTCGTTAGTAAATAGAAAGGGGAC
	Ripr-F2	GGTTCCTCATCAATGCTAATTATGTACTTACAT
	Ripr-R1	GTTGCAACATATATACGTTTAAGGATATTCC
	Ripr-R2	AGGTATATATTAAAATGAAGAAATATGCATTGG
	Ripr_FA_2	CTTGTTCCCCCCAGAAGAACAAAATTATG
	Ripr_RA_2	GATCAACTTGTTGCACAACAGTTGC
	Ripr_FA_3	GCAATGTGAAAATGGGTTCTCTATAGAAG
	Ripr_RA_3	CACCATTCTCTAGAACAAAACGTTCTG
	Ripr_FA_4	CTTTTTGTATCTTACAGCTGCTCCAATTTATG
	Ripr_RA_4	GCATGGATATATTGTTATGAGGATATTGGTTG
	Ripr_FA_5	CCACATGAATGTGTATGTAATAAACAAGGTC
	Ripr_RA_5	CATCATTATATACGTCACACATTTCATTTTCCTG
	Ripr_RA_6	CTTCATAAACAACATGAGGTTCTAATAAAAGTGG
	Ripr-F1b	CTACCATGCTTCGTTAGTAAATAGAAAGGGGAC
	Ripr-F2b	GGTTCCTCATCAATGCTAATTATGTACTTACAT
	Ripr-R1b	GTTGCAACATATATACGTTTAAGGATATTCC
	Ripr-R2b	AGGTATATATTAAAATGAAGAAATATGCATTGG
	Ripr-1FA	GAAAACATCGGCAATTGATTTAATAGAAG
	Ripr-1RA	TGAAGAAATATGCATTGGTTTAAATAAAAAGAT
	Ripr-2FA	GAACCTCATGTTGTTTATGAAGAAACATT
	Ripr-2RA	CAAGGGTTTTGTATTAAACATACACCATC
	Ripr-3FA	GAAAATTCCACATGTGAACAAATAGGAAA
	Ripr-3RA	CACACATTTCATTTGGATTTGTACAAATAT
	Ripr-4FA	GATTTCAAGAAATAGTCGAACCAACCA
	Ripr-4RA	CAAAATGGTATATTAAACCAATATCACATTCTG
	Ripr-5FA	GTTAAAGGTAAATGTGTTCCAGACAACAA
	Ripr-5RA	CATCTATTGGATCTATTGTATTTCCATAAGCA
	Ripr-6FA	GTAGCTGTCAATGGAAAATGTGTTTT
	Ripr-6RA	CCGTCACAAAATTGATTTACTGAACAT
	Ripr-7FA	GCATGTGGTATGATCGAATTTTCAT
	Ripr-7RA	TTTCTTCACTATTTGGTATTACTACTCCG

Pfgama		Primer sequence (5' to 3')
PCR primer	GAMA-F1	GAATAATGTACAAAATAAAAGCAAAGAAGACGACCT
	GAMA-F2	GTGCATTACGTTTTATATTATAGTTCATATAGCA
	GAMA-R1	AAGATGGTGTGCTATATTCATATGTACATATA
	GAMA-R2	CCTACATATAATGAAATATATTTGATAAACATACAT
Sequencing primer	GAMA-FA	CATCTTTGTACGTTGCACTTATAA
	GAMA-RA	GTGATTAATTAAACTTCCTGCCTT
	GAMA-F1	GAATAATGTACAAAATAAAAGCAAAGAAGACGACCT
	GAMA-F2	GTGCATTACGTTTTATATTATAGTTCATATAGCA
	GAMA-R1	AAGATGGTGTGCTATATTCATATGTACATATA
	GAMA-R2	CCTACATATAATGAAATATATTTGATAAACATACAT
	GAMA_FA_2	CTGAATTAACACAAAAATTATGGTCAGGTAAAATG
	GAMA_RA_2	CCTTCTTCATCAATGTTTGTGGTAGGTTC
	GAMA_FA_3	GACACCACAAAAAATCAGCAACAAAATG
	GAMA_RA_3	CATTTTGTTGCTGATTTTTTGTGGTGTC
	GAMA_FA_4	CGATAAACATGTACCAAAAAACAACCAC
	GAMA_RA_4	CATGTATACATATATTCTCATATTTTGGCTCTGAG
	GAMA_RA_5	GGCACTTACCGCCTGACATAAG
	GAMA_RA_2.1	GGTACATGTTTATCGTTTTTATTTACCTGTAGG
	GAMA_FA_3.1	GTACCAAAAAACAACCACACAACTGC
	GAMA_RA_2.2	GCAGTTGTGTGGTTGTTTTTTGGTAC
	GAMA-F1b	GAATAATGTACAAAATAAAAGCAAAGAAGACGACCTA
	GAMA-F2b	GTGCATTACGTTTTATATTATAGTTCATATAGCA
	GAMA-R1b	AAGATGGTGTGCTATATTCATATGTACATATA
	GAMA-R2b	CCTACATATAATGAAATATATTTGATAAACATACAT
	GAMA-1FA	CATCTTTGTACGTTGCACTTATAATAGC
	GAMA-1RA	ATGTTGCATAAGAGAGGTGATTAATTAAAC
	GAMA-2FA	GAGGAAGATGAAGAACAAAATACATTTAGT
	GAMA-2RA	CATTAGCATAAAGGATTGTATGACCGTT
	GAMA-3FA	GCACAATCAAAAGGAGAAGTATTAAATAATGA
	GAMA-3RA	CATGTTTATCGTTTTTATTTACCTGTAGGTT
	GAMA-4FA	GATAAACATGTACCAAAAAACAACCACA
	GAMA-4RA	CCATGCTTTGTATAATGCTTATATGCATT
	GAMA-5FA	GTAGTAACAGATTTATCTGGTGAAAAAACA
	GAMA-5RA	GTCATCCACACATGTTAATAATATTTTAAATTGTT

Pfralp1		Primer sequence (5' to 3')
PCR primer	RALP1-F1	CATGTGAACCACTTAGATTTATTCAGGTGA
	RALP1-F2	CACTTGAAACTTTCAGTGTCTATTTTTATGC
	RALP1-R1	TACACATACATATGTTATGTATGCCATATGTT
	RALP1-R2	GGGAACCTTTTCTACACGTGAAAACA
Sequencing primer	RALP1-FA	CTGATAAAGTCATCTTCCAATGAT
	RALP1-RA	GCTCAAATAAGACTTTGTATAATT
	RALP1-F1	CATGTGAACCACTTAGATTTATTCAGGTGA
	RALP1-F2	CACTTGAAACTTTCAGTGTCTATTTTTATGC
	RALP1-R1	TACACATACATATGTTATGTATGCCATATGTT
	RALP1-R2	GGGAACCTTTTCTACACGTGAAAACA
	RALP1_FA_2	CACTTCATTCAACAAAAATAAAAACATATACAAC
	RALP1_RA_2	CAATAGATTGTAGTAATTCTCCTTGTCCTTG
	RALP1_FA_3	TCTCTGTAGTAACTCATTCTTTTCTAGATATGAG
	RALP1_RA_3	GTCATCTTCATTTTTGTGGTCATCTTCATC
	RALP1_FA_4	CACCTAAAAATGTACCAAATACTGAACAGAATG
	RALP1_RA_4	GTTGTATATGTTTTTATTTTTGTTGAATGAAGTG
	RALP1_FA_1.1	GAAGAAAATGAAAATGAAGAGATTGAAAAGGAGG
	RALP1_FA_2.1	GTTTGGGAAATAATACTTTTAAAAATGACGAAAAATATAATG
	RALP1_FA_3.1	GATTTAGAAGAGGATGTATTAAATAAAGAAAAGGAAC
	RALP1_RA_3.1	CAAATGTATAAGCAATATTTTTTGGTTTCTTTCTTC
	RALP1-F1b	CATGTGAACCACTTAGATTTATTCAGGTGA
	RALP1-F2b	CACTTGAAACTTTCAGTGTCTATTTTTATGC
	RALP1-R1b	TACACATACATATGTTATGTATGCCATATGTT
	RALP1-R2b	GGGAACCTTTTCTACACGTGAAAACA
	RALP1-F2c	CACTTGAAACTTTCAGTGTCTATTTTTATGC
	RALP1-R2c	GGGAACCTTTTCTACACGTGAAAACA
	RALP1-2FA	GTTTACTGTTTTATTAGTTCATTTTACCTGATAAAGTC
	RALP1-2RA	TTCAACATAACCGTTTCTTTAACAAAAATG
	RALP1-3FA	TGATGTAAGTGCAGAAAAAAAAAATAAAGAATTACC
	RALP1-3RA	TTTGATACAATATTTTGGGCTAAATCTACACG
	RALP1-4FA	ATGAAGAAAGAAACCAAAAAATATTGCTTATACATTTG
	RALP1-4RA	TCATCAATTTTACAAAAACCCCATTTTTATCC
	RALP1-5FA	ATACCATACTCCAATCTGATGATATAACTGATG
	RALP1-5RA	TGTTGTTATTTGAACAATGTGTTTTCTTCATG
	RALP1-6FA	AGAAACACAAACAGATGATGACATAAACG
	RALP1-6RA	TTTTTTATTTACCCCTGTATGTGCCAC
	RALP1-7FA	GATCAGCAAGGGGAACTTAAAAATGTC
	RALP1-7RA	TCTTCTTCATTTTGTTGATTTTCTTCATTTTCATC
	RALP1-3FAb	CTTTCAATTTTCATAAAAATATAAACACTTCATTCAAC
	RALP1-5RAb	TTCTTCATGTCTATAGCTTCTTCTTTCTTCTC

Pfmspdbl1		Primer sequence (5' to 3')
PCR primer	MSPDBL1-F1	AATGTTAGTAGTAATTATTTAAACGCTCTTATCTTA
	MSPDBL1-F2	CACATTTAATTAAGGTTGTATTTACTGATAA
	MSPDBL1-R1	AACCTATGTAAATTTTCTATAGTAGAATAGTAT
	MSPDBL1-R2	ATTTCACTTTATGTGAAAGCATATATTAAGAACA
Sequencing primer	MSPDBL1-F2	CACATTTAATTAAGGTTGTATTTACTGATAA
	MSPDBL1-RA	TAAATCTGTCATATCTTCTGTCAA
	MSPDBL1-F1	AATGTTAGTAGTAATTATTTAAACGCTCTTATCTTA
	MSPDBL1-F2	CACATTTAATTAAGGTTGTATTTACTGATAA
	MSPDBL1-R1	AACCTATGTAAATTTTCTATAGTAGAATAGTAT
	MSPDBL1-R2	ATTTCACTTTATGTGAAAGCATATATTAAGAACA
	MSPDBL1_FA_2	CTTGTTCCCCCCAGAAGAACAAAATTATG
	MSPDBL1_RA_2	GATCAACTTGTTGCACAACAGTTGC
	MSPDBL1_FA_3	GAAGAATGGGTTAATAGAAGGAGACCTG
	MSPDBL1_RA_3	CCCACCAGTCTACACGTTTTTTAATTTTG
	MSPDBL1_FA_2.1	GTCTAAGAAATATTAACAAGGTTTGGCATCG
	MSPDBL1_FA_4	CAACAATAATTTAGAGCGTGGATTGGG
	MSPDBL1_RA_4	GCACTACCATCATCAAATCCACTATTAGTAAC
	MSPDBL1_FA_1.1	CTATCGTTGGACAAGATGTGCCTATTAC
	MSPDBL1_FA_2.2	GGCTGTTAAAGAAAGTTCAACTACAAATAAAGG
	MSPDBL1_RA_2.1	CTCGTTGATCTAGATTACTAGAATCCCTAACAC
	MSPDBL1_RA_3.1	GAATGCATCCCAGAATGCACTTTTATTAG
	MSPDBL1-F1b	AATGTTAGTAGTAATTATTTAAACGCTCTTATCTTA
	MSPDBL1-F2b	CACATTTAATTAAGGTTGTATTTACTGATAAGTTTTC
	MSPDBL1-R1b	ACCTATGTAAATTTTCTATAGTAGAATAGTATTTTTTTTC
	MSPDBL1-R2b	ATTTCACTTTATGTGAAAGCATATATTAAGAACA
	MSPDBL1-1FA	CACATTTAATTAAGGTTGTATTTACTGATAAGTTTTC
	MSPDBL1-1RA	ATTTCACTTTATGTGAAAGCATATATTAAGAACA
	MSPDBL1-2FA	GAAGGAAATAGTATTGACGATACTAAAGGTCT
	MSPDBL1-2RA	GATAGTATTCGATATGAATCTTCGTCAACTTTTG
	MSPDBL1-3FA	CATTATAAAGAAAAAAATCTGAATGCCCTTACAG
	MSPDBL1-3RA	TTGTAGTTATTATTTCCTCTTTGCGTTACAG
	MSPDBL1-4FA	GACAAATTACAAACCAGAATGATTCACAAC
	MSPDBL1-4RA	TCAAATTTATCACATTGACCTTTCCATTCAG
	MSPDBL1-5FA	GAATATAACAACATCTCAAGGAAATTCACACC
	MSPDBL1-5RA	GTCTACACGTTTTTTAATTTTGTCATTACTTGT
	MSPDBL1-6FA	CTGAAGATACGGAAGATATAGAAGAGGAAAATAA
	MSPDBL1-6RA	GACATTTTTAATACCCTTACAAAAATTTTCATCAGG
	MSPDBL1-7FA	GTAAGTTATAAGGACAATAATGAAGTAAAAAATGTTGC
	MSPDBL1-7RA	AACTATTATTTAGTAATCCGTTTCTTAGATTCGAATC

III) Citation

Ntege EH, Arisue N, Ito D, Hasegawa T, Palacpac NM, Egwang TG, Horii T, Takashima E, Tsuboi T. Identification of Plasmodium falciparum reticulocyte binding protein homologue 5-interacting protein, PfRipr, as a highly conserved blood-stage malaria vaccine candidate. Vaccine. 2016 Nov 4;34(46):5612-22.