UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

SELECTION DYNAMICS OF CIRCUMSPOROZOITE PROTEIN (CSP) VACCINE TARGET IN GHANA: THE CONTRIBUTION OF HUMAN LEUKOCYTE ANTIGEN (HLA) VARIATION

BY

JONATHAN NYUMAH KPAKA

[10703453]

This thesis/dissertation is submitted to the University of Ghana, Legon in partial fulfillment of the requirements for the award of MPHIL Degree in MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

July 2021

DECLARATION

I do hereby declare that this thesis is the result of research work done by me at Brown University (Providence, Rhode Island, USA) under the supervision of Associate Professor Dr. Jeffrey Bailey of Brown University, USA; Dr. Anita Ghansah of the West African Center for Cell Biology of Infectious Pathogens (WACCBIP) & the Department of Parasitology, Noguchi Memorial Institute of Medical Research; and Professor Gordon A. Awandare of the Department of Biochemistry, Cell, and Molecular Biology, University of Ghana. No part of this thesis has ever been previously submitted for a degree other than now.

Also, I have duly acknowledged all references cited in this thesis.

Jonathan Nyumah Kpaka	.....	July 30, 2021
(Student)	Signature	Date
Dr. Jeffrey Bailey	.....	July 30, 2021
(Supervisor)	Signature	Date
Dr. Anita Ghansah	.....	July 30, 2021
(Supervisor)	Signature	Date

DEDICATION

This thesis is dedicated to my son (Joseph S. Kpaka) and my daughters (Jonetta S. Kpaka and Scholastica K. Kpaka) who stood firmly and supported my decision to leave home (Liberia) and pursue this graduate degree in Ghana and the United States of America. They are the greatest source of my inspiration.

Also, to my deceased father and mother (Mr. Alfred F. Kpaka and Mrs. Annie K. Kpaka) who always supported my quest for higher education and wished the very best for me.

I also dedicate this thesis to my brothers (Francis F. Kpaka and Stephen T. Kandakai) for their support while I was away from home.

ACKNOWLEDGMENT

I am greatly indebted to Dr. Jeffrey Bailey whose invaluable support made this achievement a success. I am also profoundly grateful to Dr. Anita Ghansah who trusted me even before she knew me. Thank you for the support, guidance, and trust. May God bountifully reward you.

I am also indebted to the team of the Ghansah lab at Noguchi Memorial Institute of Medical Research who helped with sample collection and DNA extraction, and especially Dr. Benedicta Mensah and Ms. Elikplim A. Amegashie who helped me to analyze the data.

I sincerely thank the team at Bailey's lab, Brown University in the United States of America, especially Associate Professor Ozkan Aydemir and Deborah Chin who helped me with the DNA amplification, sequencing, and generation of the haplotypes for analysis.

I am also grateful to all my course mates, especially Jonathan Suurbaar, Harry Danwonno, and Gloria Asantewaa who made Ghana a home away from home during my studies.

I remain indebted to my family for standing by me and trusting me as I endeavored to achieve this degree. All the praises belong to God for health, grace, mental and spiritual support, and traveling mercy during my studies and hereafter.

Lastly, I wholeheartedly appreciate all the study participants whose samples made this study a success. Data would not have been generated without these blood samples. May their health be fully restored.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACT - Artemisinin-based Combination Therapy

CSP - Circumsporozoite Protein

CRR - Central Repeat Region

CHMI - Control Human Malaria Infection

DBS - Dried Blood Spot

DDT - dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

EBL - Erythrocyte binding-like protein

EMA - European Medicines Agent

ER - Endoplasmic Recticulum

GPI - Glycosylphosphatidylinositol

GTS - Global Technical Strategy

GMEP - Global Malaria Eradication Program

HBHI - High Burden High Impact

HBsAg - Hepatitis B Surface Antigen

HLA - Human Leukocyte Antigen

IPT - Intermittent Preventive Treatment

IRS - Indoor Residual Spraying

ITNs - Insecticide-treated mosquito Nets

KIRs - Killer Ig-like Receptors

MIP - Molecular Inversion Probe

MSP - Merozoite Surface Proteins

MVIP - Malaria Vaccine Implementation Program

MVTRM	Malaria Vaccine Technology Roadmap
NANP -	Asparagine; Alanine; Proline
NMCP -	National Malaria Control Program
NMIMR -	Noguchi Memorial Institute for Medical Research
NVDP -	Asparagine; Valine; Aspartic acid; Proline
PCR -	Polymerase Chain Reaction
PfEMP-1	*Plasmodium falciparum* Erythrocyte Membrane Protein-1
PfRh -	*Plasmodium falciparum* reticulocyte binding-like protein
PfSPZ -	*Plasmodium falciparum* Sporozoites
PVM -	Parasitophorous Vacuole Membrane
RBCs -	Red Blood Cells
RBM -	Roll Back Malaria
RDTs -	Rapid Diagnostic Tests
RNA -	Ribonucleic acid
SNP -	Single Nucleotide Polymorphism
TCRs -	T Cell Receptors
VIMTs-	Vaccines that Interrupt Malaria Transmission
WHO -	World Health Organization
WMR -	World Malaria Report

ABSTRACT

Implementation of RTS,S/AS01 vaccine for malaria is underway in three (3) African countries, Ghana, Kenya, and Malawi. This vaccine, which targets the *Plasmodium falciparum* circumsporozoite protein (CSP) provides partial protection for infants and children against clinical and severe malaria infections. Reasons for this reduced efficacy or immunogenicity are poorly understood, but CSP variation has been implicated. Human leukocyte antigen (HLA) has also been observed to influence RTS,S-mediated protection. This study aims to define the variants of CSP and determine its distribution between Begoro and Cape Coast in Ghana over three years. Further, the influence of HLA genotype in terms of parasite frequency and RTS,S/AS01 response was assessed.

About 50µl of peripheral blood was collected from participants in Begoro and Cape Coast in 2014, 2015, and 2016, dried blood spot (DBS) prepared and DNA was extracted. The C-terminal of *Plasmodium falciparum* CSP and the human leukocyte antigen (HLA) class II gene in humans were deep sequenced. The translated amino acid haplotypes of the CSP were aligned and compared to the reference 3D7 vaccine strain. The HLA class II haplotypes were grouped into superfamily and their association with the CSP variants was ascertained.

The CSP haplotypes are evenly distributed between Begoro and Cape Coast. There were 31 Th2R haplotypes in Begoro and 30 Th2R haplotypes in Cape Coast; 15 Th3R haplotypes in Begoro and 13 in Cape Coast. About 83.9% of Th2R and 96.5% of Th3R haplotypes in Begoro are shared with Cape Coast. The amino acid changes with reference to the 3D7 vaccine strain at the Th2R epitope range from 1 to 6 and 1 to 4 at theTh3R epitope. There is a 53% and 60% reduction in the 3D7 Th2R and Th3R haplotypes, respectively, from 2014 to 2016, but 3D7 is still common in Ghana, Kenya, and Malawi. The 3D7 haplotype does not correlate with HLA-DRB1, but there is with HLA-DQA1 and HLA-DPB1.

Begoro and Cape Coast are two different ecological zones in Ghana but the parasite population is homogenous. The Th2R epitope of CSP is polymorphic than the Th3R epitope and this higher polymorphism is driving a higher non-synonymous amino acid substitution at the Th2R epitope than the Th3R epitope which may have vaccine implication. A decline in frequency of 3D7 parasite population may also affect the performance in the vaccine in Begoro and Cape Coast. Initial correlations indicate that HLA-DPB1 (01:01/17:01) correlates with the 3D7 vaccine strain, but HLA-DPB1 (01:01/17:01) and other variants of HLA-DQA1 also correlates with other Th2R haplotypes and may compete with the vaccine haplotype for antigen presentation to CD+4 T cells. This may have implications for the efficacy of the RTS,S/AS01 vaccine in Ghana.

CHAPTER ONE

1 INTRODUCTION

1.1 Background

Malaria is still a major cause of ill health and death among under five-year-old children. A significant number of clinical cases and deaths occur in resource-limited tropical and subtropical countries. The World Health Organization World Malaria Report 2021 estimated 241 million cases of clinical malaria in 2020 in 85 countries, an increase from 227 million cases in 2019, 228 million in 2018, and 231 million in 2017. This estimate is a decline from 251 million cases in 2010. Majority of the clinical malaria cases (228 million, representing 95%) in 2020 occurred in Africa. About 55% of the global malaria cases in 2020 occurred in six (6) African countries: Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%), Angola (3.4%), and Burkina Faso (3.4%) (WHO. 2021).

Over the past decade, malaria has continued to claim about half a million lives yearly. According to WHO, malaria deaths increased by 12% (estimated 627,000 deaths) in 2020 compared with 2019 due to service disruptions during the COVID-19 pandemic. Children under the age of five years accounted for 77% of deaths in 2020 compared to 67% 2018 and 87% in 2000. In the WHO African Region, malaria deaths reduced by 36%, from 840,000 in 2000 to 534,000 in 2019, then increasing to 602,000 in 2020. About 96% of the global malaria deaths in 2020 were in 29 countries, and six (6) countries in Africa – Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%), Angola (3%), and Burkina Faso (3%) accounted for over half of all malaria deaths globally (WHO. 2021). India was responsible for about 82% of all malaria deaths in the WHO South-East Asia Region. Since 2018, Cabo Verde and Sao Tome and Principe have not reported any malaria death.

Between 2000 and 2019, the case incidence of malaria infection in the WHO African Region reduced from 368 to 222 per 1000 population at risk, but increased to 232 in 2020 due to disruptions in services during the COVID-19 pandemic. An estimated 1.7 billion cases and 10.6 million malaria deaths were averted in the period 2000-2020 (WHO. 2021). Majority of the cases (82%) and deaths (95%) averted were in the WHO African Region.

Also, between 2015 and 2018, thirty-one (31) malaria-endemic countries reduced case incidence significantly and could reduce malaria cases by 40% or more by 2020 (WHO. 2019). Despite the considerable progress made since 2000, the Global technical strategy (GTS) for malaria 2020 milestones for morbidity and mortality were not achieved globally (WHO. 2021). The GTS for malaria 2016-2030 aims to reduce malaria case incidence and death rate of at 40% by 2020, 75% by 2025, and 90% by 2030 from a 2015 baseline of 224 million estimated malaria cases.

In Ghana, there were 4.9 million confirmed cases of malaria and 29,517 deaths in 2017, according to the Ghanaian National Malaria Control Program (NMCP). Children under five years account for 54.6% of these deaths (Ghana NMCP, 2017). Ghana is among the 15 highest burden malaria countries with 2.1% of global malaria cases and 1.9% global malaria deaths. Ghana accounted for 4.3% of malaria cases in West Africa between 2017-2020 (WHO. 2021).

Five Plasmodium species infect humans with malaria; they include *Plasmodium falciparum, P. malariae, P. ovale, P. vivax,* and *P. knowlesi*. *Plasmodium falciparum* is the most prevalent species in Africa and it was responsible for about 99.7% of the clinical malaria cases in 2018. In the Americas, *P. vivax* accounted for 75% of malaria cases in 2018. *Plasmodium vivax* is also the predominant species in South-East Asia, causing 53% of malaria infections in that region (WHO. 2019).

Early diagnosis and treatment of malaria are important to reduce clinical malaria and preventing deaths and reduce parasite transmission. The WHO recommended first line treatment for uncomplicated malaria best available treatment for is artemisinin-based combination therapy (ACT).

Malaria is transmitted when infected female *Anopheles* mosquitoes take their blood meal from their human host, usually between dusk and dawn. Of the more than 100 species of *Anopheles* that exist, only 50-60 species can transmit malaria infection. Malaria is rarely transmitted through transfusion of contaminated whole blood products (Kitchen et al., 2006), organ transplant (Mejia et al.), and congenitally from infected mother to fetus during pregnancy or delivery (Menendez et al.).

Malaria control, elimination, and eradication require effective interventions. At the community level, controlling vectors is a mainstay of reducing parasite transmission, and personal protection against bites of mosquitoes signifies the first line of defense for an individual against malaria. Vector control relies on two interventions: indoor residual spraying (IRS) and the use of insecticide-treated bed nets. The increased use of both interventions over the past decade has helped propel the decline of cases and deaths in some endemic regions, saving hundreds of thousands of lives. Other key interventions decreasing malaria burden that have been scaled up over the past decade include the use of ACT as the first-line therapy, intermittent preventive treatment (IPT) for pregnant women, early and improved diagnosis using rapid diagnostic tests (RDTs). But the global impact of these interventions on malaria is still not enough (Bhatt et al., 2015) and the burden of malaria remains high particularly in Africa (Mukonka et al., 2014).

An intervention we lack is a vaccine. An effective malaria vaccine that is well-tolerated will significantly reduce the morbidity and mortality associated with malaria when implemented along with these control interventions.

RTS,S/AS01 is the only malaria vaccine that has been approved by the European Medicines Agency and is now being used on a small scale in some regions in Ghana, Kenya, and Malawi (EMA, 2015). RTS.S/AS01 is a monovalent recombinant protein vaccine made by fusing 19 of the NANP repeats and the C-terminal of the CSP with hepatitis B surface antigen (HBsAg) (Casares et al., 2010; Gordon et al., 1995). AS01 is a novel adjuvant system that is administered with the vaccine.

Circumsporozoite protein (CSP) is abundant on the surface of the sporozoites. It consists of an amino (N)-terminal conserved region, a central repeat region (CRR) of 37-42 NANP repeats, and a carboxyl (C)-terminal region that is highly polymorphic (Pringle et al., 2018). The Th2R and Th3R sub-regions of the C-terminal are most polymorphic and elicit T-cell responses (Malik et al., 1991). The CRR region holds the immunogenic B-cell epitopes.

Anti-sporozoite vaccine development has long been of interest since when a study showed that an individual exposed to bites of irradiated infected Anopheles mosquitoes acquired protective immunity against malaria for more than 380 days (Clyde et al., 1973) and from observation of the unique immunogenic properties of CSP (Zavala et al., 1983). After many decades of research and experiment, the first human malaria vaccine (RTS,S/AS01) by GlaxoSmithKline (GSK) was licensed in 2015.

The RTS,S/AS01 vaccine efficacy in 5-17 months old children was modest at 36.3%. This intention-to-treat group received three doses of the vaccine on months 0, 1, and 2, and a booster dose on month 20 (RCTP, 2015). This represents a reduction from the 50.4% that was observed in a smaller phase 3 trial (RCTP, 2011). For infants, 6-12 weeks old, no significant efficacy against severe malaria was observed, even when a booster was administered.

Plasmodium falciparum circumsporozoite protein variation poses a serious threat to the success of this vaccine and has been implicated for the low vaccine efficacy. The gene encoding CSP

is globally diverse (Jalloh et al., 2009; Zeeshan et al., 2012) but the vaccine does not encompass this diversity and only contains one haplotype (3D7 strain). The vaccine has been shown to have greatest effect haplotypes most similar to 3D7 and according to Neafsey et al., (2015), the proportion of the CSP haplotype in the local parasite population that matches that target will determine the overall vaccine efficacy. The cause of CSP variation is not well understood; however, balancing selection has been thought to be a potential factor, whether it is driven by the host and/or vector (given CSP is expressed in both), remains an open question.

The human leukocyte antigens (HLA) regulate the human immune system. HLA genes are also highly polymorphic and HLA variation is well documented to contribute to vaccine response (Z.-K. Li et al., 2013; Posteraro et al., 2014). HLA haplotypes potentially influence how each individual responds to a vaccine (Moorthy et al., 2009). Nielsen et al., (2018) suggested that there was an association between HLA allele groups and protection mediated by RTS,S/AS01 across multiple Control Human Malaria Infection (CHMI) study as most of the alleles that were associated with the vaccine protection were less prevalent in people living in sub-Saharan Africa. Better powered studies are needed to determine these interactions in the African population.

1.2 Rationale

A detailed assessment of CSP variation in Ghana across time and space, particularly at the haplotypic level is lacking. Further, the interplay of HLA with CSP as well as RTS,S response, and efficacy has not been addressed in Africa. It is not known whether these variations impact the efficacy of the RTS,S/AS01 vaccine for malaria being rolled out in Ghana on a pilot basis. Examining the parasite and human together will allow us to determine these interactions as well as provide a baseline for vaccine effects on the parasite population in Ghana. It is important to define these CSP haplotypes/variations across space and time in Ghana, and the

association between HLA and CSP variants in infections to understand the mechanisms of action of this vaccine and to make a better judgment for future vaccine design.

1.3 Hypothesis

This study sought to test the hypothesis that the variability of CSP in Ghana is driven by host HLA haplotypes and specific HLA variants will demonstrate increased RTS,S-associated protection against malaria.

1.4 Aim

The main aim of this study was to better define the spatial and temporal distribution of CSP variants and their haplotypic combinations in Ghana. In addition, a comparison was made to the stability of the human HLA haplotype.

1.5 Specific Objectives

The specific objectives of this study included:

1. To map the spatial distribution of the parasite CSP haplotype within Ghana.
2. To determine the temporal stability of the CSP haplotypes within Ghana.
3. To compare CSP variants in Ghana to Kenya and Malawi.
4. To determine the contribution of HLA class II haplotypes to the control of CSP variants in Ghana.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Malaria

Malaria is a mosquito-borne and acute febrile disease caused by a protozoon of the genus *Plasmodium*. The disease is mostly endemic in the tropical and subtropical regions of the world and remains the major cause of sickness and death among children who are under 5 years old in these regions. Pregnant women in these regions are also at greater risk of losing their unborn babies or giving birth to premature or underweight babies due to malaria.

Five species of *Plasmodium* are known to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi*. Symptoms, which include fever, headache, chills, anorexia appear about a week after bites from infected female *Anopheles* mosquito, but incubation and symptomatology may vary depending on the causative species and host factors. Malaria has a good prognosis when it is diagnosed early and treated appropriately, but if not treated, can become complicated with anemia, convulsion, and death.

Severe malaria infection in children is usually in the form of severe anemia, metabolic acidosis with respiratory distress, or cerebral malaria. There can be a multi-organ failure in children and adults. These complications are the leading cause of malaria deaths amongst children. Prompt diagnosis and treatment with appropriate medication can revert severe cases of the disease and lead to a better outcome.

Artemisinin-based combination therapy (ACT) is the first recommended treatment for *P. falciparum* malaria in most malaria-endemic regions.

As people grow older in these malaria-endemic regions, they develop partial immunity against the disease, which allows asymptomatic infection to occur and they may only experience mild

infection (Doolan et al., 2009). But other population groups such as children under 5 years old, pregnant women, and people living with HIV/AIDS, as well as non-immune migrants and travelers from regions with no malaria transmission are at higher risk of contracting the disease (Cuadros et al., 2011; Desai et al., 2007; Semakula et al., 2016). These vulnerable groups should be highly prioritized in malaria control strategies.

2.2 Epidemiology

2.2.1 Malaria cases

Malaria is still a disease of public health concern. The World Health Organization (WHO) reported an estimated 228 million cases of clinical malaria globally in 2018 and the African region accounted for the highest number of estimated cases, that is, 213 million cases which constitute 93% (WHO. WMR, 2019). The Democratic Republic of the Congo (DRC), Côte d'Ivoire, Uganda, Mozambique, Nigeria, and Niger were responsible for half of the worldwide estimated cases.

According to the World Health Organization World Malaria Report of 2019, 85% of the worldwide malaria burden was shared by nineteen (19) sub-Saharan African countries and India. South-East Asia was responsible for 3.4% of the estimated cases in 2018, while the Eastern Mediterranean region was responsible for 2.1% of cases during the same period.

The number of malaria infections dropped from 71 to 57 cases per 1000 population worldwide between 2010 and 2018, but the decline has since stalled. When compared to 251 million cases in 2010 and 231 million estimated cases in 2017, the 228 million estimated cases in 2018 did not show a significant reduction in the number of yearly cases of malaria in the world. But the WHO indicated that 31 countries significantly reduced their incidence of malaria in endemic regions between 2015 and 2018 and that these countries could further reduce their incidence up to 40% or more by 2020.

2.2.2 Malaria deaths

Malaria kills about half a million persons every year. WHO estimated 405,000 malaria deaths globally in 2018 and 67% of these deaths occurred in under five (5) years old children. About 94% of the malaria deaths in 2018 occurred in sub-Saharan Africa. In Ghana, there were 29,517 deaths as a result of malaria in 2017 and under five-year-old children represented 54.6% of those deaths (Ghana NMCP, 2017).

Globally, there were fewer deaths in 2018 (405,000) compare to 585,000 estimated malaria deaths in 2010 and 416,000 in 2017. Although the WHO African region reported the highest number of malaria cases and deaths in 2018, the region also reported the largest decrease in the number of malaria-related deaths, that is, 533,000 deaths in 2010 to 380,000 in 2018 (WHO WMR 2019).

2.2.3 Distribution

Malaria transmission is still ongoing in 92 countries and over 100 countries are at risk of malaria transmission because more than 125 million tourists and international travelers visit these regions every year, according to the World Health Organization. The disease is highly confined to the tropical regions of the world (Figure 1), and children under 5 years old, pregnant women, and immunocompromised individuals living in these regions, as well as travelers from countries with no malaria transmission are at greater risk of acquiring malaria. Since travelers from no transmission areas have never been exposed to malaria and have no malaria immunity, the consequences of getting infected with malaria are more severe; they need to be given prophylactic drugs ahead of their visits to these regions with active malaria transmission.

The World Health Organization divided malaria transmission zones into five (5) regions: Africa, South-East Asia, Eastern Mediterranean, Europe, and Western Pacific. Among these regions, Africa reported 93% (213 million) of the cases in 2018. This is in part due to the

presence of the most efficient vector (*Anopheles gambiae*), which is associated with high malaria transmission in the region. Also, *Plasmodium falciparum* which causes the most complicated form of the disease resulted in 99.7% of estimated cases in 2018 and is the predominant species in the WHO African region (WHO. WMR, 2019). Most of sub-Saharan Africa has weather conditions that promote transmission all year round, and socio-economic instability and insecurity make it more difficult to effect efficient malaria control interventions and policies.

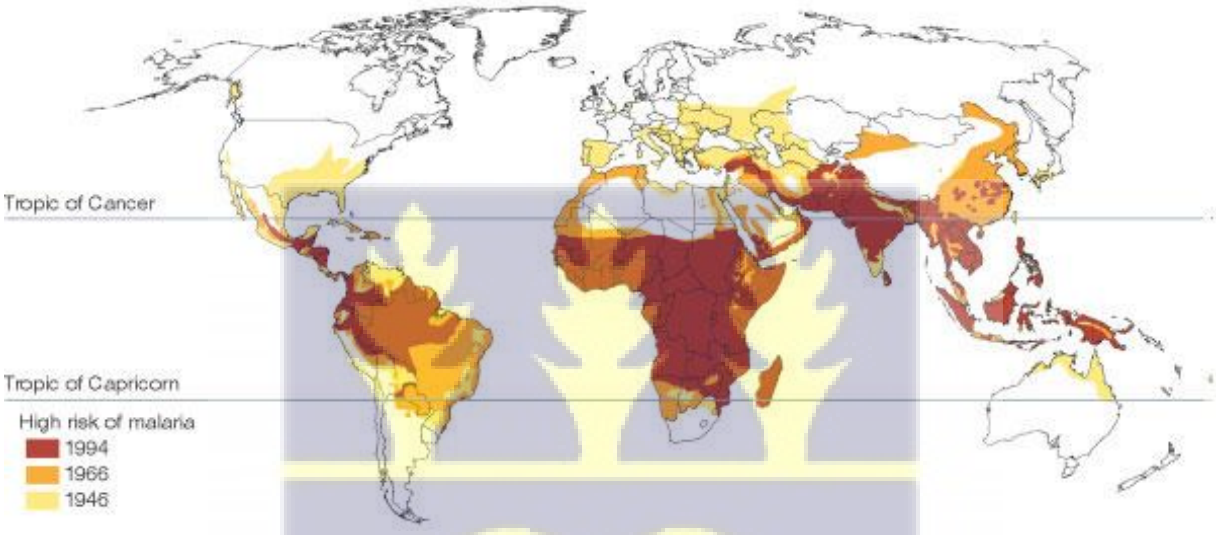


Figure 1: The global distribution of malaria

Malaria has become a disease of the tropical regions from 1946 to 1994. Image adapted from Sachs et al., (2002)

The WHO South-East Asia region is the second-largest infected region, accounting for 3.4% of malaria morbidity in 2018. According to the WHO, India bears the majority of the burden of the disease in South-East Asia, and *P. vivax* is the predominant species, accounting for 50% of infectivity. The WHO Eastern Mediterranean Region reported 2.1% of malaria cases in 2018 and *P. vivax* was responsible for 29% of cases.

2.3 Malaria Burden

Africa continues to bear a disproportionately high burden of malaria infection every year, as 93% of morbidity and 94% of mortality due to malaria occurred in Africa in 2018. According to WHO reports of 2017 and 2018, 11 countries bore the highest burden of the disease and were offtrack to achieve the 2025 Global Technical Strategy (GTS) milestones. These countries include 10 African countries (Burkina Faso, Cameroon, the Democratic Republic of the Congo, Ghana, Mali, Mozambique, Niger, Nigeria, Uganda, and Tanzania) and India. In November 2018, WHO launched the high burden high impact (HBHI) approach in Mozambique to mobilize governments to coordinate malaria responses and help reduce the impact of malaria, drive strategic information, and better guide policies and strategies. Most of these countries are among the poorest in the world (Figure 2).

Malaria imposes a substantial social and economic burden on households and governments of affected regions. Household expenditure includes the out-of-pocket purchase of antimalarial drugs for treatment, fare to a health center, consultation and laboratory fees, purchase of insecticide for IRS, absence from school or work, and the cost to bury the dead. The costs on governments include keeping the health system functional by ensuring staffing, drug and supplies, maintenance, and control interventions are maintained.

Despite malaria services being provided for free in many regions, households still incur high direct and indirect costs. A study by Alonso et al., (2019) found that the median household costs among individuals of all ages in Mopeia, Mozambique were US$3.46 and US$81.08 per uncomplicated and complicated malaria, respectively, and the economic malaria burden per year in Mopeia was US$332,286.24.

The toll is also high on businesses; about US$6.58 million was lost to malaria by businesses in Ghana in 2014, of which 90% were direct costs (Nonvignon et al., 2016). Businesses in Ghana

lost a total of 3913 workdays to malaria from 2012-2014. Lack of education, low income, low wealth, living in poorly constructed houses, and occupation in farming may increase risk of malaria in Sub-Saharan Africa, and strong public policy is needed to reduce inequalities in health coverage, improve economic and educational opportunities for the poor to improve socioeconomic status and reduce the burden of malaria (Degarege et al., 2019)

According to WHO, total funding for malaria control and elimination in 2020 was estimated at US$ 3.3 billion, compared with US$ 3.0 billion in 2019 and US$ 2.7 billion in 2018. This amount in 2020 did not meet the expected US$ 6.8 billion estimated to stay on track towards the GTS milestones (WHO. 2021).

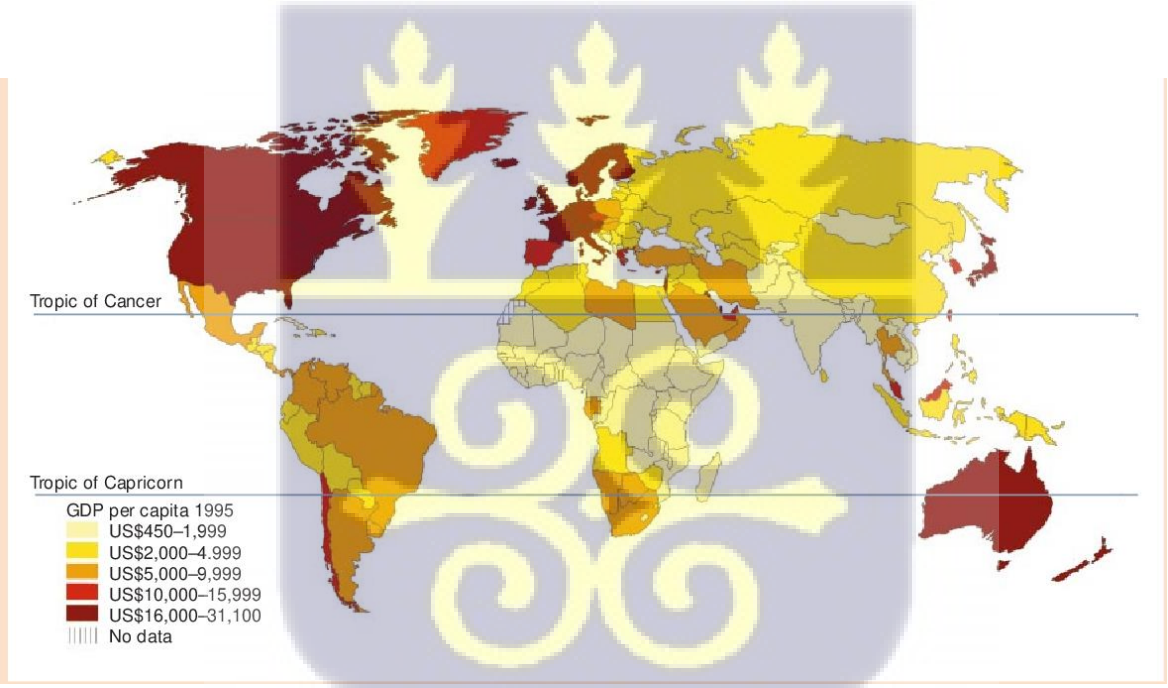


Figure 2: GDP per capita

Income is unevenly distributed and countries in tropical regions are the poorest. Image adapted from Sachs et al., (2002)

2.4 *Plasmodium* Species

Plasmodium is the causative agent of malaria. The parasites are transmitted between an insect host and a vertebrate host for their life cycle to be completed. Some species infect a single vertebrate and insect host, others infect multiple vertebrate and insect hosts. Five species cause clinical malaria in humans: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi.*

2.4.1 Plasmodium falciparum

Plasmodium falciparum is by far the deadliest species and the cause of malignant tertian malaria. It destroys red blood cells and causes acute anemia, especially in children. It is most prevalent in sub-Saharan Africa and accounted for 99.7% of the malaria infection in this region in 2018 (WHO. WMR, 2019).

2.4.2 Plasmodium vivax

Plasmodium vivax is most prevalent in South-East Asia and Latin America and mostly causes benign tertian malaria. Compare to *P. falciparum*, it is the most widespread geographically but the second most important species because its symptoms are less severe. *Plasmodium vivax* and *P. ovale* can become dormant in the liver, as hypnozoites, and lie in hepatocytes for months or years, and become reactivated in the absence of mosquito bites, causing clinical symptoms (White, 2011). The mechanisms that lead to their reactivation are not fully understood. *Plasmodium vivax* was responsible for 75% of malaria cases in the Americas, a third of the cases in WHO Western Pacific, 18% of cases in WHO Eastern Mediterranean, and a third of the cases in WHO South-East Asia in 2020 (WHO. 2021).

2.4.3 Plasmodium ovale

Plasmodium ovale is less frequent and the cause of benign tertian malaria. Along with *P. malariae,* they represent only a small percentage of malaria infection worldwide. Although rare, it can cause relapses (CDC., 2005) and generally occurs in sub-Saharan Africa.

2.4.4 Plasmodium malariae

Plasmodium malariae is the cause of benign quartan malaria. It causes fewer cases and less severe forms of malaria, but it is still significant. It can persist in the blood for a very long time without producing symptoms. It is responsible for the asymptomatic spread of malaria infection through mosquito bites and blood donation. *P. malariae* can be found in sub-Saharan Africa.

2.4.5 Plasmodium knowlesi

Plasmodium knowlesi is a major cause of human malaria in the forest region of Southeast Asia, resident to the macaques who are the natural host of the parasite. *Plasmodium knowlesi* was thought to only infect monkeys until Chin et al., (1968) demonstrated its transmission from monkeys to humans. The parasite is no longer rare in humans as was previously thought because identification by nested PCR conclusively showed that *P. knowlesi* infection had been misidentified as *P. malariae* (Cox-Singh et al., 2008; B. Singh et al., 2004). *P. knowlesi* can also cause severe malaria disease.

2.5 Genome

The malaria genome is composed of about 23-27 megabases (Mb) that are arranged among 14 chromosomes that range from 0.643 to 3.29 Mb (Gardner et al., 2002). There are about 5,500 genes organized in 36 synteny blocks (Kooij et al., 2005), most of whose protein functions are unknown. The genome is composed of 80.6% (A + T) and is even higher (approximately 90%) in introns and intergenic regions.

The mean length of *P. falciparum* genes is 23 Mb, excluding introns, which is substantially larger than other organisms. The majority of these genes encode proteins that may be cytosolic proteins with signal peptides that are unknown (Gardner et al., 2002).

The transfer RNA (tRNA) genes are contained in the nuclear genome and exhibit minimal redundancy in *P. falciparum*. The nonnuclear genomes are found in the mitochondrion and apicoplast. The mitochondrial genome does not encode tRNAs, so they must be imported from the cytoplasm. It is about 6 kb. Unlike the mitochondrial genome, the apicoplast genome encodes sufficient tRNAs for protein synthesis. It is about 35 kb in size (Denny et al., 1996; Vaidya et al., 1989).

The ribosomal RNA (rRNA) genes of *Plasmodium* species are not long arrays of repeated tandem; they are distributed on different chromosomes as single 18S-5.8S-28S rRNA units (Gardner et al., 2002). The rRNA units are different from one another and different sets are expressed at different stages of the *Plasmodium* life cycle (J. Li et al., 1994; Waters, 1994) . There are two types of rRNA genes in *Plasmodium*: the A-type is expressed in the human host while the S-type is expressed in the mosquito vector (Gardner et al., 2002).

2.6 Transmission

Malaria is transmitted when an infected female *Anopheles* mosquito feeds on its human host between dusk and dawn. The mosquito injects the parasite in a form known as sporozoite in the dermis of the human hosts (Sidjanski et al., 1997). The sporozoites enter the bloodstream and go to the liver to begin the hepatic or pre-erythrocytic stage of their life cycle. People can also contribute to malaria transmission by moving from one place to another (Prothero, 1977), and this is leading to widespread drug resistance in malaria (Rajagopalan et al., 1986).

Malaria may also be transmitted through the transfusion of malaria-infected whole blood. This is very common in high transmission areas where blood donors are not properly screened or

not screened at all for malaria. Malaria can also be transmitted rarely through organ transplants and congenitally from pregnant mothers to their babies during delivery.

Malaria transmission is high in areas where mosquitoes live longer and feed on humans for their egg production. Transmission in sub-Saharan African is the highest in the world, with approximately 90% of global cases, because the vectors in this region have a strong habit of feeding on humans and a longer lifespan.

Also, human immunity tends to play a role in influencing malaria transmission. Adults living in areas with moderate or intense malaria transmission develop partial immunity from repeated exposure to malaria parasites over a long period (Doolan et al., 2009). These adults are at reduced risk of infection and they become asymptomatic carriers of matured gametocytes which are picked up when mosquitoes take their blood meal.

Climatic conditions such as rainfall patterns, temperature, and humidity also favor malaria transmission. In places with seasonal malaria transmission, malaria cases peak during and after the rainy season. In some parts of Africa, there is a year-round transmission of malaria (Hellmann et al., 2011).

2.6.1 Asymptomatic transmission

In areas with high malaria transmission, varying population with repeated exposures to *Plasmodium* parasites becomes asymptomatic carriers who are reservoirs of low parasitemia and contribute to the persistence of malaria transmission. These repeated exposures give this population group partial immunity against malaria and limit parasite density and symptoms but do not necessarily prevent infection (Hviid, 2005). These asymptomatic carriers harbor matured gametocytes in their peripheral blood circulation and are responsible for infecting mosquitoes with matured gametocytes and viciously continuing the transmission of the parasite. This low-density parasitemia in the absence of clinical symptoms is detected by

molecular diagnostic techniques (Golassa et al., 2013), and not by conventional microscopic techniques or rapid diagnostic tests (RDTs).

The gametocyte is the form of the parasite that is transmitted from an infected human host to the mosquito when the mosquito sucks human blood. The transmission of gametocytes depends on the density of mature gametocytes circulating in peripheral blood. A study in Cameroon, Gambia, Mali, and Senegal found that approximately 5% of mosquitoes are infected by 27.6% of asymptomatic gametocyte carriers (Bousema et al., 2012). The high prevalence of these carriers in malaria-endemic areas suggests their contribution to human asymptomatic transmission and there is a need for interventions to halt human-mosquito transmission (Churcher et al., 2013).

2.7 Vectors

Human blood-sucking mosquitoes are the vectors of malaria. Those belonging to the genera *Anopheles, Culex, Culiseta, Mansonia,* and *Aedes* are the main vectors of *Plasmodium*. They transmit *Plasmodium* to mammals and birds, but only *Anopheles* can transmit malaria to humans. *Anopheles* is the best-studied mosquito, with about 465 species recognized. Of those 465 species, 70 cause human malaria, and 41 species can transmit severe human malaria infection (Sinka et al., 2012).

Anopheles mosquitoes are distributed worldwide, although they are most found in tropical and subtropical regions (Figure 3). Female *Anopheles* mosquitoes feed nearly exclusively on human blood and are the ones that are infected with *Plasmodium* (Crompton et al., 2014). The *Anopheles* genus is divided into six subgenera, of which four causes disease. *Anopheles gambiae* and *An. funestus* are two main species that transmit human malaria in Africa. *Anopheles amharicus, An. arabiensis, An. bwambae, An. gambiae, An. coluzzii, An. melas,* and *An. merus* are almost indistinguishable and make up the *An. gambiae* complex of sibling

species found in tropical Africa (Coetzee et al., 2013). None of these species are found in North America.

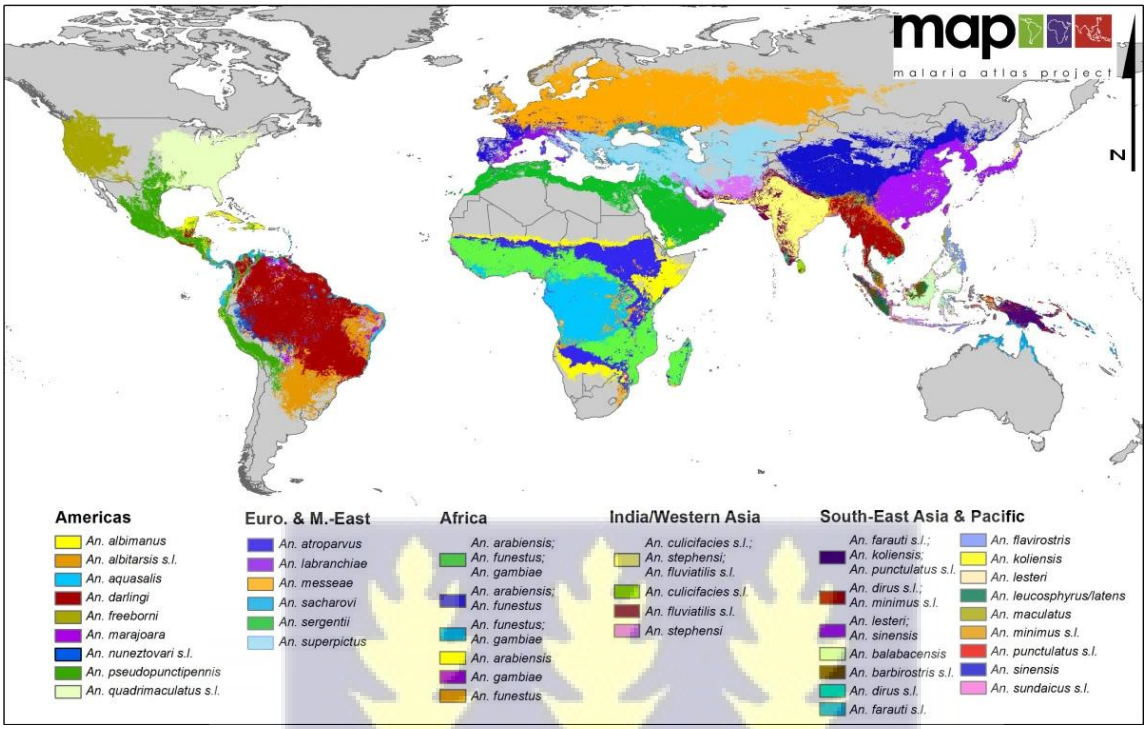


Figure 3: Distribution of Anopheles mosquitoes

Image adapted from Sinka et al., (2012)

While it is true that a range of intrinsic physiological and biological processes are responsible for the natural susceptibility of malaria infection in a particular mosquito species, these processes are not very well understood. However, factors like the feeding habit and lifespan of the species, as well as the population density of mosquitoes to humans are all important determinants of the transmission potential of a species (Warrell et al., 2017). Mosquitoes undergo four distinct metamorphic stages during development - egg, larva, pupa, and adult. The early and immature stages depend on free water for their development and survival.

2.8 Diagnosis and treatment

Early diagnosis and treatment are two important elements in the fight to reduce morbidity and mortality. The "WHO Guidelines for the treatment of malaria" recommends that people who are suspected of having malaria should have a malaria smear done by microscopy or malaria rapid diagnostic test (RDT) to confirm the diagnosis before they are given antimalarial treatment (WHO, 2015). However, treatment can be given based on symptoms when malaria diagnosis is not available. Artemisinin-based combination therapy (ACT) is considered the drug of choice for uncomplicated *P. falciparum* malaria. Severe or complicated cases of malaria require hospitalization and treatment with parenteral artesunate for at least 24 hours. Once the patient can orally tolerate medication, treatment must be completed with three (3) day of ACT. If parenteral artesunate is not available, parenteral artemether is preferred to quinine for treating complicated malaria.

2.9 Malaria Life Cycle

The life cycle of *Plasmodium* takes place in two hosts, the human host, and female *Anopheles* mosquito (vector) host, and without one host the cycle will not be completed (Figure 4). The parasite goes through two (2) reproductive stages: the sexual stage occurs in the mosquito and the asexual reproductive stage in the human host. For malaria transmission to continue, the parasite must complete these two reproductive stages of its life cycle.

The origin of malaria infection in humans is when an infected female *Anopheles* mosquito injects sporozoites into the skin of the human host while feeding (Sidjanski et al., 1997). The injected sporozoites glide their way through the skin to reach nearby blood circulation by chemotaxis, and through a process known as transmigration, make their way to the liver by blood flow to avoid phagocytosis and destruction by non-phagocytic cells (Amino et al., 2008). But not all of the sporozoites inoculated into the skin get to the liver; only 35% transmigrate to

the liver. About 50% of them are phagocytized in the skin and 15% become trapped and phagocytized by dendritic cells in the nodes of the lymphatic system (Sturm et al., 2006).

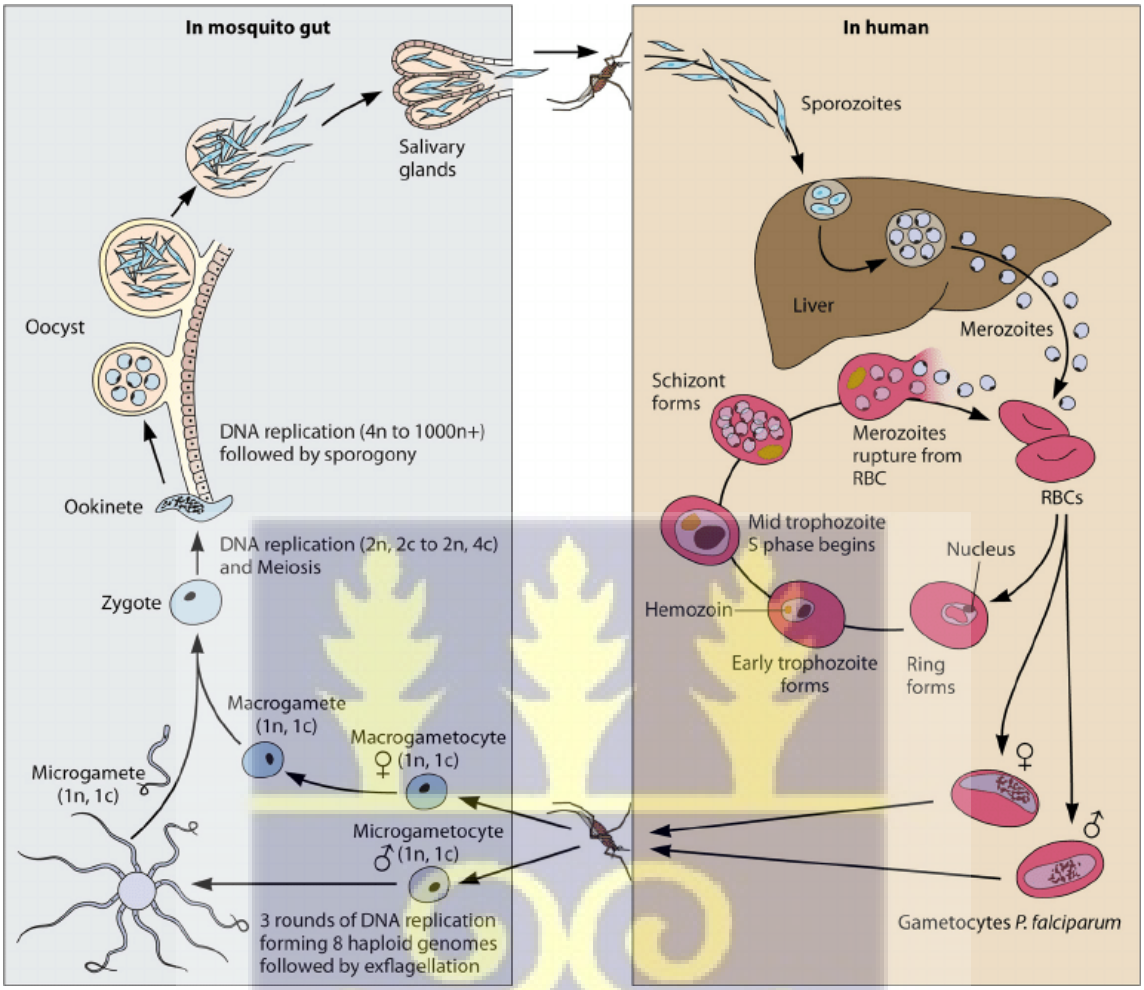


Figure 4: The malaria life cycle

Human malaria infection begins when an infected female Anopheles mosquito with sporozoites in its salivary gland (left) injects those sporozoites into humans while taking blood meal (right). The sporozoites infect the liver and red blood cells, before transforming into gametocytes that can be transmitted to another mosquito. The Parasites replicate sexually in the mosquito and can be injected into another human host when the mosquito feeds. Image adapted from A. H. Lee et al., (2014).

When they reach the liver, the sporozoites use their circumsporozoite proteins (CSP) to attach to heparin sulfate proteoglycan (HSPG) molecules on the surfaces of hepatocytes through tiny channels called fenestrae (Ménard et al., 2013). This triggers calcium-dependent protein kinase

6 (CDPK-6) and other kinase signaling cascades in the sporozoites. CSP then cleaved as the result of progressive proteolytic signaling events, thereby exposing the adhesive thrombospondin repeat (TSR) which is used to attach to the sinusoidal endothelium (Coppi et al., 2011). The interactions between the CSP and liver-specific glycosaminoglycans cause the plasma membrane of hepatocytes to invaginate at the entry point (Pradel et al., 2001). This invagination leads to the formation of the parasitophorous vacuole membrane (PVM), a highly permeable compartment with a vital supply of host nutrients that supports the growth of the parasites in the cytoplasm of the hepatocytes, close to the host nucleus, endoplasmic reticulum (ER) and mitochondria (Bano et al., 2007). The parasites rapidly differentiate to a round trophozoite in the hepatocyte (Jayabalasingham et al., 2010). They then develop into multi-nucleated liver schizonts approximately 20 hours post-invasion and replicate asexually, inhibiting apoptosis of the host cells while they develop (Van De Sand et al., 2005).

After about one to two weeks of development in the liver, the parasites initiate a signaling cascade that eventually leads to atypical apoptosis of the host cells, releasing merosomes, a merozoite-filled vesicle. One infected hepatocyte can contain as much as 40,000 merozoites (Miller et al., 2013). The merosomes quickly go to the capillaries of the lungs and rupture, releasing merozoites into the peripheral circulation, and this marks the beginning of the blood stage (Baer et al., 2007). The liver stage starts from the invasion of hepatocytes up to when merozoites enter into the bloodstream. Depending on the species, the liver stage might take a week to years; *P. falciparum* takes up to 10 days, *P. malariae* up to 15 days, while *P. vivax* and *P. ovale* hypnozoites can take months to years, resulting in relapsing human malaria (Richter et al., 2010).

The blood stage involves the invasion and destruction of red blood cells and causes all associated symptoms of clinical malaria. Merozoites that are released into blood circulation are programmed to invade RBCs. They attach and invade over 20% of circulating red blood cells,

growing and dividing rapidly, and destroying the blood cells in the process. Parasitized RBCs rupture and release more merozoites. They release diverse antigenic surface proteins on the surfaces of these RBCs which reorientate the RBCs and cause them to stick together or adhere to the inner surfaces of the blood vessels.

Hundreds of proteins mediated the attachment of merozoites to RBCs. Some of these proteins include merozoite surface proteins (MSP), erythrocyte binding-like (EBL) proteins, *P. falciparum* reticulocyte binding-like (PfRh) proteins (Cowman et al., 2006; Tham et al., 2012). When merozoites attach, they reorient themselves so that their apical end is on the red blood cell plasma membrane, forming a tight junction. Once the parasite has entered, the membrane is sealed and the processes aiding the invasion are immediately terminated. It takes about 27.6 seconds after the parasite makes contact to enter into the red cell (Gilson et al., 2009).

Inside the red cell, the merozoite is encapsulated in a parasitophorous vacuole membrane (PVM), which originates from the plasma membrane of the red blood cell but is changed by lipid material secreted by the rhoptries (Dluzewski et al., 1992). The PVM is useful for nutrient uptake and is the channel for the export of unfolded proteins into the host cytoplasm (Gehde et al., 2009). The parasite uses the PVM to hide from the host immune response while it asexually replicates.

It takes 24-72 hours from the invasion and rupture of a red blood cell to the reinvasion of another red blood cell, depending on the species of *Plasmodium*. *Plasmodium knowlesi* takes 24 hours, *P. falciparum*, *P. vivax*, and *P. ovale* take 48 hours, and *P. malariae* takes 72 hours. A merozoite can go from the ring to the trophozoite stage, and then replicate 8-32 daughter merozoites during schizogony.

Some merozoites produce sexual progeny that then goes on to become male and female gametocytes. Gametocytogenesis takes place between 8-10 days in the extravascular space of

the bone marrow, gametocytes can mature in other parts of the human body (for example, the spleen). After maturing, the male and female gametocytes get back into the peripheral circulation, and will only mate when they enter the gut of the mosquitoes.

When the mosquito ingests the male and female gametocytes while feeding on an infected human host, they mature quickly and form gametes, by a process called gametogenesis; each male gametocyte differentiates into eight microgametes and each female gametocyte becomes a macrogamete (Carter et al., 1977). The macrogamete and microgamete mate to produce a zygote, which then develops into an ookinete. The ookinete develops into oocyst which will asexually replicate to form thousands of sporozoites by sporogony. When the oocyst ruptures, the sporozoites move to the mosquito salivary glands, waiting to be transmitted to the human host when the infected mosquito takes a blood meal.

2.10 Malaria Control Interventions

Several malaria control interventions have been scaled up over the past decades to reduce malaria morbidity and mortality. They include distributing insecticide-treated bed nets, scaling up indoor residual spraying (IRS), diagnosing and treating early-onset clinical malaria with ACT, and initiating intermittent preventive treatment in pregnancy (IPTp) (Sutcliffe et al., 2012).

According to WHO, 50% of people at risk of malaria infection in sub-Saharan Africa are now sleeping under an insecticide-treated net (ITN), which protected 50% of the population in 2018 as compared to 29% in 2010. IPTp coverage for eligible pregnant women increased to 31% in 2018, compared to 22% in 2017. Although 19 million children were protected through seasonal malaria chemoprevention (SMC) programs in 2018, 12 million more who could have benefitted were not covered because of lack of funding. Also, protection provided by indoor residual

spraying dropped from a peak of 5% in 2010 to 2% in 2019, according to the WHO World Malaria Report of 2019.

Despite the progress in scaling-up interventions to reduce malaria morbidity and mortality, a high burden of the disease is still reported in other regions and this is threatening gains made in other regions (Karema et al., 2012; Kouyate et al., 2007).

2.10.1 Vector control

Controlling malaria vectors is important for malaria prevention and should target mosquitoes that have the capacity of transmitting the parasite. It is an effective way of interrupting and reducing malaria transmission, especially in high transmission areas. According to WHO, there are 2 main ways to control malaria vectors: using insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS). You can supplement these two vectors' control mechanisms by managing the sources of larvae and using personal protection.

Insecticide-treated nets provide physical protection and a barrier against insects and reduce contact with mosquitoes. WHO claimed that since 2000, nearly 2 billion ITNs have been delivered to countries with high malaria transmission, thereby increasing the proportion of children under the age of 5 years old who sleep under ITNs in sub-Saharan Africa from about 2% in 2000 to 61.2% in 2018 (WHO. WMR, 2019). According to WHO, the incidence of malaria has dropped over the past decade because of the effectiveness of ITNs as a control strategy and ease of use, and its high population coverage protected about 50% of high-risk individuals in Africa from malaria in 2018, compared to 29% in 2010.

Indoor residual spraying (IRS) is spraying the inside of a house with an insecticide. This is another powerful and effective way of rapidly reducing malaria transmission. Notwithstanding, WHO reported that protection against malaria by IRS reduced to 3% in 2017 from 5% in 2010. This could be due to the introduction of more expensive pyrethroids. Pyrethroids,

organochlorines, carbamates, organophosphates, and neonicotinoids are the 5 classes of IRS recommended by the WHO. These insecticides are prequalified by the WHO to ensure they meet global standards of safety, quality, and efficacy. Dichlorodiphenyltrichloroethane (DDT) is not prequalified by WHO for use.

Malaria control and elimination programs must prioritize the delivery of either ITNs and IRS at high coverage for all their population in malaria-endemic countries. Vector control involves the collection of data on local vector species periodically to study their susceptibility to insecticides and understand their behaviors. All countries should ensure the gains made in controlling malaria vectors are not derailed by emerging insecticide resistance.

2.10.2 Antimalarial drugs as a control intervention

Antimalarial drugs are also used to prevent and control malaria infection. Chemoprophylaxis can prevent malaria infection by suppressing the blood stage of the parasite life cycle. Travelers from regions with no or low malaria transmission are given chemoprophylaxis as a way of protecting them from contracting the disease. The World Health Organization recommends that sulfadoxine-pyrimethamine be given to pregnant women who are in their second and third trimesters as intermittent preventive treatment in areas where malaria transmission is moderate to high. Also, three doses of sulfadoxine-pyrimethamine are recommended for infants in high transmission areas in Africa, to be administered with routine vaccinations.

As a prevention strategy, WHO also recommends that monthly amodiaquine plus sulfadoxine-pyrimethamine should be administered to all children in the Sahel sub-region of Africa during high transmission season as chemoprevention therapy.

2.10.3 Malaria Elimination and Eradication

According to the World Health Organization, malaria elimination is the interruption of local transmission of a specific malaria parasitological species in a defined geographical area while

malaria eradication is permanently reducing the incidence of malaria infection to zero worldwide. Both elimination and eradication require deliberate actions, but once eradication is realized, interventions are no more necessary.

A lot of countries are moving toward eliminating malaria. More countries are now reporting less than 100 indigenous cases of malaria. According to WHO, the total number of malaria endemic countries that reported fewer than 10,000 cases increased from 26 in 2000 to 47 in 2020, while those reporting fewer than 100 indigenous cases also increased from 6 to 26 during the same period (WHO. 2021). A country can apply to WHO for a malaria elimination certificate when it reports no indigenous cases of malaria for at least 3 consecutive years. The World Health Organization has certified 10 countries over the last decade for eliminating malaria.

Malaria eradication is still important for the global public. It became a global strategy with the launch of the Global Malaria Eradication Program (GMEP) by WHO in 1955. At the time, dichlorodiphenyltrichloroethane (DDT) was presented as a transformative tool and effective insecticide to eradicate malaria, but it failed when it was not very effective in every part of the world and there was re-emergence of malaria after long periods of no transmission in some areas. Although GMEP eradicated malaria in many regions of the world, the program was ended in 1969 after its primary objective failed (Nájera et al., 2011).

At the African Summit on Roll Back Malaria (RBM) in April 2000, a renewed call to eliminate malaria was made. Heads of 44 malaria-endemic countries, along with international partners committed to reducing malaria infection and deaths by half in Africa by 2010. This RBM initiative was aimed at ensuring that at least 60% of people in Africa got malaria prevention and access to treatment.

2.11 Vaccine for Malaria

Although there have been gains in controlling vectors since the 2000s, about half a million people are still dying from malaria yearly. This death toll is still significantly high and is felt mostly in sub-Saharan Africa (WHO. 2021).

Vaccination is an effective way of preventing and controlling infectious diseases. Vaccines have been successfully used to eliminate the health burden posed by smallpox, polio, and measles and improve economies (Fenner, 1989; Rivière et al., 1997; Tebbens et al., 2010). An effective malaria vaccine that can be used alongside current control interventions will most likely reduce the death toll and hence, boost the fight to eliminate and eradicate malaria. A vaccine aims to induce protective memory immune responses in individuals before they can encounter the disease-causing agent. This acquired immunity is specific to the agent against which it is developed and it prevents infection.

It has taken more than three decades to develop an effective vaccine against malaria. Many factors have made the development of a malaria vaccine challenging: the complex parasite life cycle, immune evasion mechanism of the parasite, lack of sterile immunity against the parasite, among others.

The complexity of the life cycle is a challenge to the development of a malaria vaccine. The cycle composes of three stages: pre-erythrocytic, erythrocytic, and sexual. The pre-erythrocytic stage is the liver stage (the stage when the sporozoites infect hepatocytes). The erythrocytic stage is when the merozoites infect red blood cells and multiply. The sexual stage takes place in the mosquito gut after it has taken up gametocytes from infected human blood while feeding. During each stage, the parasite changes its form and mechanism of host invasion. Researchers have been studying to understand and determine the stage of the life cycle that an effective vaccine can target - whether one or more stages of the cycle (Theisen et al., 2014). Because of

this complexity, some scientists think a multistage vaccine will be a better candidate than a vaccine that targets only one stage.

Antigenic variation is one immune evasion strategy a lot of parasites use for survival. Some of them randomly mutate exposed antigens that have become the targets of the host defense mechanism, and by so doing create an unsurmountable invasion of the host. Others regularly replace targeted antigens with new variants that make it difficult for the host to effectively clear them. Malaria parasites are constantly using antigenic variation to escape the host immune response against them. One way they evade their host immune response is by expressing Plasmodium falciparum Erythrocyte Membrane Protein-1 (PfEMP-1) on the surfaces of infected red blood cells to stick on the endothelium of blood vessels to avoid splenic destruction. PfEMP-1 is a polymorphic protein and encoded by the var gene. Each parasite expresses a single var gene at a time and maintains the other copies. When the host antibody responds to the expressed PfEMP-1, sub-populations of the parasites will switch to another form of the gene and continue to infect the host. PfEMP-1 is important for the *Plasmodium falciparum* evasion mechanism (Pasternak et al., 2009) and antigenic variability results in diverse parasite populations (Kirkman et al., 2012), posing sufficient challenge to malaria vaccine development.

Another major challenge is that malaria does not confer sterile immunity in humans. Unlike other infectious diseases, a person who gets infected with malaria is not prevented from future infection. But there is evidence of some degree of naturally acquired immunity in people who live in malaria-endemic areas after they are repeatedly infected (Hviid, 2005). However, this naturally acquired immunity only lessens the severity of the infection and does not prevent it. Natural immunity against malaria varies with age and is reduced in pregnancy (Desai et al., 2007). People who traveled from malaria-endemic region to areas with no transmission and stay there for a long time tend to lose their natural immunity.

The Malaria Vaccine Technology Roadmap (MVTRM) has led the initiative to develop a vaccine against malaria from 2006 till now. The main goal was to develop a malaria vaccine (mainly against *P. falciparum*) that is more than 50% efficacious against the severe form of the disease, with immunity lasting more than a year. The goal later included developing vaccines against *P. vivax* and transmission-blocking vaccines (also known as vaccines that interrupt malaria transmission, VIMTs). Not much research has been done on VIMTs to date, and a study by Miura et al., (2014) suggested that RTS,S does not inhibit the formation of sporozoites.

Malaria vaccine development strategies target different forms of *Plasmodium* species in the life cycle: sporozoite, merozoite, and gametocyte. Some of the recent vaccine candidates targeting the sporozoites, merozoites, and gametocytes are summarized in Table 1.

2.11.1 Vaccines targeting sporozoites

The sporozoites, which are transmitted by mosquitoes, infect hepatocytes, and develop into thousands of merozoites. Vaccines that target sporozoites or infected hepatocytes prevent or impair infection. The PfSPZ vaccine (radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* [Pf] sporozoites [SPZ]), induced sterilizing immunity in six adults in Tanzania against controlled human malaria infection (CHMI), and appeared safe in African children (Jongo et al., 2020; Steinhardt et al., 2020).

The RTS,S vaccine, a subunit vaccine based on a truncated form of the circumsporozoite protein (CSP), expressed as a virus-like particle, and administered with an adjuvant system (AS01) is the most successful so far. A phase 3 clinical trial in many African sites resulted in a pilot implementation of a four-dose booster regimen in 5-month old children in Ghana, Kenya, and Malawi to further assess safety and efficacy (RCTP, 2011).

The R21 malaria vaccine, similar to RTS,S, is expressed as a virus-like particle but with higher density of CSP antigen and Matrix-M adjuvant. R21 demonstrated 76% vaccine efficacy against malaria in children (5-17 months) over 12 months in a phase 2 clinical trial in Nanoro, Burkina Faso (Datoo et al., 2021). A phase 3 trial is currently ongoing at multiple sites in Africa.

2.11.2 Vaccines targeting merozoites

Malaria vaccines that target merozoites or infected red blood cells aim to prevent clinical illness and control parasitemia. Developing vaccines against merozoites have been challenging, because they present multiple antigens, interactions, and events during the invasion of red blood cells and most vaccine candidates evaluated in phase 2 clinical trials have not further progressed (Beeson et al., 2016).

The RH5.1 vaccine, consisting of the full-length RH5 protein with AS01 adjuvant was found to be safe, well tolerated, and highly immunogenic in malaria-naïve volunteers but showed limited efficacy (Minassian et al., 2021).

2.11.3 Vaccines that block malaria transmission

Some merozoites can develop into gametocytes and be picked up by mosquito during feeding and be transmitted. Transmission-blocking vaccines target the gametocytes by preventing their transmission to the mosquito and, hence, blocking malaria transmission to the population. These vaccines will be important components of effective control and malaria elimination strategies.

Although much progress has not been made in developing transmission-blocking vaccines, one candidate, Pfs230 has been promising in early clinical trials. Pfs230, also Pfs230D1M, is based on the gametocyte surface antigen. Phase 2 clinical trial is ongoing in Mali (Healy et al., 2021).

Table 1 Summary of recent and ongoing malaria vaccine clinical trials. Adopted from Kurtovic et al., (2021)

Vaccine	Dosage	Phase; trial no.	Population	Outcome; follow-up	Reference
Vaccine targeting sporozoites					
PfSPZ	3 doses of $9x10^5$	1; NCT02613520	18-45 years, Tanzania (n=6)	100% efficacy against CHMI at 23-79 days	(Jongo et al., 2020)
	3 doses of $9x10^5$	1; NCT02687373	5-12 months, Kenya (n=8)	Safe and immunogenic over 29 days	(Steinhardt et al., 2020)
PfSPZ-CVas	2 doses of $2x10^5$ with chloroquine	1; NCT03083847	Malaria-naïve adults (n=6)	100% efficacy against CHMI at 3 months	(Mwakingwe-Omari et al., 2021)
GAP3KO	Bites from 200 infected mosquitoes	1; NCT03168854	Malaria-naïve adults (n=16)	Vaccine efficacy pending (unpublished)	NA
RTS,S	3 doses of 25µg	3; NCT00866619	5-17 months, multiple African sites (n=4296)	56% efficacy against first or only malaria episode over 12 months	(RCTP, 2011)
	2 doses of 50 µg, 1 delayed dose of 10 µg	2; NCT01857869	Malaria-naïve adults (n=30)	87% efficacy against CHMI at 3 weeks	(Regules et al., 2016)
	2 doses of 25 µg, 1 delayed dose of 5 µg	2; NCT03276962	5-17 months; Ghana and Kenya	Vaccine efficacy pending (unpublished)	NA
	RTS,S with seasonal malaria chemoprevention	3; NCT03143218	5-17 months; Burkina Faso and Mali	Vaccine efficacy pending (unpublished)	NA
R21	3 doses of 5 µg	2; NCT03896724	5-17 months; Burkina Faso (n=146)	71-76% efficacy against at least one malaria episode over 12 months	(Datoo et al., 2021)

				(depending on adjuvant dosage)	
	3 doses of 5 µg	3; NCT04704830	5-36 months; multiple African sites	Vaccine efficacy pending (unpublished)	NA
Vaccines targeting blood stages					
RH5.1	3 doses of 10 µg	2; NCT02927145	Malaria-naïve adults (n=14)	1-day delay in parasitemia following CHMI at 14 days	(Minassian et al., 2021)
	Dose escalation study	1; NCT04318002	5-17 months, 18-45 years, Tanzania	Vaccine safety and immunogenicity pending (unpublished)	NA
PAMVAC	3 doses of 50 µg	1; NCT02647489	Malaria-naïve adults (n=27)	Safe and immunogenic over 6 months	(Mordmüller et al., 2019)
PRIMVAC	3 doses of 100µg	1; NCT02658253	Burkina Faso (n=20)	Safe and immunogenic over 35 days	(Sirima et al., 2020)
Transmission-blocking vaccines					
Pfs23OD1M	2 doses of 40 µg	1; NCT02334462	Malaria-naïve adults (n=5)	Safe and immunogenic over 56 days	(Healy et al., 2021)
	3 doses of 40 µg	2; NCT03917654	1 year and older; Mali	Vaccine efficacy pending (unpublished)	NA

Table summarizes the vaccine dosage, population, and outcomes from recent and ongoing clinical trials. Note that this is not a comprehensive list of all completed or ongoing vaccine trials. Not all groups or results from each trial are included in the table. For full details, refer to the referenced publications. NA not available, CHMI controlled human malaria infection

2.12 RTS,S/AS01

The RTS,S vaccine is formulated with GSK proprietary liposome-based Adjuvants Systems (AS), AS01. It is a recombinant protein vaccine from *P. falciparum* CSP, fused to hepatitis B

surface antigen (HBsAg). The HBsAg is the carrier for the epitopes. It is the only vaccine to date that has been shown to partially protect young African children against malaria. It prevented 4 in 10 cases of malaria for young children who got 4 doses of the vaccine (RCTP, 2012).

RTS,S/AS01 is aimed at stopping the liver or pre-erythrocytic stage of the *P. falciparum* life cycle from happening. It does this by targeting the circumsporozoite protein (CSP). The protein is made up of 412 amino acids and is most plentiful on the surface of the sporozoites.

The European Medicines Agency (EMA) positively opined and, along with WHO, recommended that RTS,S/AS01 be introduced in few regions with moderate to high transmission in Ghana, Kenya, and Malawi as part of their routine childhood vaccination program (EMA 2015). The safety profile of the vaccine will continue to be monitored by GSK and PATH during this small-scaled implementation. The RTS,S/AS01 vaccine implementation is coordinated by the Malaria Vaccine Implementation Program (MVIP), and data from MVIP will be used to recommend widespread use of the vaccine.

2.12.1 Development of RTS,S

The development of RTS,S started in 1987 by GSK scientists, with clinical development done at the Walter Reed Army Institute for Research. The development is led by GSK Biologicals and the PATH Malaria Vaccine Initiative (MVI) and with funding from the Bill and Melinda Gates Foundation. After series of clinical trials, the final phase 3 trial for efficacy and safety was conducted at 11 sites in Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and Tanzania, and included 15,459 infants and children (RCTP, 2015).

2.13 Circumsporozoite protein (CSP)

Circumsporozoite protein coats the surface of sporozoite and plays important roles during the parasite life cycle (Coppi et al., 2011). The primary structure of the protein is the same across

species but the amino acid sequences differ. The protein consists of three regions: the N-terminal region, the central repeat region (CRR), and the carboxyl-terminal region (Adams et al., 2000) (Figure 5).

The N-terminal region lies upstream of the CRR. This is the region that binds to heparin sulfate proteoglycans (HSPGs) on hepatocytes. It encodes a signal peptide sequence and contains Region I with five (5) conserved amino acid (KLKQP) and Pexel motifs (A. P. Singh et al., 2007).

The CRR is the middle third of the CSP with species-specific amino acid repeats. It contains 37-42 NANP (Asparagine; Alanine; Proline) repeats, NVDP (Asparagine; Valine; Aspartic acid; Proline) repeats, and B-cell epitopes (Kumar et al., 2006). The CRR supports the development of sporozoites (Ferguson et al., 2014).

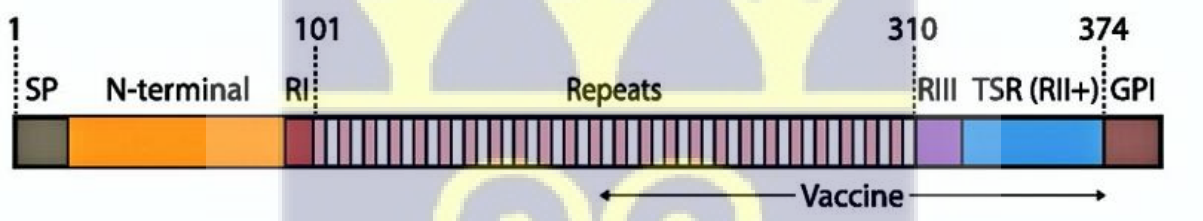


Figure 5: Schematic of the circumsporozoite protein gene.

The N-terminal region contains the signal peptide (SP) sequence and region I; central repeat region (CRR) contains NANP and NVDP repeats, and B-cell epitopes; C-terminal region contains TSP-like domain (region II), region III, and a GPI anchor sequence. Image adapted from Doud et al., (2012)

The C-terminal of the CSP consists of region II, also known as the thrombospondin-like (TSP-like) type I repeat domain (TSR) and the glycosylphosphatidylinositol (GPI) anchor sequence (Doud et al., 2012; Wang et al., 2005b). The variable CD4+ and CD8+ T-cell epitopes, and the conserved CD4+ T-cell epitope are also found in the C-terminal region (Crompton et al., 2010). Region III is immediately downstream of the CRR. The C-terminal is highly polymorphic in the Th2R and Th3R sub-regions (Zeeshan et al., 2012).

Circumsporozoite protein is important for the development of sporozoite in the midgut of mosquitoes (Ménard et al., 1997), the release of sporozoites from the oocyst (Thathy et al., 2002; Wang et al., 2005a) and invade salivary glands (Kariu et al., 2002; Kojin et al., 2016). In humans, CSP enables the sporozoite to attach to heparin sulfate proteoglycans (HSPGs) on liver cells. The adhesive properties of the TSR domain help the sporozoites to attach to the liver cells which then results in a series of signaling events in the parasite that accelerate the invasion of these liver cells (Tewari et al., 2002).

2.13.1 CSP Variation

The surface antigens of most parasitic organisms are under intense human immune pressure, which subjects their loci to positive selection. *Plasmodium falciparum* has shown substantial genetic variability, especially in their surface antigens, and this is the leading cause of the rapid emergence of drug-resistant strains and failure of most vaccine targets. The resistance of selected *Plasmodium* parasite population to naturally acquired or vaccine-induced immunity has alarmed the scientific community and is a serious threat to drug and vaccine development.

The circumsporozoite (cs) gene encoding the protein is diversely polymorphic. Most polymorphisms are observed in the NANP repeat and non-repeat regions (Jalloh et al., 2009). The genetic diversity of CSP appears to be locally restricted as polymorphism is high in areas of high malaria transmission (Lockyer et al., 1989), whereas in areas of low transmission, minor and major allelic variants are representative of the population and the number of NANP repeats is even (Tanabe et al., 2004). Nucleotide substitutions in the non-repeat regions at the T-cell epitopes, namely Th2R and Th3R, are non-synonymous and there seems to be no apparent association between the patterns of the sequences seen in the repeat region and T-cell epitopes of the non-repeat region (Jongwutiwes et al., 1994).

Although CSP has long been the target of the human immune response (Clyde et al., 1973), some level of polymorphism in the repeat region that encodes the B cell immunodominant epitope has been described but its evolution remains unexplained (Galinski et al., 1987), although selection could be a factor. It is argued that single nucleotide polymorphism in the non-repeat regions is the result of positive selection to avoid being recognized by T cells since these regions contain peptides that HLA molecules can present to T cells (Good et al., 1988).

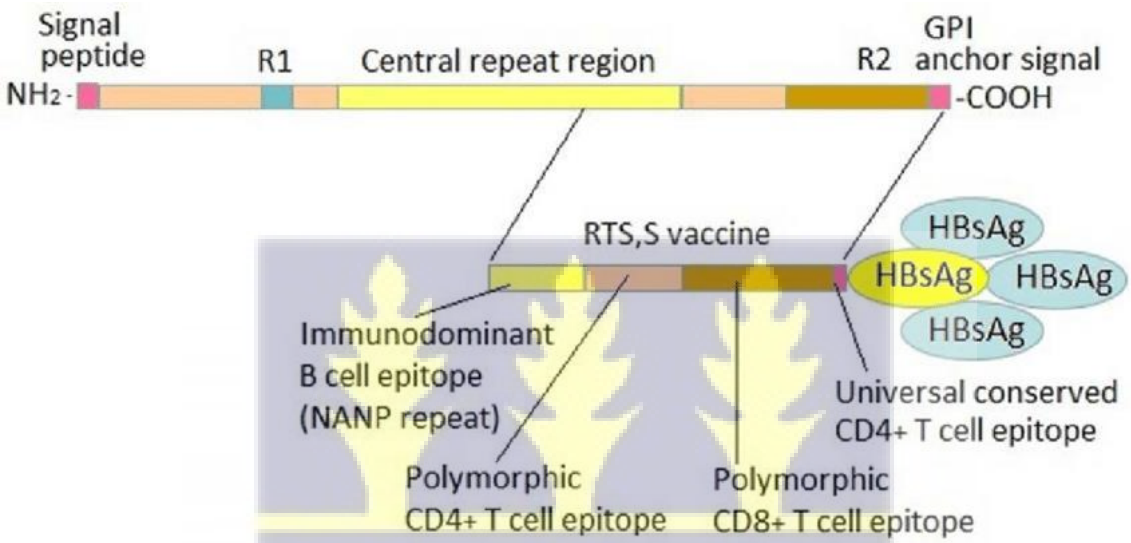


Figure 6: Scheme of CSP and RTS,S/AS01.

Top: The three regions of CSP; Bottom: Construct of the RTS,S vaccine: the last 18 NANP of the CRR which is the B cell epitopes; C-terminal contains two sub-regions, Th2R (a highly variable CD4+ T cell epitope) and Th3R (a highly variable CD8+ T cell), and a conserved universal CD4+ T cell epitope (CS.T3); fused to HBsAg virus-like particles (VLPs). Images adapted from N. Wang et al., (2019).

2.13.2 CSP as a vaccine target

For many years, scientists attempted eliciting an immune response against malaria sporozoites, which they hoped would result in the development of a vaccine against malaria. After promising results from experiments with rodents and monkeys, the next aim was to replicate the experiment in human volunteers. One out of the three volunteers who were exposed 6 times

each to bites of 54-137 irradiated infected *Anopheles* over 84 days didn't develop parasitemia; after repeated exposure to bites, he was immunized against malaria for up to 380 days (Clyde et al., 1973). Irradiated sporozoites did confer some level of immune protection but a large number of mosquito bites was required.

2.13.3 CSP and RTS,S/AS01

The RTS,S/AS01 vaccine construct is made of 189 amino acids from CSP (NF54 199-387 aa), which includes the last 18 NANP repeats and the C-terminus. It does not include the GPI anchor addition sequence. This truncated CSP is combined with hepatitis B surface antigen (HBsAg) monomers, which serve as the protein carriers (Figure 6).

Although CSP was fused to HBsAg virus-like particles in RTS,S, its immunogenicity was weak and needed an adjuvant system (Kaslow et al., 2015). The two adjuvant systems evaluated with RTS,S (AS01 and AS02) improved CS-specific immunogenicity than non-adjuvanted RTS,S, but RTS,S/AS01 was significantly higher than RTS,S/AS02 (Leroux-Roels et al., 2014). The immunostimulants in the formulations of the adjuvant systems promote protective immune responses following the administration of the vaccine. The innate immune system is stimulated by the immunostimulants, which in term enhances the adaptive immunity to intensify the response to the vaccine antigens. According to Didierlaurent et al., (2014), AS01 works by activating a high number of dendritic cells that are present in draining lymph nodes.

RTS,S lacks the N-terminus of CSP in its construct. Although antibody responses have been directed towards domains in the N-terminal region, it is not yet established whether these responses can prevent the parasite from binding to hepatocytes (Bongfen et al., 2009). The N-terminal region is not as highly diverse as the CRR and the C-terminal region (Gandhi et al., 2014). The high genetic diversity observed in these regions has been implicated for the reduced efficacy associated with RTS,S (Neafsey et al., 2015), so validating the less polymorphic

region I for vaccine development may address issues of genetic diversity. The vaccine efficacy for 5-17 months old children who got three vaccine doses plus a booster was 36.3% in the 2015 study (RCTP, 2015), compared to 50.4% from the first dose over 14 month period in 2011 (RCTP, 2011).

2.14 Human Leukocyte Antigens (HLA)

The human leukocyte antigen (HLA) is a component of the adaptive T cell immune system. It presents host and foreign peptides to their specific CD4+ and CD8+ T-cell receptors found on antigen presenting cells (APCs). HLA, also called major histocompatibility complex (MHC), spans 4-6 Mb with more than 200 genes on the short arm of chromosome 6 (Figure 7) and varies from one human to another because of the low crossover rate (Marsh et al., 2010). It is the most intensively studied segment of the human genome because it influences transplantation rejection and antigen-specific immune responses (P.-C. Lee et al., 2002; Lim et al., 2016). HLA genotyping for polymorphism identification is widely used to screen organ donors and recipients for transplantation in clinical medicine, and to assess the risk of diseases such as narcolepsy, ankylosing spondylitis, hereditary hemochromatosis, and autoimmune disorders (Thorsby et al., 2005).

The HLA complex has six (6) major loci, divided into two (2) classic groups, HLA class I and class II, based on their interactions with T cell subsets, structure, source of the peptide, and distribution. These molecules are co-dominantly inherited.

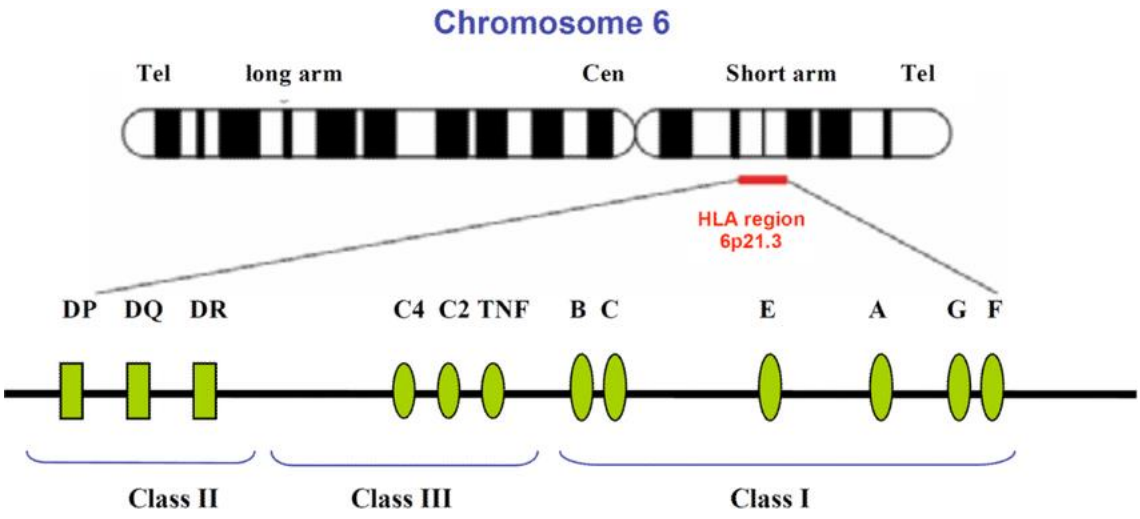


Figure 7: HLA Complex on Chromosome 6.

Human leukocyte antigen, also called major histocompatibility complex (MHC) contains more than 200 genes on the short arm of chromosome 6, divided in Class I, Class II, and Class III. Image adapted from X. Li et al., (2009)

2.14.1 HLA class I

HLA class I molecules are HLA-A, HLA-B, and HLA-C. They are encoded by three (3) distinct loci, spanning approximately 1Mb of the MHC region. They are found on nucleated cells which degrade endogenous peptides and antigens and present them on their surface so they can be recognized by T cell receptors (TCRs) of CD8+ T cells. Additionally, these molecules can be recognized by killer Ig-like receptors (KIRs). The general structure of the class I molecules is similar, but their primary amino acid sequences differ. Each molecule comprises a heavy chain (α chain) and a β2 microglobulin chain (Natarajan et al., 1999). The α chain is made of α1, α2, and α3 domains. The α1 and α2 domains form the antigen-binding cleft, while α3 and β2 microglobulin form the Ig-like region for CD8+ attachment (Figure 8).

2.14.2 HLA class II

HLA class II molecules are found on immune-competent cells such as macrophages, mature B cells, and dendritic cells, recognized by TCRs of CD4+ helper T cells which binds to

exogenously-derived peptides. These degraded exogenous proteins enter the cell via phagocytosis. The majority of the peptides presented by class II molecules do not trigger T cell response because the APCs bearing these molecules can phagocytose host proteins. But T cells become activated and differentiate when the peptides are foreign to the host. The class II molecules are the classic HLA-D genes on different loci, about 1 Mb: DP (DPA1, DPB1), DQ (DQA1, DQB1), and DR (DRA1, DRB1). Each class II molecule comprises two heavy chains, α and β that are non-covalently associated (Madden, 1995). The α heavy chain is divided into α1 and α2, and the β heavy chain is also divided into β1 and β2 (Figure 8). The α1 and β1 domains form the binding site for peptides. The main difference between HLA class I and class II binding grooves is that the bottom of the groove in HLA class I is closed, while it is open in class II, allowing for the binding of longer peptides by class II molecules.

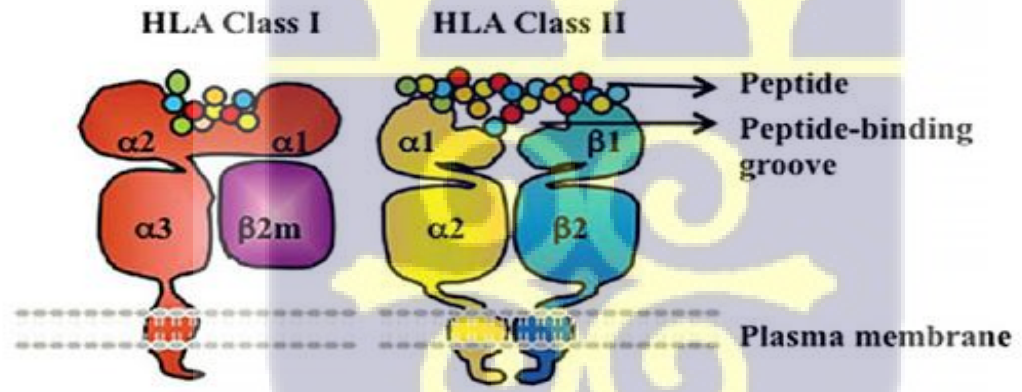


Figure 8: Structure of HLA class I and HLA class II..
Image adapted from Hickey et al., (2016).

2.14.3 HLA Variation

There are nine (9) classic HLA genes, all of them have allelic variants. About 28,320 HLA alleles (20,597 class I and 7,723 class II alleles) have been recorded in the IPD-IMGT/HLA

database. The HLA-B locus is the most polymorphic (7,562 alleles) in class I while HLA-DRB is the most polymorphic (3,536 alleles) in class II.

The transmembrane region of the α chains is conserved. The α3 domain is less polymorphic, while the α1 and β1 domains of class II are most polymorphic (Rodey et al., 1987).

Every individual is heterozygous for most of the genes, and there are several genetic loci with different alleles for each class of HLA molecules. The alleles show a high degree of sequence variation with up to 56 amino acids difference. Diversity at these loci is important because heterozygosity increases the chance of successfully presenting changing antigens. Polymorphism ensures variant of HLA alleles is distributed in the population and makes it more likely for individuals within the population to respond to any given antigen and the species as a whole can survive (Kaufman, 2000).

2.14.4 HLA and RTS,S/AS01 vaccine response

Vaccines have been effectively used to control and eradicate infectious diseases of public health concern, such as measles, polio, smallpox, hepatitis B, anthrax, influenza, etc. Whereas a greater portion of the population elicits an immune response to vaccines, studies have shown healthy individuals failed to respond to vaccination (Sjogren, 2005). The causes of vaccine nonresponse are not known but chronic illnesses, immunosuppression, and genetic factors may be responsible (Dhiman et al., 2007; Poland et al., 2007). This heterogeneity of immune response is still a problem for vaccine design and subsequent distribution in the population.

HLA alleles are diverse at the population level and their variable response to antigens plays a significant role in vaccine response (Posteraro et al., 2014). The specificity of the peptide-binding sites for these HLA variants can limit which peptides can bind and be presented to T cells and therefore influence how each individual will respond to an antigen or a vaccine.

Association between HLA molecules and CSP has been demonstrated in different studies and regions (Kollars Jr et al., 2004; Storti-Melo et al., 2012).

As it is with most vaccines, there is a likelihood that HLA plays a major part in RTS,S-mediated immune responses. Immunogenic associations have been shown between hepatitis B vaccine and specific HLA alleles (Z.-K. Li et al., 2013; L.-Y. Wang et al., 2019), which is important to note because the RTS,S vaccine contains CSP fused to HBsAg polymers. In fact, Nielsen et al., (2018) observed significant protective associations between RTS,S, and some HLA allele groups and lack of protection with other groups, which indicate HLA genes may influence immune protection mediated by RTS,S vaccine against malaria infection.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Ethical statement

This study has obtained ethical approval from Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, and the study has followed the guidelines and regulations of the ethical committee. The samples were collected with the consent of the parents or guidance of the participants.

3.2 Study design

This study was part of a four-year prospective study that was designed to collect blood samples on filter paper or dried blood spot (DBS) from symptomatic and asymptomatic children, aged 6 months to 14 years old in Cape Coast and Begoro, Ghana. The samples were divided into two categories: those collected from children with symptoms of malaria, brought to the health centers by their parents or guardians, and tested positive for malaria by microscopy are called symptomatic or hospital-based samples; samples collected from children who were in school without symptoms of malaria, but tested positive for malaria by microscopy are asymptomatic or community-based samples. The hospital-based samples were collected from Ewim Polyclinic in Cape Coast and Begoro District Hospital in Begoro. The community-based samples were collected from three randomly selected schools within each site. For this study, only symptomatic or hospital-based samples were used, and a total of one thousand and forty (1040) samples were processed, 474 from Begoro and 566 from Cape Coast.

3.3 Study area

This study took place in Begoro and Cape Coast in Ghana. Begoro is a forested zone located in Fanteakwa District in the Eastern region of Ghana. The yearly rainfall is 1,500 to 2,000 mm

in the district, maximum in June and October every year. According to the Ghana Statistical Service (2010) population and housing census, the population of Begoro is 86,154 inhabitants. Malaria transmission is present all through the year, but most intense in June. where malaria transmission is high. Most of the malaria cases are treated at Begoro District Hospital, which is the only state-owned hospital in the district.

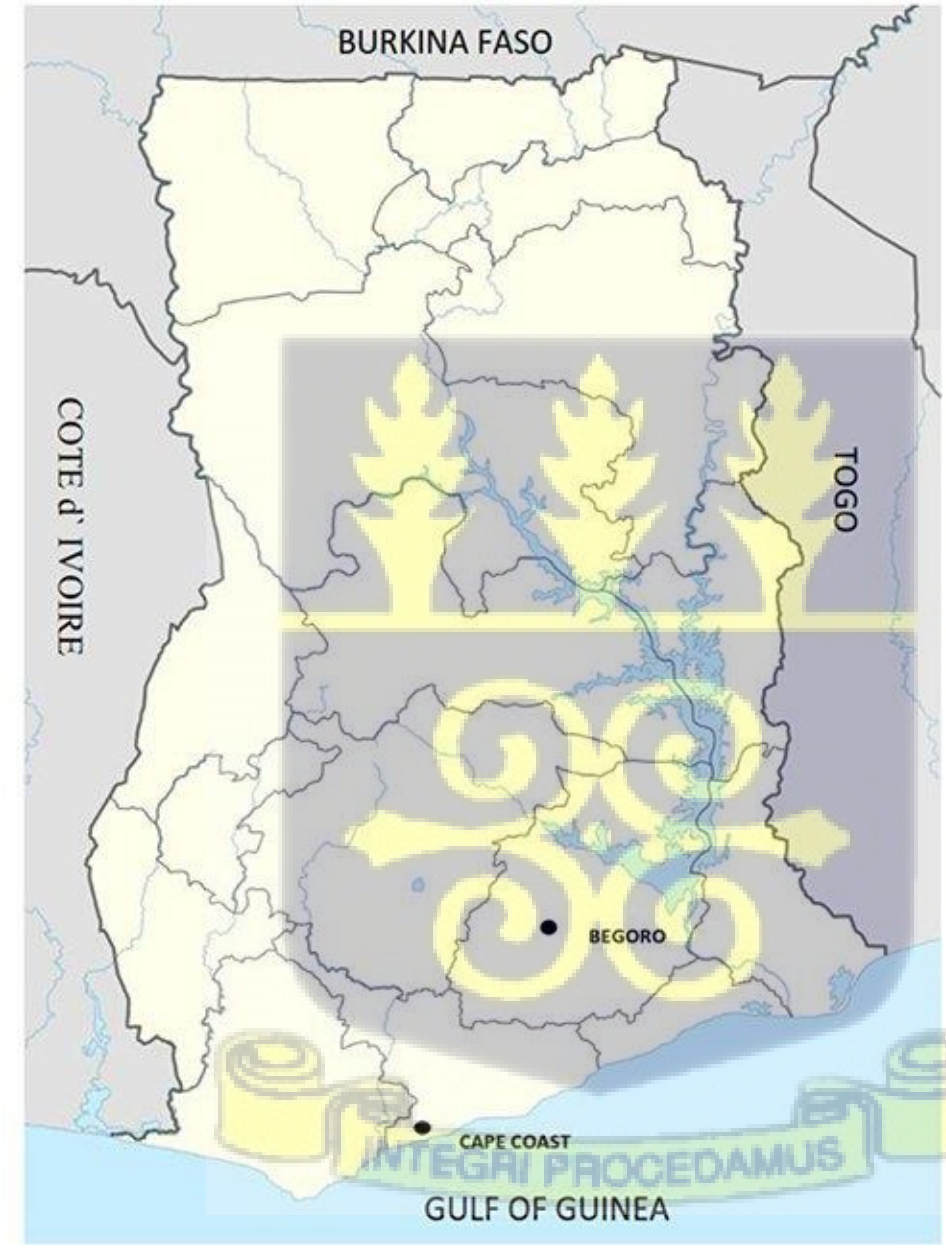


Figure 9: Location of the sites in Ghana

Map of Ghana with Cape Coast (lying in the Gulf of Guinea on the Atlantic Coast) and Begoro (in the forest of Fanteakwa District). Cape Coast and Begoro are ~ 255km (158.45 miles) apart. Image adapted from Ofori et al., (2021)

Cape Coast is a coastal city along the Atlantic Ocean in the Gulf of Guinea. It lies in the Central region, 165 km west of the capital city of Ghana (Accra). The population of Cape Coast is 169,894 inhabitants (Service., 2010) with yearly rainfall of 750 to 1,000 mm. Although malaria transmission takes place throughout the year, it is low to moderate and intense in June. Cape Coast is approximately 255 km away from Begoro (Figure 9).

3.4 Study Inclusion Criteria

The following are the inclusion criteria: age between 6 months and 14 years, infection with *P. falciparum* only (no other species of *Plasmodium*) detected by microscopy, asexual parasitaemia of 1,000-250,000/µl, presence of axillary temperature ≥ 37.5^{o}C or history of fever during the past 24 hours, ability to swallow oral medications, and parent/guardian giving consent and willing to comply with the study protocol for the duration of the study including study visit schedule.

3.5 DNA extraction

DNA was extracted from punched dried blood spots (DBS) placed in 200 µl of eluent. The DNA extraction was done in the Ghansah lab at NMIMR with QIAamp DNA mini kit (Qiagen) DNA extraction kit, following the manufacturer's instruction. The quality of the extracted DNA was estimated by Qubit (version 2.0) fluorometer and quantified by Quant-iT PicoGreen double-stranded DNA (dsDNA) assay (catalog number P11496; Invitrogen). The extracts were placed in 96-welled PCR plates, sealed, and shipped to Bailey's lab at Brown University, Providence, Rhode Island (USA) under controlled temperature to maintain the integrity of the DNA extract.

3.6 Molecular Inversion Probe (MIP) Capture, Amplification, and Sequencing of *Plasmodium falciparum* CSP

For targeted deep amplicon sequencing, molecular inversion probes (MIPs) were designed to capture the circumsporozoite protein gene in the malaria parasite. These single-stranded oligos were about 100 nucleotides, with sequences specifically complementary to the region of interest. This technique includes three steps (capture, exonuclease and PCR) as described by Aydemir et al., (2018). See Figure 10 for overview of MIP workflow and Figure 11 for bioinformatic processing of sequences.

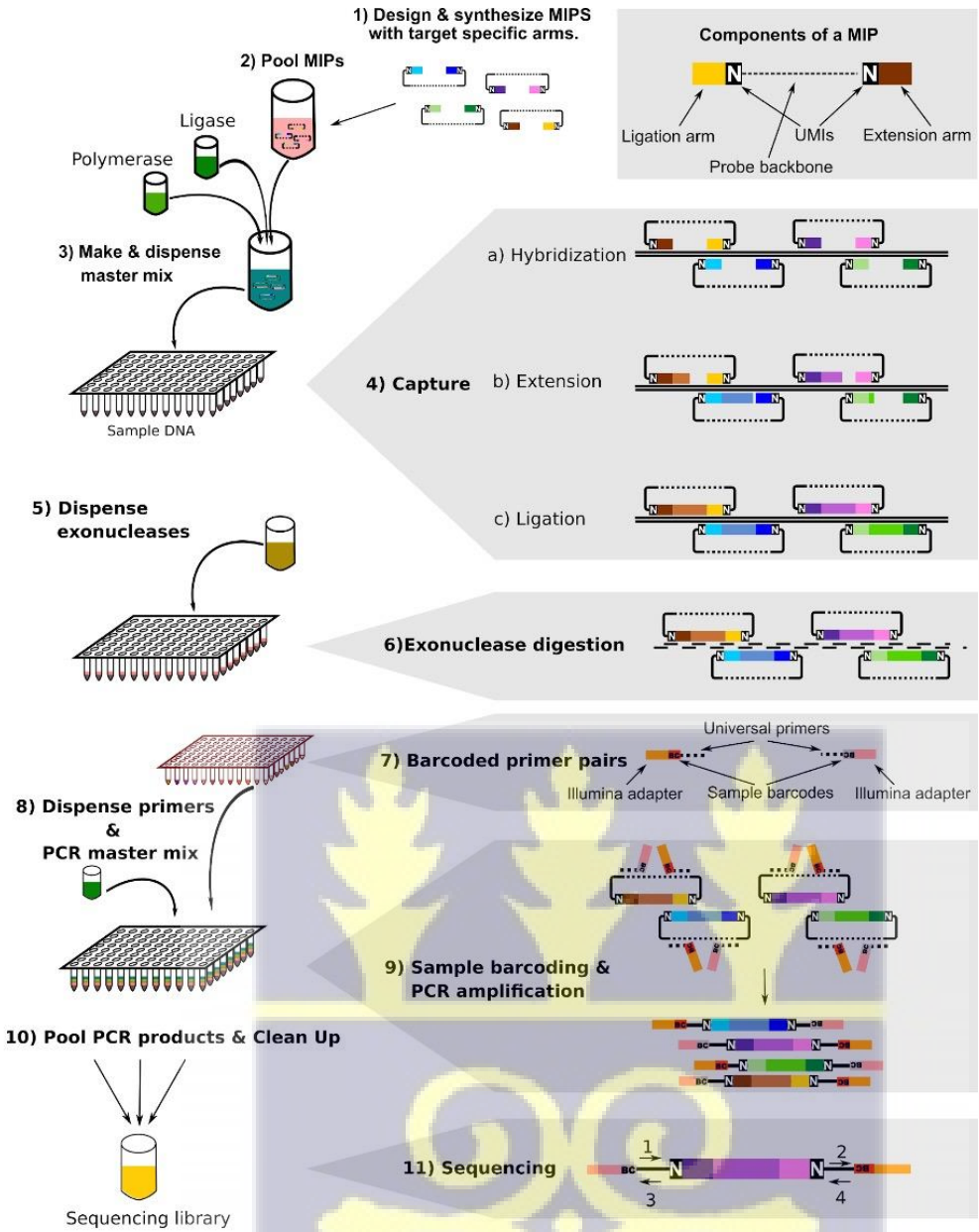


Figure 10: MIP workflow.

Molecular inversion probes (MIPs) are designed to target specific genomic region of interest (1). These MIPs are pooled (2) and added to a master mix that is then added to the DNA template (3). The first step is to capture the genomic region of interest when these probes hybridize to the targeted sequence and form circularized DNA by extension and ligation (4). The second step is the exonuclease digestion step where exonuclease is dispensed (5) to digest linear templates and unbounded probes (6), leaving only captured DNA. PCR forward and reverse primers in a PCR master mix (8) plus illumina sequencing barcodes (7) and unique sample barcode (9) are added during the third step, the PCR amplification step. Because the samples have been barcoded, they can be pooled in a single tube and cleaned up (10) to prepare a sequencing library for illumine sequencing (11). Aydemir et al., (2018)

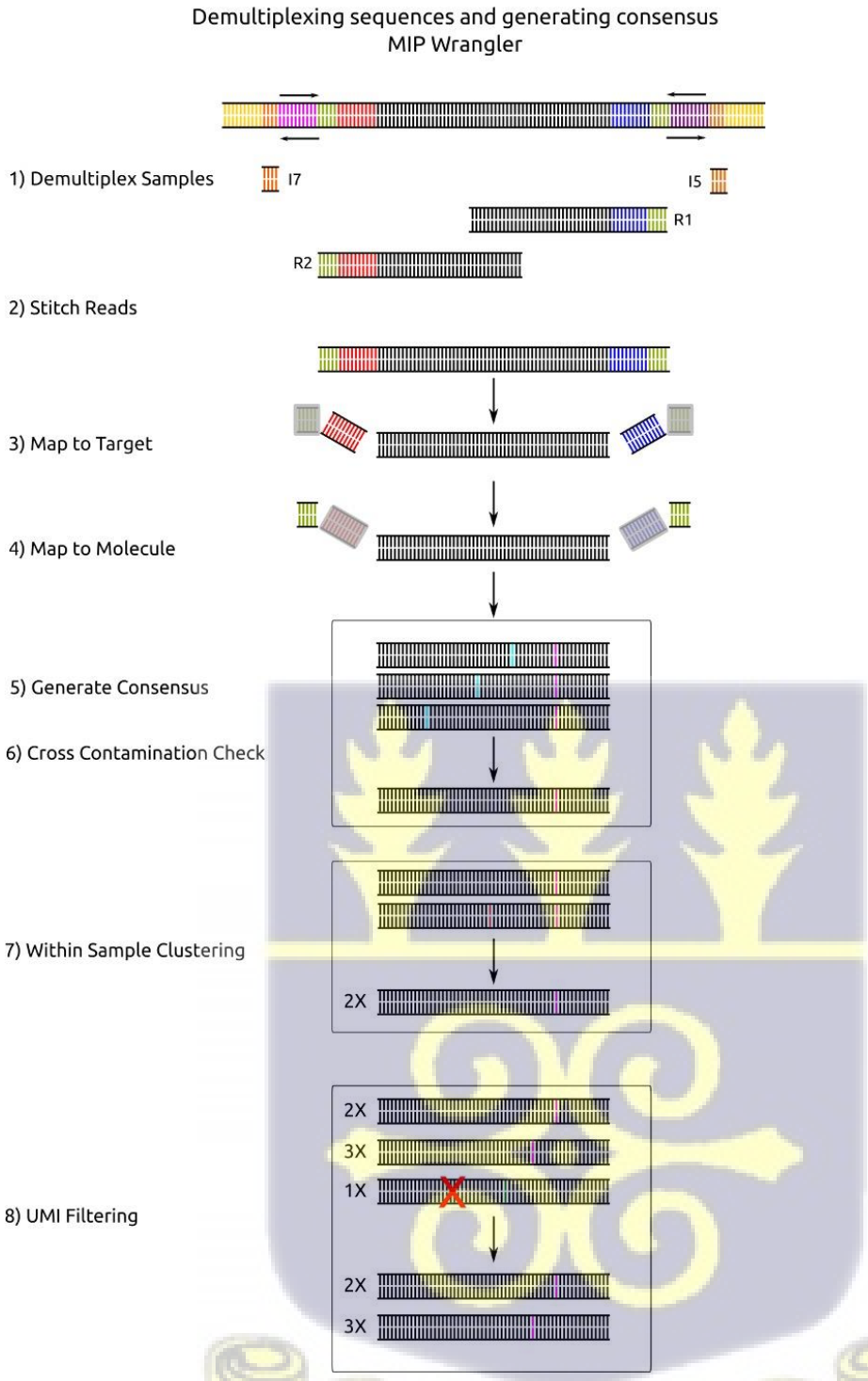


Figure 11: Bioinformatic demultiplexing of sequences.
Samples are demultiplexed based on the forward and reverse sample barcodes (orange) after illumine sequencing (1), and paired end reads are stitched to generate a single sequence for each read using FLASH software (2). The extension and ligation arms (blue and red, respectively) are used to identify the target (3). The UMIs (green) used on each side of capture can identify sequences that are PCR duplicates originating from the same DNA template (4), and a consensus sequence is generated by collapsing reads with the same UMIs which removes PCR errors (aqua) and leaves only true variants (pink) (5). A cross contamination check is performed (6). UMI collapsed sequences are further clustered within sample and collapsed based on sequencing quality, removing likely sequencing errors (red), (7). A minimum UMI criteria (>=2) is used to remove rare errors occurring in the initial rounds of PCR (green) (8). Aydemir et al., (2018)

3.6.1 Capture step

The capture step was to allow MIPs to bind to their targets, followed by polymerase extension at the extension arm and ligase at the ligase arm. This results in the formation of single-stranded circular products. The capture was done by mixing 5 µL of DNA template with 5 µL of capture master mix of Molecular Biology grade water, Ampligase DNA Ligase (Lucigen A0110K), Ampligase 10X Reaction Buffer (Lucigen A1905B), dNTP solution mix (NEB N0447L), the working concentration of MIP mix, Q5 High-Fidelity DNA Polymerase (NEB M0491L) (listed in order in Appendix 2) in 384 well plates. The 384 well plates were sealed with plastic seal and isothermally incubated in a thermocycler (Eppendorf models) programmed to pre-heat the cycler lid at 100°C and held at 95°C, followed by 20 cycles each, of denaturation at 95°C for 10 min, annealing at 60°C for 60 min, and the reaction held at 4°C.

3.6.2 Exonuclease step

After capturing the targets, the exonuclease step was done to treat the reaction with exonuclease master mix to remove all linear DNA template and unbound probes. 2 µL of exonuclease master mix of Molecular Biology grade water, Ampligase 10X Reaction Buffer (Lucigen A1905B), Exonuclease I (E. coli) (NEB M0293L), Exonuclease III (E. coli) (NEB M0206L) (listed in order in Appendix 3) was added into each capture in the 384 well plates, sealed and transferred to a cycler (Eppendorf models) preheated at 100°C and held at 37°C. The cycler condition was 37°C (60 min), 95°C (2 min), and held at 4°C.

3.6.3 PCR step

During the PCR step, one forward primer and one reverse primer were used with the circularized captured DNA. These primers bind to the universal primer site on the DNA. The samples were also tagged with Illumina sequencing adapters and sample barcodes as described

(Aydemir et al., 2018). These barcodes make it possible to pool the samples into a single tube to create a library for sequencing.

The PCR was done by transferring 1.25 µL of 10 µM forward primers to their assigned wells and the 1.25 µL of 10 µM reverse primer to all wells. After barcoding the samples, 10.75 µL of PCR master mix of Phusion High-Fidelity DNA Polymerase 5X Reaction Buffer, 5X MMC (in-house prepared reagent), dNTP solution mix (10mM) (NEB N0447L), Phusion High-Fidelity DNA Polymerase (NEB M0530L) (listed in order in Appendix 3) was added to each captured reaction, sealed and transferred to a cycler (Eppendorf models) preheated at 105°C and initial denaturation at 98°C; followed by 20 cycles of denaturation at 98°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, and held at 4°C.

3.6.4 Control Samples

Serially diluted DNA isolated from laboratory strains 3D7, 7G8, HB3, DD2, HC, and LC, NTC (no template capture), and NTP (no template PCR) were used to validate the MIP panel. Water (5 µL) was added to the NTC in place of the DNA template during the capture step, and12 µL of water was added in place of the capture and exonuclease during the PCR step to the NTP.

3.6.5 Post-capture Quality Control Check

The quality of the capture and amplification was checked by running selected reactions (including controls) on a 1.5% agarose 0.5X TBE gel. 5 µL of amplicons mixed with 1.3 µL of previously prepared SYBR Gold + Orange loading dye solution were loaded on a 1.5% agarose 0.5X TBE gel and ran for 45 minutes. A GeneRuler 1 kb DNA ladder was used.

3.6.6 PCR product pooling and bead cleaning

To create a sequencing library, 5 µL of each PCR product was pooled into one tube and then cleaned with solid phase reversible immobilization (SPRI) beads. The bead volume was 0.8X

the pool volume and was separated from the solution using a magnetic separation rack and washed with 80% ethanol. After washing, the bead was eluted with 20 µL of 1X TE (low EDTA), separated on the magnetic rack, and 20 µL of library was collected.

3.6.7 Final Sequencing Library Gel Extraction

For the final library gel extraction, the 20 µL of the bead cleaned library product plus 5 µL of SYBR Gold + Orange loading dye solution was run on a 2% agarose 0.5X TBE gel until the MIP capture band and the self-ligated MIP band were separated. The capture band was excised from the gel and gel extraction was done with NEB Monarch DNA Gel Extraction kit (NEB T1020S), following the manufacturer's instruction. The final library was eluted to a final volume of 6 µL.

3.7 Molecular Inversion Probe (MIP) Capture, Amplification, and Sequencing of Human Leukocyte Antigen (HLA) Class II

3.7.1 Capture step

For HLA capture, 5 µL of DNA template with 5 µL of the capture master mix which contained molecular biology grade water, Ampligase DNA Ligase (Lucigen A0110K), Ampligase 10X Reaction Buffer (Lucigen A1905B), dNTP solution mix (NEB N0447L), the working concentration of HLA class II MIP pool, 5X Q Solution, Q5 High-Fidelity DNA Polymerase (NEB M0491L) (listed in order in Appendix 5) were mixed in 384-well PCR plates and isothermally incubated in a thermocycler (Eppendorf models) under the following condition: [Lid at 100°C: 95°C for 10 minutes → 60°C for 60 min → 4°C hold].

3.7.2 Exonuclease step

After capture, 2 µL of exonuclease master mix of molecular biology grade water, Ampligase 10X Reaction Buffer (Lucigen A1905B), Exonuclease I (E. coli) (NEB M0293L), Exonuclease

III (E. coli) (NEB M0206L) (listed in order in Appendix 3) was added into each capture and transferred to a cycler (Eppendorf models) with the following condition: [Lid 100°C, 37°C for 60 minutes → 95°C for 2 minutes → 4°C hold].

3.7.3 PCR step

For the PCR step, 1.25 µL of 10 µM forward primers was dispensed to their assigned wells and the 1.25 µL of 10 µM reverse primer to all wells. After barcoding, 10.75 µL of PCR master mix of 5X Q5 Reaction Buffer, 5X MMC (in-house prepared reagent), dNTP solution mix (10mM) (NEB N0447L), Q5 DNA Polymerase (NEB M0530L) (listed in order in Appendix 6) was added to each captured reaction and transferred to a cycler (Eppendorf models) with this condition: [Lid 105°C; 98°C for 30 sec → 20 x (98°C for 10 sec → 63°C for 30 sec → 68°C for 30 sec) → 68°C for 2 min → 4°C]. Illumina sequencing adapters and sample barcodes were also included during the PCR step.

3.7.4 Control Samples

NTC (no template capture) and NTP (no template PCR) were used as negative controls, while MGP000019a_CTLS_D1 was a positive control.

3.7.5 Post-capture Quality Control Check

To check the quality of the capture and PCR steps, 5 µL of selected amplicons was mixed with 1.3 µL of previously prepared SYBR Gold + Orange loading dye solution, cast on a 1.5% agarose 0.5X TBE gel and ran for 45 minutes. A GeneRuler 1 kb DNA ladder was used.

3.7.6 PCR product pooling and bead cleaning

The sequencing library was created by pooling 5 µL of PCR product from each well, including controls, into a single tube, and the library was cleaned using solid phase reversible immobilization (SPRI) beads, whose volume was 0.8X the volume of the pool. The solution

was separated on a magnetic separation rack and washed with 80% ethanol. Elution was done with 20 µL of 1X TE (low EDTA).

3.7.7 Final Sequencing Library Gel Extraction

The 20 µL of library product plus 5 µL of SYBR Gold + Orange loading dye solution was run on a 2% agarose 0.5X TBE gel. The excised band was extracted with NEB Monarch DNA Gel Extraction kit (NEB T1020S), following the manufacturer's instruction. The final library was eluted to a final volume of 15 µL.

3.8 Quality Control Check of CSP and HLA sequencing libraries

For quality control purposes, Qubit fluorometer with Qubit 1X dsDNA HS Assay Kit (Invitrogen Q32854) was used, following the manufacturer's instructions. We used 1 µl of gel purified library of CSP and HLA to assess the qualities of the libraries. Each library was then checked with Fragment Analyzer so that no fragment was less than 200 bp.

3.9 Sequencing of CSP and HLA II libraries

The CSP MIP library was sequenced on the Illumina NextSeq platform at Brown University while HLA class II MIP library was sent to Genewiz (genewiz.com) to be sequenced by MiSeq.

3.10 Bioinformatics and population genetics

After sequencing, the CSP data was processed with MIPWrangler software, as described (Aydemir et al., 2018). Using R software, the CSP sequences were filtered out and monoclonal sequences were aligned to the vaccine reference strain (3D7 0304600.1, PlasmoDB) and translated to amino acid sequences by Molecular Evolutionary Genetics Analysis (MEGAX) software (version 0.1). After aligning the sequences, a customized python script was used to generate and analyze the haplotypes across the C-terminal region (937-1140 nucleotides position). The frequencies of the overall Th2R and Th3 haplotypes, haplotypes that are shared

in both study sites, and differences in amino acid positions with reference to the 3D7 vaccine strain were calculated. With DnaSP software (version 6.12.03), the following diversity parameters of the CSP C-terminal within our parasite populations were determined: number of sequences (n), number of haplotypes (h), segregating sites (S), the average number of pairwise nucleotide differences (K), haplotype diversity (Hd) and nucleotide diversity (π), Tajima's D and Fu & Li's F* test. Also, a haplotype network to assess the relationship between the CSP C-terminal haplotypes in Begoro and Cape Coast was constructed using PopArt (Leigh et al., 2015).

For Kenya and Malawi, CSP sequences were mined from the Malaria Genomic Epidemiology Network (MalariaGEN) *Plasmodium falciparum* community project database in variant call format (https://www.malariagen.net/parasite/p-falciparum-community-project). The sequences were filtered out using R software. With a customized python script, the overall haplotypes were determined. PopArt was used to construct the haplotype network to show the relationship among the haplotypes in Ghana, Kenya and Malawi. The genetic indices were determined for Ghana, Kenya, and Malawi with DnaSP (Ver. 6.12.03), across the C-terminal region (937-1140 nucleotides position).

For the temporal analysis, the CSP sequences for both study sites were grouped by year (2014, 2015, and 2016). With a customized script, the frequencies of the Th2R and Th3R haplotypes in each year (2014, 2015, and 2016) were determined, and Microsoft Excel (version 64.0) conditional formatting tool was used to generate the heat maps for the temporal stability of Th2R and Th3R haplotypes in 2014, 2015, and 2016 across the study sites.

For HLA class II, the sequences were demultiplexed and processed using MIPTool. HLA class II genotype calling was done on Brown's Oscar server (https://docs.ccv.brown.edu/oscar/) as described (Dilthey et al., 2016). For quality control purposes, HLA sequences with quality

score below 90% were excluded from the analysis. Sequences with average coverage below 10 were also excluded. A customized python script was then used to generate the haplotypes which were grouped into superfamily for Begoro and Cape Coast, and their frequencies determined. Using R software, Th2R and HLA class II haplotypes were merged on sample ID and analyzed for correlation between HLA class II and Th2R haplotypes, and the heat maps were generated. An analysis of association was preferred but the lack of statistical power could only allow for a correlation of the data.

Although studies have shown that immunity against malaria liver stages involves induction of CD8+ T cells and production of IFN-γ, and CSP-derived peptides presented on the surface of hepatocytes in the context of HLA class I molecules, data analysis for this study is focused on HLA class II and Th2R due to limited time for submission. A future study is hoped to analyze HLA class I and Th3R.

CHAPTER FOUR

4 RESULTS

4.1 CSP Sample and Data Analysis

A total of one thousand and forty (1040) samples [474 samples (45.6%) from Begoro and 566 samples (54.4%) from Cape Coast] were processed and sequenced for CSP analysis using Illumina NextSeq technology. For quality control, sequences with coverage of >=10 were filtered, leaving four hundred and eleven (411) individual sequences for further analysis. Those sequences were aligned with the malaria vaccine reference strain using MEGA X and translated into amino acid sequences. The analysis was focused on the polymorphic Th2R (amino acid position 313 – 327) and Th3R (amino acid position 352 – 363) sub-regions of the C-terminus. A customized python script was designed to generate and analyze the haplotypes for Begoro and Cape Coast.

4.2 CSP Spatial Analysis

A total of eighty-nine (89) Th2R and Th3R haplotypes were observed. Of these, 68.5% were Th2R haplotypes and 31.5% were Th3R haplotypes. The Th2R represented 67.4% (31/46) of the haplotypes in Begoro and 69.8% (30/43) of the haplotypes in Cape Coast. For the number of sequences analyzed, 34.8% were from Begoro, while 65.2% from Cape Coast (Table 2).

Table 2. Overall Th2R and Th3R haplotypes for Begoro and Cape Coast

	Begoro	Cape Coast	Total
No. of Sequences	143	268	411
Th2R haplotypes	31	30	61
Th3R haplotypes	15	13	28

4.2.1 Th2R Haplotype frequency and Amino acid difference

There were 31 Th2R haplotypes (50.8%) in Begoro and 30 (49.2%) in Cape Coast. The 3D7 vaccine strain Th2R haplotypes make up 8.4% of the haplotypes in Begoro and 10.1% in Cape Coast. DKHIEQYLKTIQNSL is the most prevalent Th2R haplotype in Begoro and Cape Coast, representing 10.5% and 13.1%, respectively (Figure 12). The least prevalent Th2R haplotype in both Begoro and Cape Coast is DKHIKEYLTKIQNSL, representing 0.7% and 0.4%, respectively. The frequency of every Th2R haplotype can be found in Appendix 6.

There are changes observed in the amino acid position in the Th2R haplotypes with reference to the 3D7 vaccine strain. These changes range from 1 to 6 amino acids in both Begoro and Cape Coast. See Appendix 6 and Figure 13.

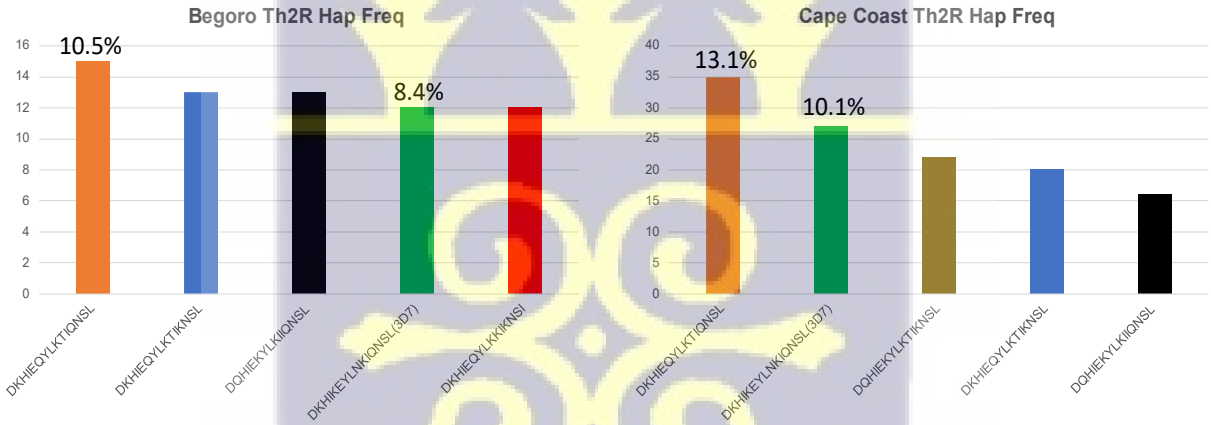


Figure 12. Th2R haplotype frequency for Begoro and Cape Coast

DKHIEQYLKTIQNSL is the most prevalent Th2R haplotype in both Begoro and Cape Coast, 10.5% and 13.1%, respectively. The 3D7 Th2R haplotype is more prevalent in Cape Coast than in Begoro.

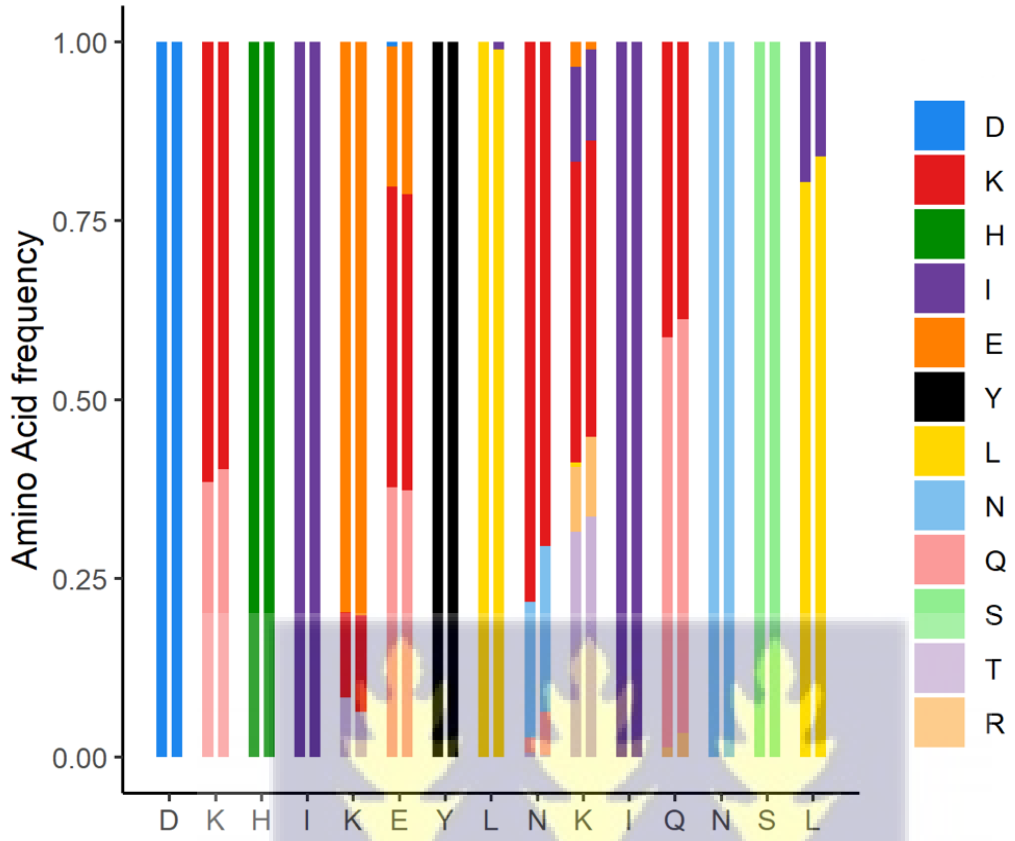


Figure 13. Th2R amino acid difference with reference to the 3D7 vaccine strain

The amino acids (on the horizontal axis) have two bars, Begoro (left) and Cape Coast (right). While some amino acids remained stable or unchanged, others have two (2) or more amino acid changes.

Twenty-six (26) Th2R haplotypes are found in both Begoro and Cape Coast (Appendix 7). These shared haplotypes represent 83.9% of the Th2R haplotypes in Begoro and 86.7% of the Th2R haplotypes in Cape Coast (Figure 14). The top five (5) Th2R haplotypes are in Begoro and Cape Coast are DKHIEQYLKTIQNSL, DKHIKEYLNKIQNSL(3D7), DKHIEQYLKTIKNSL, DQHIEKYLKIIQNSL, and DKHIEQYLKKIKNSI.

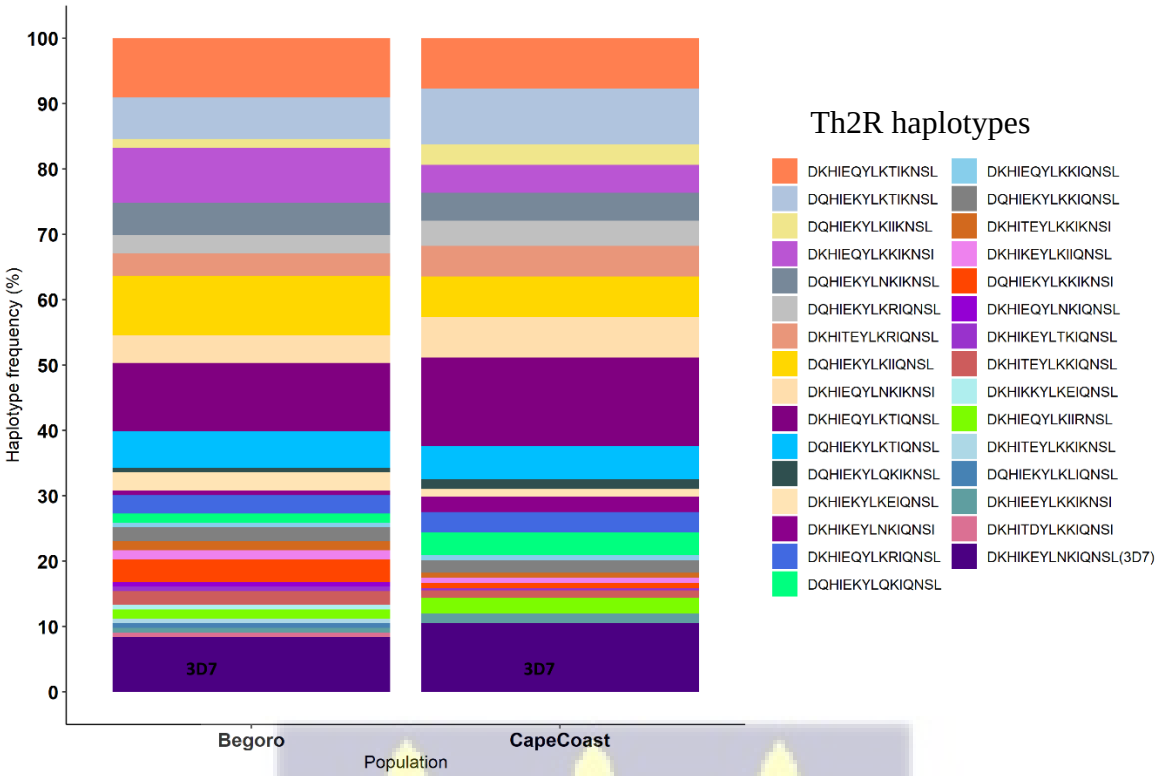


Figure 14. Shared Th2R haplotype for Begoro and Cape Coast
Most of the Th2R haplotypes (83.9%) in Begoro (left) are shared in Cape Coast (right).

4.2.2 Th3R Haplotype Frequency and Amino Acid Difference

There are twenty-eight (28) Th3R haplotypes, 53.6% in Begoro and 46.4% in Cape Coast (Table 2). The 3D7 vaccine strain (Th3R haplotypes) represent 13.3% of the haplotypes in Begoro and 14.2% in Cape Coast. The most prevalent Th3R haplotype in both Begoro and Cape Coast is NKPKDQLDYAND, which represents 33.6% and 34.7%, respectively (Figure 15). The least prevalent Th3R haplotype is NKPKEELDYEND, representing 0.7% in Begoro and 0.7% in Cape Coast. All of the Th3R haplotype frequencies are in Appendix 8.

The changes in amino acid positioning of the Th3R haplotypes with reference to the 3D7 vaccine strain range from 1 to 4 amino acids in both Begoro and Cape Coast (see Appendix 8 and Figure 16).

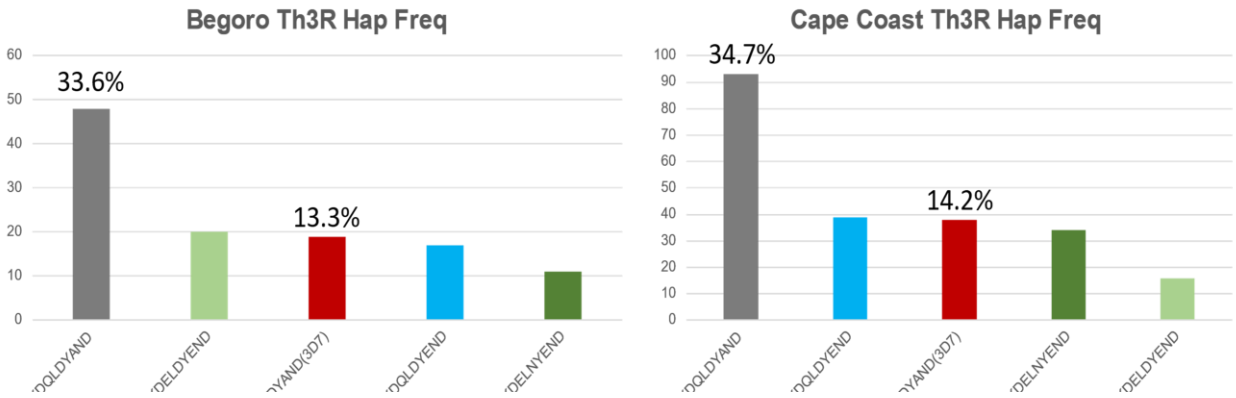


Figure 15. Th3R haplotype frequency for Begoro and Cape Coast

The 3D7 Th3R haplotype represents 13.3% in Begoro and 14.2% in Cape Coast. The most prevalent Th3R haplotype in both Begoro and Cape Coast is NKPKDQLDYAND.

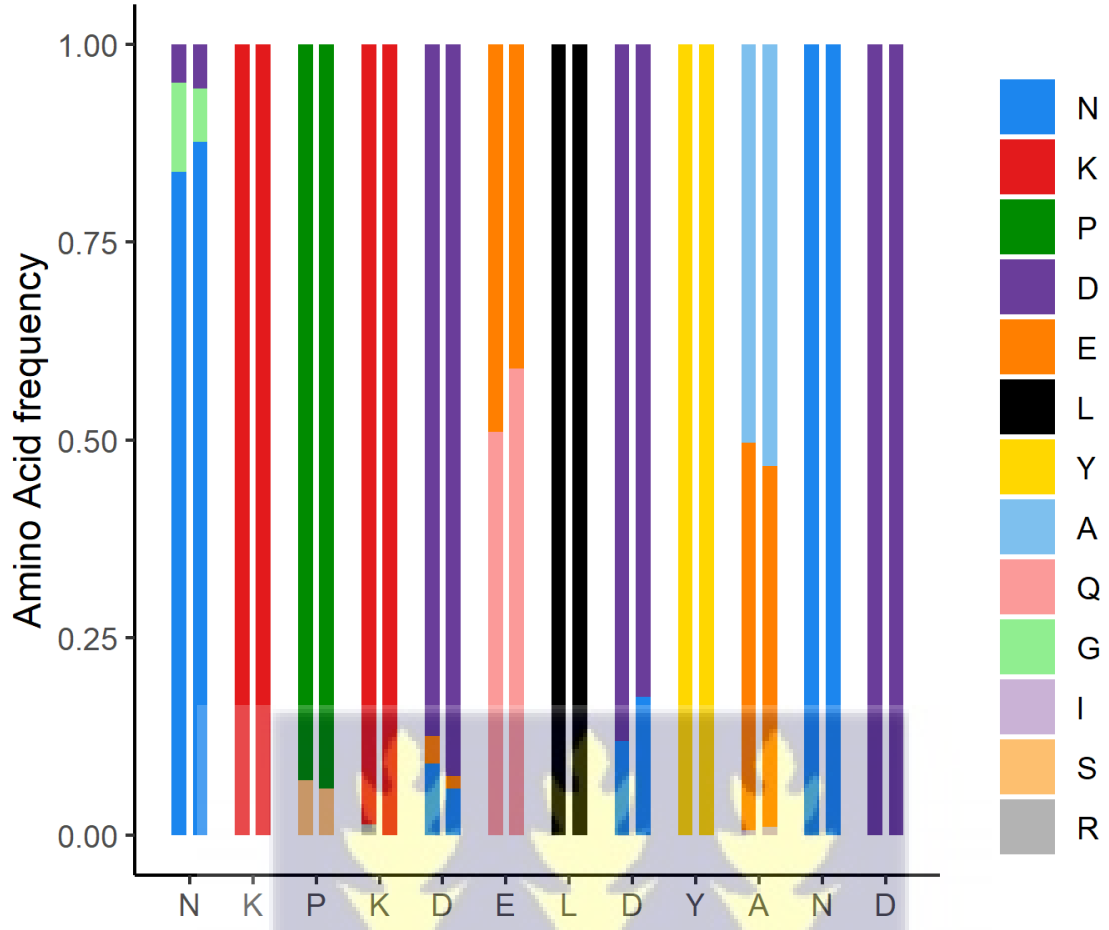


Figure 16. Th3R amino acid difference with reference to the 3D7 vaccine strain

The amino acids (on the horizontal axis) have two bars, Begoro (left) and Cape Coast (right). Change in the color of the bar indicates change in amino acid. Some amino acids remained stable or unchanged, while others have two (2) or more changes.

Twelve (12) Th3R haplotypes found in Begoro are also in Cape Coast (Appendix 9); 96.5% of the Th3R haplotypes in Begoro are shared with Cape Coast and 99.3% of the Th3R haplotypes in Cape Coast are also in Begoro (Figure 17).

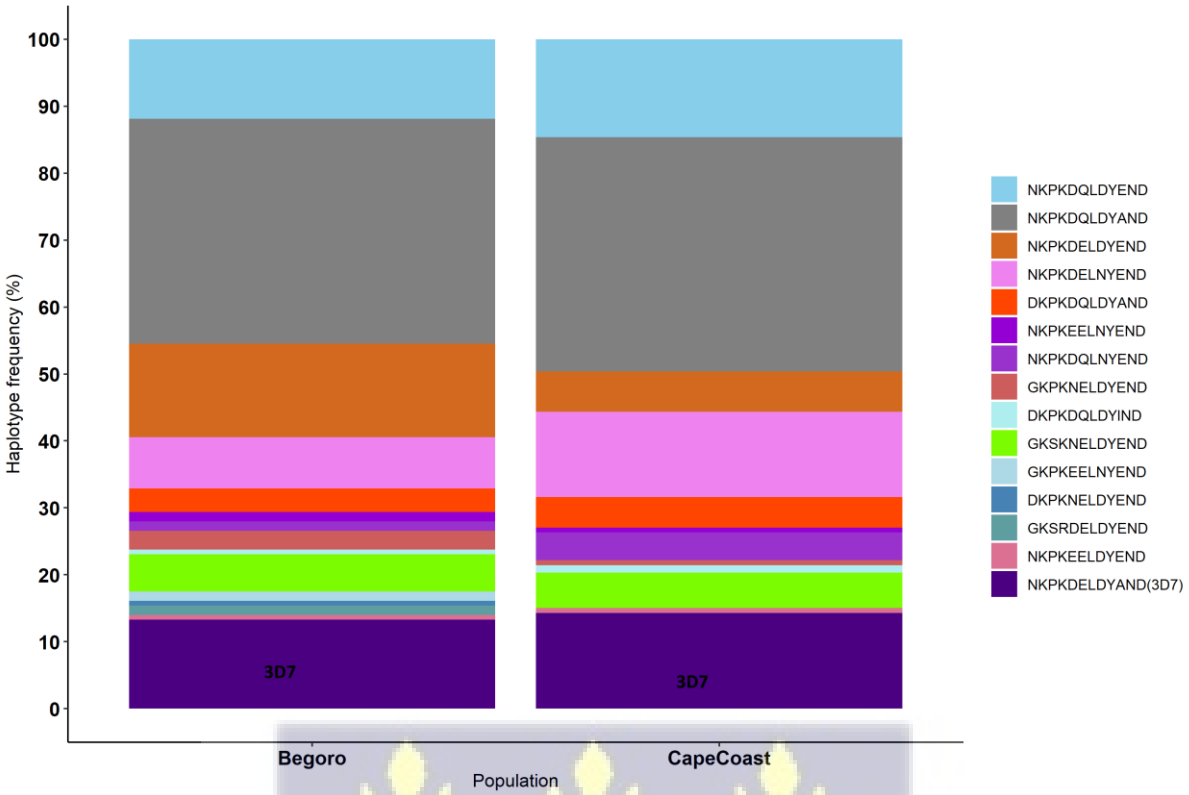


Figure 17. Shared Th3R haplotypes for Begoro and Cape Coast
About 96.5% of the Th3R haplotypes in Begoro are also found in Cape Coast.

4.2.3 Network Analysis of the C-terminal

The haplotype diversity of CSP in Begoro and Cape Coast was also examined across the C-terminal region (937-1144 nucleotides) for all 411 DNA sequences. A total of sixty (60) haplotypes were observed, 44 haplotypes in Begoro, 50 haplotypes in Cape Coast, and 33 haplotypes shared in both sites. A CSP haplotype network of the C-terminal region for Begoro and Cape Coast was constructed by POPART (Leigh et al., 2015) (Figure 18).

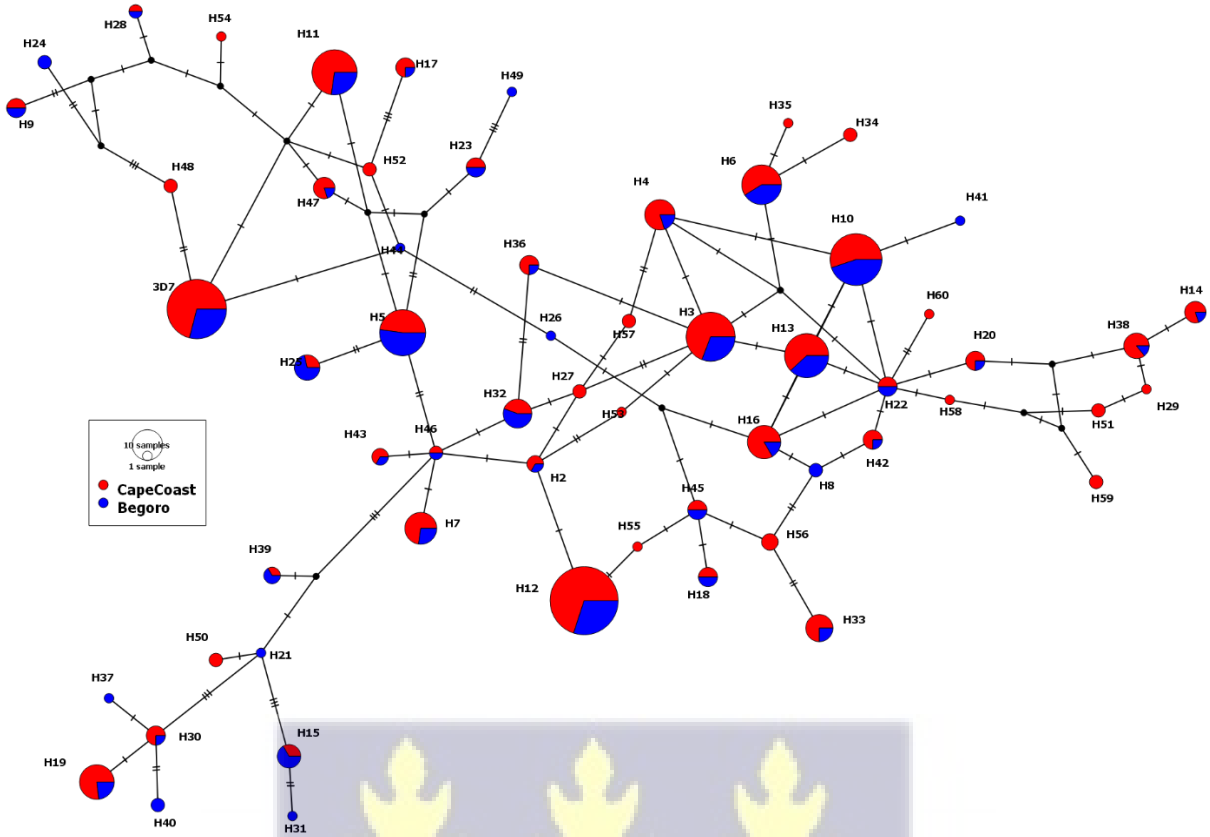


Figure 18. Haplotype Network for Begoro and Cape Coast

Haplotypes are labeled 3D7, H2 through H60. Most of the haplotypes are shared between Begoro (blue) and Cape Coast (red). The size of the circle depicts the frequency of the haplotype in the population.

Tajima's D and Fu & Li's F* were done using DnaSP (version 6.12.03) to test for neutrality of the C-terminal region (Table 3). The fixation index was calculated to examine genetic subdivisions between the two studied populations. The fixation index is -0.00112, which shows no population subdivision.

Table 3. Test of Neutrality

Study sites	N	H	S	Hd ± SD	π ± SD	Fu & Li's F*	Tajima's D
Begoro	143	44	23	0.957 ± 0.006	0.0273 ± 0.00104	0.18953	0.24033
Cape Coast	268	50	23	0.951 ± 0.005	0.0264 ± 0.00076	1.06459	0.57350

N, Number of isolated analyzed per site; H, number of haplotypes; S, number of segregating sites; Hd, Haplotype diversity; pi, observed average pairwise nucleotide diversity; SD, standard deviation

4.3 Temporal Stability of CSP Haplotype within Ghana

A customized python script was used to filter out the sequences and then generate and distribute Th2R and Th3R haplotypes for the two sites into three time periods in which the samples were collected (2014, 2015, and 2016). A total of 272 sequences were obtained and used for the temporal analysis (Table 4).

Table 4. Temporal Analysis

Number of sequences and Th2R and Th3R haplotypes for Begoro and Cape Coast in 2014, 2015, and 2016.

	2014	2015	2016
No. of Sequences	119	71	82
Th2R haplotypes	23	20	20
Th3R haplotypes	12	10	11

4.3.1 Th2R Temporal Analysis

A total of 31 Th2R haplotypes were observed from 2014 to 2016; 23 of these haplotypes were in 2014, 20 in 2015, and 20 in 2016. A decreasing trend is observed 54.8% (17/31) of the

haplotypes, while 35.5% (11/31) of the haplotypes in 2014 and 2015 did not prevail in 2016. The 3D7 reference strain decreased in frequency from 15 to 7 during the study period; 13% (4/31) of the haplotypes only appeared for the first time in 2016 (Appendix 10). The heat map was constructed to illustrate the trend of CSP Th2R haplotypes over the three years of this study (Figure 19).

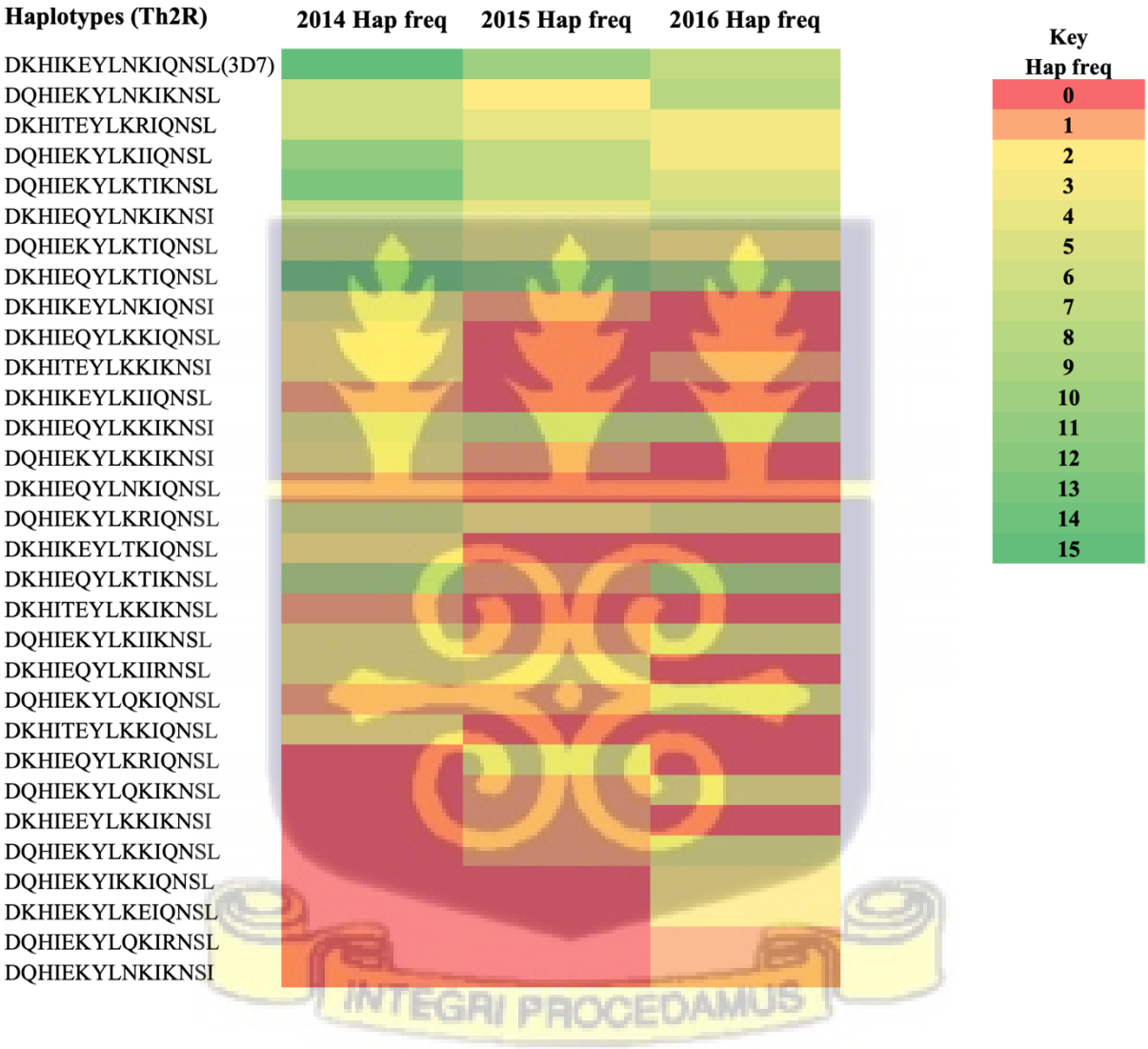


Figure 19. Heat map of Th2R haplotype frequency in 2014, 2015, and 2016

The heat map shows the trend of Th2R haplotype frequency from 2014 to 2016 in Begoro and Cape Coast. Most haplotypes (54.8%) are decreasing in frequency. (Key shows the color code for each haplotype frequency).

4.3.2 Th3R Temporal Analysis

There were 14 Th3R haplotypes observed from 2014 to 2016 (12 in 2014, 10 in 2015, and 11 in 2016). About 64.3% (9/14) of these haplotypes were decreasing in frequency over the three-year period, while 21.4% (3/14) of the haplotypes observed in 2014 and 2015 were no longer in the population in 2016 (DKPKDQLDYIND, GKPKEELNYEND, and GKSKDELDYEND). GKPKNELDYEND appeared only for the first time in 2016. The frequency of the 3D7 reference strain decreased 20 in 2014 to 8 in 2016 (Appendix 11). The heat map below illustrates the trend of the frequency of Th3R haplotypes from 2014 to 2016 (Figure 20).

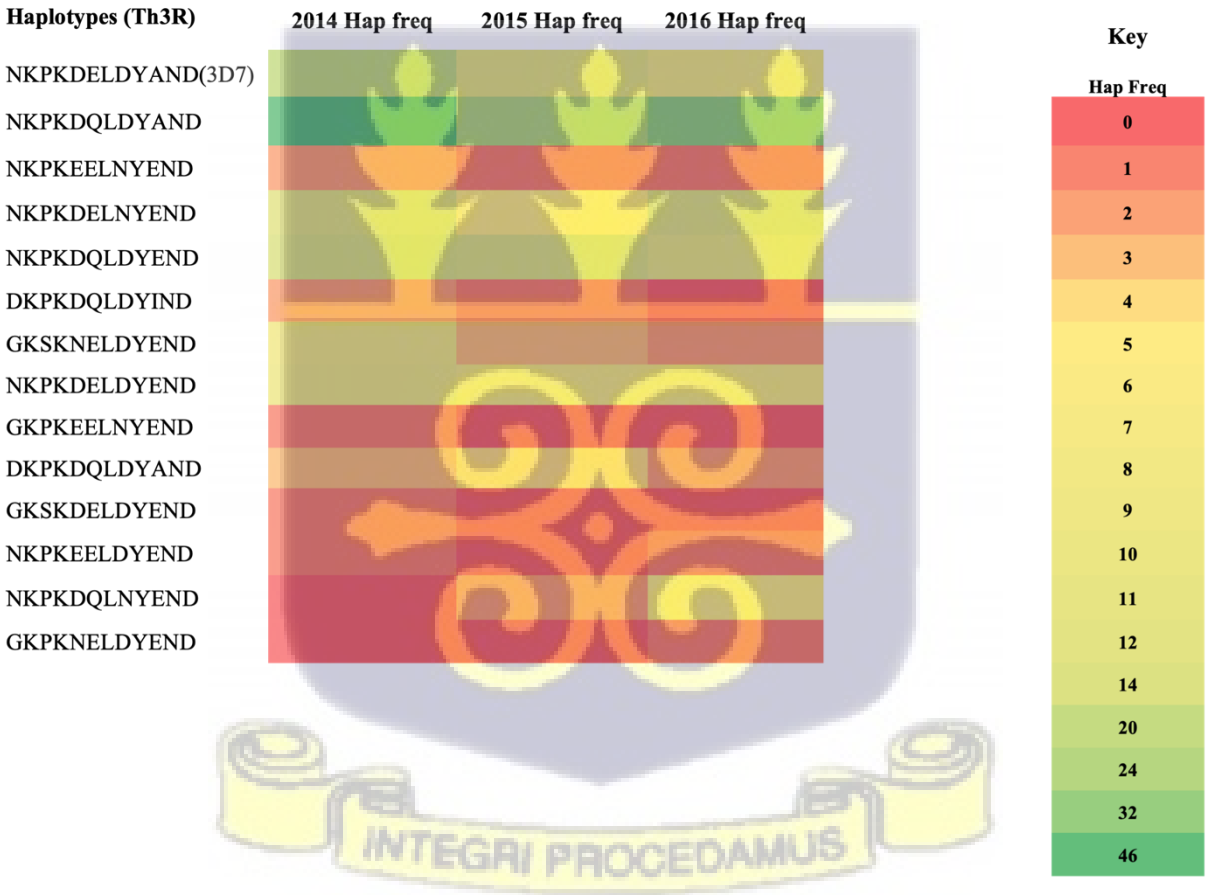


Figure 20. Heat map of Th3R haplotype frequency in 2014, 2015, and 2016

The heat map shows the trend of Th3R haplotype frequency from 2014 to 2016. A decrease in frequency for 64.3% of haplotypes is observed, indicated by color change in the heat map. (Key shows the color code for each haplotype frequency).

4.4 To compare CSP variants in Ghana to Kenya and Malawi

The RTS,S/AS01 malaria vaccine is currently being rolled out on a small scale in selected regions of Ghana, Kenya and Malawi as part of the routine vaccination program for eligible children. This study sought to compare the CSP haplotypes in Ghana to CSP haplotypes in the other two countries that are currently implementing the malaria vaccine. To do so, CSP sequenced data for Kenya and Malawi were mined from Malaria Genomic Epidemiology Network (MalariaGEN; www.malariagen.net). A customized Python script was used on monoclonal aligned sequences to generate and analyze the haplotypes across the C-terminal region (937-1140 nucleotides position) (Appendix 12), and DnaSP (version 6.12.03) to determine the CSP diversity statistics for the three (3) populations. The haplotypes are represented in a network constructed by POPART (Leigh et al., 2015).

The average number of nucleotide difference (K) and the number of segregating sites (S) is more in Ghana (K=5.55258; S=25) than in Kenya (K=2.19718; S=11) and Malawi (K=3.03173; S=13). The nucleotide diversity is also higher in Ghana (π =0.02722) than in Kenya (π =0.01077) and Malawi (π =0.01486). Ghana has 60 haplotypes, while Kenya has 14 and Malawi has 27 (Table 5). The haplotype diversity is higher in Ghana (Hd=0.95301) than in Kenya and Malawi (Hd=0.85681 and Hd=0.89224), respectively. The genetic variability between and within population was calculated for evidence of population differentiation and the fixation index (F_{ST}) value was 0.31425. This means there is no clear evidence of population structure.

Table 5. CSP diversity for the three (3) populations (Ghana, Kenya and Malawi)

	Ghana	Kenya	Malawi
n	411	72	185
S	25	11	13
h	60	14	27
Hd	0.95301	0.85681	0.89224
K	5.55258	2.19718	3.03173
π	0.02722	0.01077	0.01486

n = number of sequences; S = number of segregating sites; h = number of unique haplotypes; Hd = haplotype diversity; K = average number of nucleotide differences; π = nucleotide diversity

In order to examined the extent of the CSP diversity and how similar the three populations are, 668 sequences from Ghana (n=411), Kenya (n=72) and Malawi (n=185) were analyzed across the C-terminal region of the CSP. There were 60 haplotypes in Ghana, 14 in Kenya, and 27 in Malawi. A haplotype network was constructed to show the relationship among these haplotypes (Figure 21). Except the shared 3D7 vaccine strain, the CSP haplotypes are unique to each country.

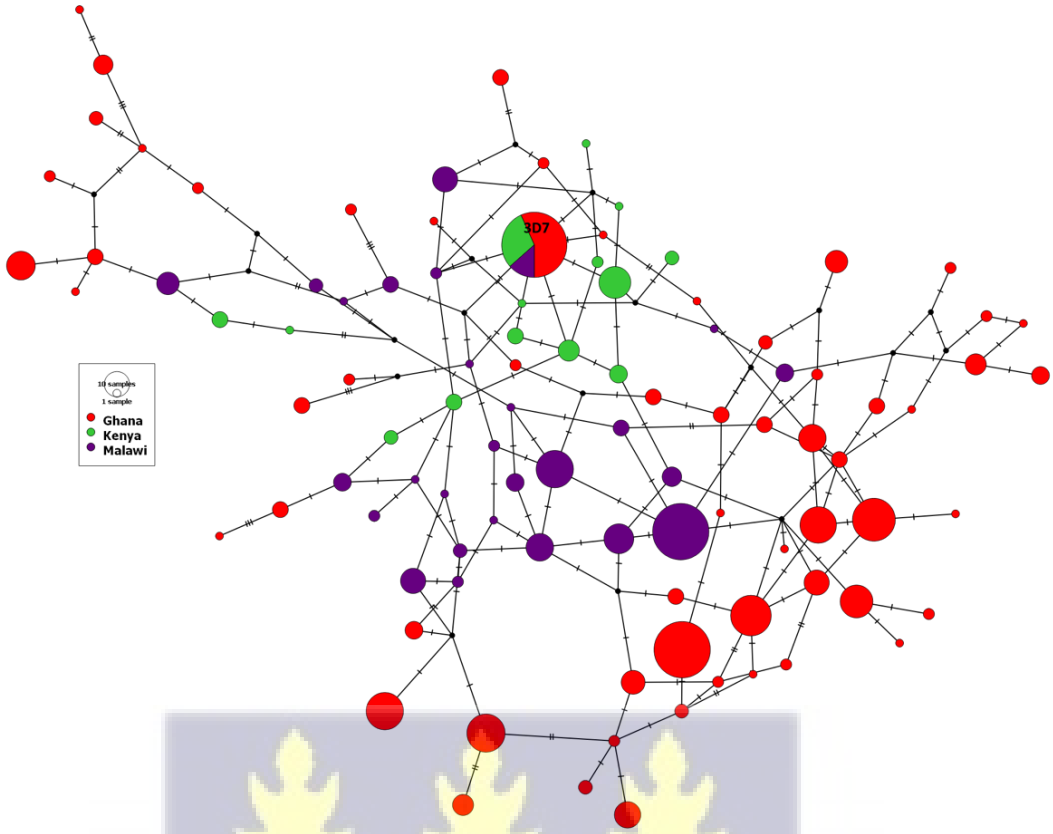


Figure 21. Haplotype Network of CSP C-terminal for Ghana, Kenya, and Malawi.

Expect the 3D7 vaccine haplotype, no other haplotype is shared among the three countries. Each circle represents a haplotype and scaled based on its frequency. The 3D7 vaccine strain is labeled "3D7" and the haplotypes are color-coded (red for haplotypes from Ghana, green

4.5 Contribution of HLA class II haplotypes to control of CSP variants

A total of 235 DNA sequences from Begoro (n=160) and Cape Coast (n=75) were analyzed to determine the contribution of human leukocyte antigen (HLA) class II to control of CSP variants. A customized python script was used and HLA sequences with quality score below 90% and average coverage below 10 were excluded from the analysis.

First, HLA class II haplotypes were grouped into superfamily for Begoro and Cape Coast and their frequencies determined (Appendix 13).

Second, the Th2R and HLA class II haplotypes were merged on sample ID and analyzed for correlation between HLA class II and Th2R haplotypes. A heat map was plotted using R package.

The heat map (Figure 22) showed no correlation between the Th2R3D7 vaccine strain and HLA-DRB1 subtypes. However, strong correlation is observed between other Th2R haplotypes and HLA-DRB1 subtypes.

The Th2R 3D7 vaccine haplotype is observed to weakly correlate with HLA-DQA1 subtypes (01:01/01:01, 01:02/02:01, 01:02/08:04, 03:01/04:01, and 03:01/05:01) and strongly correlate with (01:01/05:01) in the heat map represented in Figure 23. DQHIEKYLKIIKNSL also correlates strongly with subtype (01:01/05:01). Strong correlation is also observed between HLA-DQA1 subtypes and other Th2R haplotypes. For example, DQHIEKYLQKIQNSL correlates strongly with both 01:02/02:01 and 04:01/04:01 subtypes (Figure 23).

There is a stronger correlation observed between the Th2R 3D7 vaccine haplotype and HLA-DPB1 subtype (01:01/17:01), which also shows a strong correlation with DKHIEQYLKTIKNSL and a weak correlation with DKHIEQYLKTIQNSL (Figure 24).

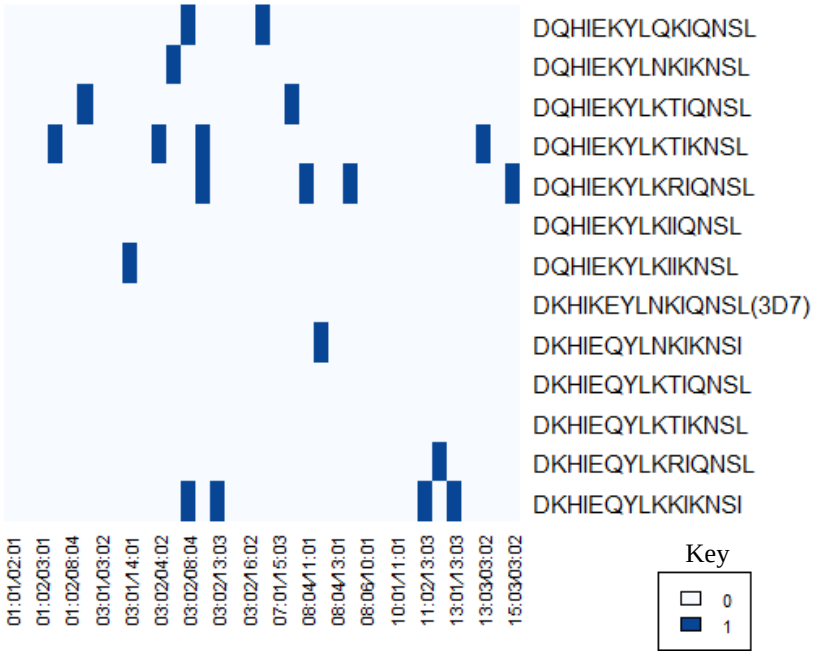


Figure 22. Correlation between HLA-DRB1 and Th2R haplotypes

A heat map showing the relationship between Th2R haplotypes (vertical) and HLA-DRB1 subtypes (horizontal). No correlation is observed between HLA-DRB1 subtypes and Th2R 3D7 haplotype. [Key: No correlation = 0; Correlation = 1]

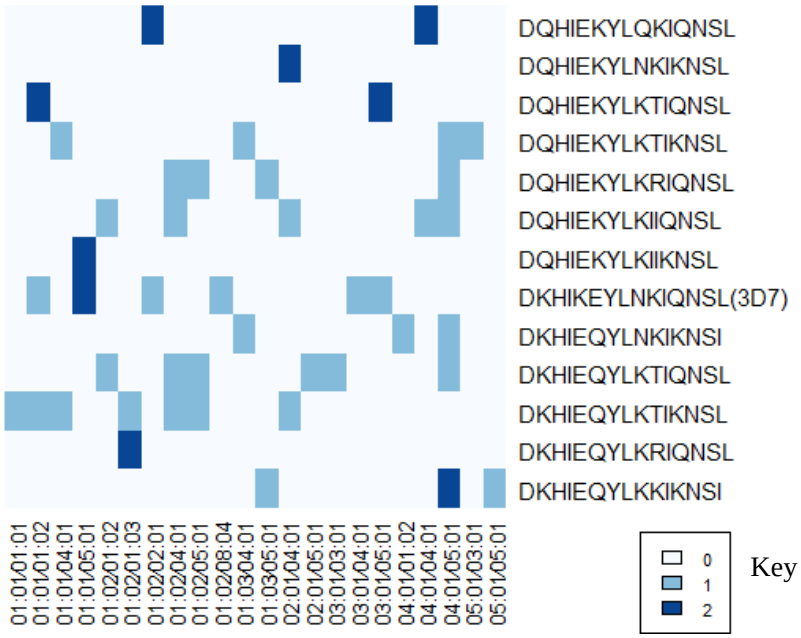


Figure 23: Correlation between HLA-DQA1 and Th2R haplotypes
A heat map showing the relationship between Th2R haplotypes (vertical) and HLA-DQA1 subtypes (horizontal). Correlation is observed between some HLA-DQA1 subtypes and Th2R 3D7 haplotype, but at different degree (colored bars) [Key: No correction = 0; Correction = 1 (weak) & 2 (strong)]

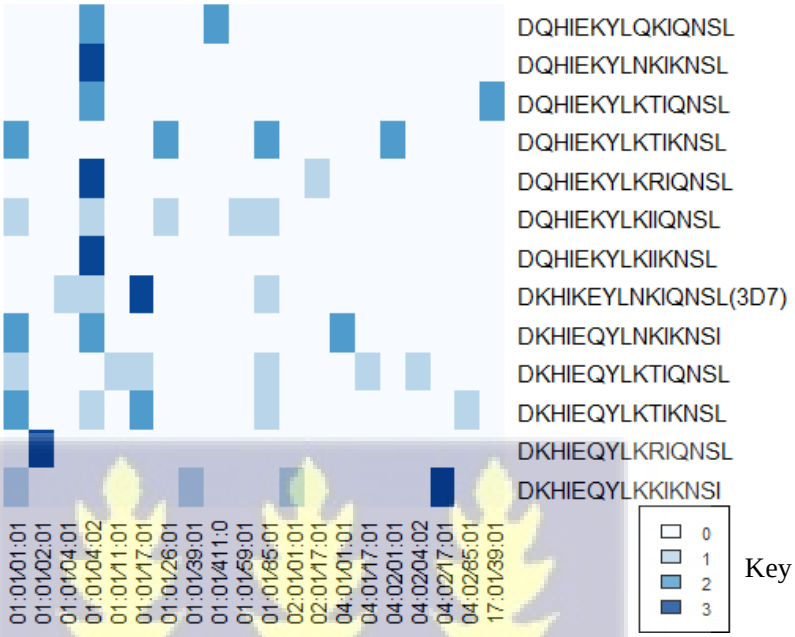


Figure 24: Correlation between HLA-DPB1 and Th2R haplotypes

A heat map showing the relationship between Th2R haplotypes (vertical) and HLA-DPB1 subtypes (horizontal). Correlation is observed between some HLA-DPB1 subtypes and Th2R 3D7 haplotype at different degree (colored bars) [Key: No correction = 0; Correction = 1 (weak), 2 (strong) & 3 (stronger)]

CHAPTER FIVE

5 DISCUSSION, CONCLUSION, AND RECOMMENDATION

5.1 Discussion

Malaria is an infectious disease that has existed for centuries and continues to take half of a million lives every year. In 2020, there were 241 million cases and 627,000 deaths from malaria, according to the WHO 2021 World Malaria Report. The main victims of malaria are children younger than five (5) years old in the tropical regions of the world. Pregnant women in these regions are also at risk of malaria due to reduced immunity in pregnancy.

The fight to end malaria goes back more than 100 years, with failed attempts in most regions of the world. In 1955, global effort to eradicate malaria gained a lot of momentum when WHO launched the Global Malaria Eradication Program (GMEP), only to be abandoned 14 years later after malaria reemerged in some regions with no transmission. The World Health Assembly recognized that eradication was not feasible in the short term for many countries and recommended control of malaria be encouraged (WHO, 1973). We have seen renewed efforts to reduce the mortality and burden associated with malaria infection over the past two decades. The use of mosquito bed net as a strategy to reduce transmission has scaled up since 2000, but transmission among bed net and non-net users got higher because the mosquitoes have changed their biting habits (Thomsen et al., 2017).

Vaccine is an effective tool for the control and elimination of infectious diseases. Vaccine has been used to eradicate smallpox (Henderson, 1987) and polio (John, 2009) and eliminate measles (Moss et al., 2006). There is a need to add an effective malaria vaccine to current interventions in order to eliminate and eradicate malaria.

The Malaria Vaccine Initiative (MVI) developed the malaria vaccine roadmap which outlines a path for accelerating the development of malaria vaccines. The vision of the MVI is to develop and license safe and effective vaccines that prevents disease and death caused by *Plasmodium falciparum* and *Plasmodium vivax* malaria, and prevent the transmission of malaria parasite, thereby leading to elimination and eradication of malaria. The two objectives of the roadmap are developing malaria vaccines with at least 75 percent protective efficacy against clinical malaria and malaria vaccines that reduce transmission of malaria parasite to substantially reduce the incidence of human malaria infection (Moorthy et al., 2013).

RTS,S/AS01, which was created by scientists in GSK laboratories in 1987, has been licensed as a vaccine against malaria caused by *P. falciparum* but is still being piloted on a small scale in selected regions of Ghana, Kenya, and Malawi after a positive opinion by the European Medicines Agency (EMA, 2015). But the efficacy of the current vaccine is low (36.3%) and ongoing research is investigating this suboptimal protection. The target of RTS,S/AS01 is *Plasmodium falciparum* circumsporozoite protein (CSP), but this polymorphic protein may be a reason for the poor performance of the vaccine (Gandhi et al., 2014; Zeeshan et al., 2012). A study by Neafsey et al., (2015) showed that RTS,S/01 performed well against parasite population that matched the vaccine haplotype, suggesting the vaccine may be haplotype-specific. Predicated on this knowledge, this study aimed to map the spatial distribution of CSP haplotypes in Begoro and Cape Coast in order to predict the performance of RTS,S/AS01 in these regions.

To do so, blood samples collected from 6 months old to 14 years old children who were brought to Begoro District Hospital in Begoro and Ewin Polyclinic in Cape Coast by their parents/guardians and tested positive for malaria by microscopy were used. The samples were collected in 2014, 2015, and 2016.

Sixty-one (61) Th2R and 28 Th3R haplotypes were observed out of 411 sequences analyzed (Table 2). The Th2R haplotype is almost evenly distributed between Begoro and Cape Coast, 50.8% (31/61) and 49.2% (30/61), respectively. Th3R haplotypes are also almost even between Begoro and Cape Coast, 53.6% (15/28) and 46.4% (13/28), respectively. Notably, 83.9% (26/31) of Th2R haplotype in Begoro are found in Cape Coast population and 80% (12/15) of Th3R haplotypes in Begoro are also in Cape Coast (Figure 14 and Figure 17). Although these two populations are in two different ecological zone, the population is homogenous, with no clear evidence of population structure (Figure 18 and Table 3). There is movement of people between these two regions and this frequent movement could be responsible for the flow of genetic materials which can result to the spread of drug-resistant gene and parasite population that is resistant the vaccine. The two sites are approximately 255 km apart (Figure 9).

Difference in malaria transmission corresponds with parasite diversity (Babiker et al., 2000; Yuan et al., 2013). This is consistent with our results which showed higher parasite diversity in Begoro than Cape Coast. Malaria transmission in Begoro is high while it is low to moderate in Cape Coast.

For the RTS,S/AS01 to make a significant impact in a region, the 3D7 vaccine haplotype must be the most prevalent haplotype since the vaccine is haplotype-specific. The results showed that the 3D7 haplotype in the Th2R and Th3R epitopes in Begoro represents 8.4% (12/143) and 13.3% (19/143), respectively. In Cape Coast, it represents 14.2% (27/268) in the Th2R and Th3R epitopes, respectively (Figure 12 and Figure 15). This means the RTS,S/AS01 vaccine may not be an effective vaccine against malaria in Begoro and Cape Coast. This finding is consistent with previous findings (Gandhi et al., 2014; Zeeshan et al., 2012).

There are non-synonymous amino acid substitutions ranging from 1 to 6 at the Th2R epitope and 1 to 4 at the Th3R epitopes (Figure 13 and Figure 16). This result is the same in both of

the studied populations. Protein structure may influence the polymorphism of the circumsporozoite gene and render CS-based vaccines ineffective (Escalante et al., 2002). We know the Th2R epitope is more polymorphic than the Th3R, and this is why there are more amino acid substitutions at the Th2R epitope than the Th3R epitope (Malik et al., 1991; Zeeshan et al., 2012).

RTS,S/AS01 performs better when the circulating parasite population matches the vaccine haplotype (Neafsey et al., 2015). As such, it is also important to monitor the frequency of the circulating parasite haplotypes to give an insight on the performance of the vaccine over time. Malaria-positive samples were collected in 2014, 2015, and 2016, and 272 sequences analyzed to determine the temporal stability of CSP.

There was more sequence diversity in 2014 than 2015 and 2016 but this did not impact the haplotype frequencies (Table 4), similarly observed in previous study (Jalloh et al., 2006). A 53% decline in the 3D7 Th2R haplotype and 60% decline in 3D7 Th3R haplotype were observed from 2014 to 2016 (Figure 19 and Figure 20). The higher the 3D7 haplotype that matches the RTS,S/AS01 vaccine haplotype in the population, the higher the efficacy of the vaccine (Neafsey et al., 2015). A decline in 3D7 Th2R and Th3R haplotypes over a period of time may also result to decline of vaccine efficacy, which will have a serious implication for this novel vaccine.

Interestingly, 35.5% and 21% of Th2R and Th3R haplotypes, respectively, observed in 2014 and 2015 were no longer circulating in the population in 2016 (Figure 19 and Figure 20). This is as a result of selection on these parasites which may be caused by host immunity and transmission patterns (Duffy et al., 2015). Such selection will definitely impact the efficacy of a vaccine that is suboptimal if the 3D7 Th2R and Th3R haplotypes are affected.

It is also important to compare the CSP haplotypes in Ghana to Kenya and Malawi since these are the countries that are currently piloting the RTS,S/AS01 vaccine. Sequences were mined from MalariaGEN for Kenya and Malawi and analyzed. The results showed that other than the vaccine 3D7 strain, the three countries do not share any other haplotypes (Figure 21). This could be due to the low nucleotide diversity that was observed in these countries. Ghana has 59 unique haplotypes, Kenya has 13, and Malawi has 26. Such haplotypes were previously observed in Malawi (Bailey et al., 2012) and their uneven geographic polymorphism may impact CS-based vaccine (Escalante et al., 2002).

Although the haplotype diversity is higher in Ghana compared to Kenya and Malawi (Table 5), the RTS,S/AS01 vaccine may perform better in Ghana compared to Kenya and Malawi because the frequency of the vaccine 3D7 haplotype is higher in Ghana than in Kenya and Malawi. Previous studies suggest that RTS,S/AS01 has a better performance against malaria parasites that matched the CSP allele.

The human leukocyte antigens (HLA) mediate an individual's response to antigens. HLA class I presents endogenous peptides to CD+8 cells while class II presents exogenous peptides to CD+4 cells. HLA, like CSP, is highly polymorphic and we do not know what drives this polymorphism. The study aimed to investigate whether any HLA class II variant was associated with a specific Th2R haplotype. The study was however not powered for further statistical analysis of association. However, heat map showed correlation between HLA class II haplotypes and Th2R haplotype.

Particularly, there was no correlation between the 3D7 vaccine haplotype and HLA-DRB1 which means HLA-DRB1 may not present the 3D7 haplotype to CD+4 T cells (Figure 22). They will rather present other Th2R haplotypes with which they correlate and the vaccine may underperform in population with higher frequency of HLA-DRB1 haplotype.

There is a correlation between the Th2R3D7 haplotype and HLA-DQA1 and HLA-DPB1 (Figure 23 and Figure 24). However, other Th2R haplotypes showed a much stronger relationship with HLA-DQA1 and HLA-DPB1, and these Th2R haplotypes with much stronger relationship with HLA-DQA1 and HLA-DPB1 will compete with 3D7 haplotype for antigen presentation, thereby affecting vaccine efficacy.

Mining sequences for Kenya and Malawi from the repository (MalariaGEN) in order to compare the CSP variants in Ghana to these countries is a limitation to this study. These mined sequences may have affected our result for that specific objective. Another limitation is the lack of statistical power to associate the HLA class II variants with the Th2R haplotypes.

5.2 Conclusion

The Th2R and Th3R haplotypes are almost evenly distributed in Begoro and Cape Coast. Although the two regions are ecologically different, the parasite population is homogenous with no evidence of population structure. The Th2R epitope is more polymorphic than the Th3R and this high polymorphism has resulted in increased non-synonymous substitution of 1 to 6 amino acid positions at the Th2R epitope, and 1 to 4 at the Th3R epitope.

The 3D7 vaccine haplotype is not the most prevalent haplotypes in Begoro and Cape Coast. Its Th2R haplotype is the fourth most prevalent in Begoro and second in Cape Coast, while its Th3R haplotype is the third most prevalent haplotype in Begoro and Cape Coast.

The frequency of the 3D7 Th2R haplotype reduced by 53% from 2014 to 2016, while Th3R reduced by 60% during the same period. Such decline in the haplotype that is the target of the RTS,S/AS01 do have vaccine implication. The Th2R and Th3R epitopes are under selection which may be as a result of host immunity or transmission intensity. This need to be further investigated.

The vaccine 3D7 haplotype is the only haplotype common in Ghana, Kenya, and Malawi, but difference in haplotype frequency, haplotype diversity and nucleotide diversity could see the vaccine perform better in one region than the other.

The vaccine 3D7 Th2R haplotype does not correlate with HLA-DRB1 but it does correlate with HLA-DQA1 and HLA-DPB1. Although 3D7 Th2R haplotype correlates with HLA-DQA1 and HLA-DPB1, other Th2R haplotypes showed a much stronger correlation with these HLA-II haplotypes than the 3D7 Th2R haplotype. They will compete with the 3D7 Th2R haplotype for presentation by HLA-II to CD4+ T cells, which could have implication on RTS,S/AS01 efficacy.

5.3 Recommendation

Continuous monitoring of circulating CSP variants, especially the 3D7 vaccine haplotype is highly recommended. This will track what impact of RTS,S/AS01 will have on the population. Also, this study must be expanded to other regions in Ghana, which will help to predict the performance to the RTS,S/AS01 and to obtain a larger sample set for the relationship between HLA type and CSP variants.

BIBLIOGRAPHY

Adams, J. C., & Tucker, R. P. (2000). The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. *Developmental dynamics: an official publication of the American Association of Anatomists, 218*(2), 280-299.

Alonso, S., Chaccour, C. J., Elobolobo, E., Nacima, A., Candrinho, B., Saifodine, A., . . . Zulliger, R. (2019). The economic burden of malaria on households and the health system in a high transmission district of Mozambique. *Malaria journal, 18*(1), 1-10.

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J.-F., . . . Ménard, R. (2008). Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell host & microbe, 3*(2), 88-96.

Aydemir, O., Janko, M., Hathaway, N. J., Verity, R., Mwandagalirwa, M. K., Tshefu, A. K., . . . Reimonn, T. (2018). Drug-resistance and population structure of Plasmodium falciparum across the Democratic Republic of Congo using high-throughput molecular inversion probes. *The Journal of infectious diseases, 218*(6), 946-955.

Babiker, H. A., Abdel-Muhsin, A. A., Hamad, A., Mackinnon, M. J., Hill, W. G., & Walliker, D. (2000). Population dynamics of Plasmodium falciparum in an unstable malaria area of eastern Sudan. *Parasitology, 120*(2), 105-111.

Baer, K., Klotz, C., Kappe, S. H. I., Schnieder, T., & Frevert, U. (2007). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. *PLoS pathogens, 3*(11), e171.

Bailey, J. A., Mvalo, T., Aragam, N., Weiser, M., Congdon, S., Kamwendo, D., . . . Juliano, J. J. (2012). Use of massively parallel pyrosequencing to evaluate the diversity of and selection on Plasmodium falciparum csp T-cell epitopes in Lilongwe, Malawi. *The Journal of infectious diseases, 206*(4), 580-587.

Bano, N., Romano, J. D., Jayabalasingham, B., & Coppens, I. (2007). Cellular interactions of Plasmodium liver stage with its host mammalian cell. *International journal for parasitology, 37*(12), 1329-1341.

Beeson, J. G., Drew, D. R., Boyle, M. J., Feng, G., Fowkes, F. J. I., & Richards, J. S. (2016). Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS microbiology reviews, 40*(3), 343-372.

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., . . . Eckhoff, P. A. (2015). The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature, 526*(7572), 207.

Bongfen, S. E., Ntsama, P. M., Offner, S., Smith, T., Felger, I., Tanner, M., . . . Silvie, O. (2009). The N-terminal domain of Plasmodium falciparum circumsporozoite protein represents a target of protective immunity. *Vaccine, 27*(2), 328-335.

Bousema, T., Dinglasan, R. R., Morlais, I., Gouagna, L. C., van Warmerdam, T., Awono-Ambene, P. H., . . . Tchuinkam, T. (2012). Mosquito feeding assays to determine the infectiousness of naturally infected Plasmodium falciparum gametocyte carriers. *PLoS One, 7*(8), e42821.

Carter, R., & Nijhout, M. M. (1977). Control of gamete formation (exflagellation) in malaria parasites. *Science, 195*(4276), 407-409.

Casares, S., Brumeanu, T.-D., & Richie, T. L. (2010). The RTS, S malaria vaccine. *Vaccine, 28*(31), 4880-4894.

Centers for Disease & Prevention. (2005). Late relapse of Plasmodium ovale malaria--Philadelphia, Pennsylvania, November 2004. *MMWR. Morbidity and mortality weekly report, 54*(48), 1231-1233.

Chin, W., Contacos, P. G., Collins, W. E., Jeter, M. H., & Albert, E. (1968). Experimental mosquito-transmission of Plasmodium knowlesi to man and monkey. *American Journal of Tropical Medicine and Hygiene, 17*(3), 355-358.

Churcher, T. S., Bousema, T., Walker, M., Drakeley, C., Schneider, P., Ouédraogo, A. L., & Basáñez, M.-G. (2013). Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. *Elife, 2*, e00626.

Clyde, D. F., Most, H., McCarthy, V. C., & Vanderberg, J. P. (1973). Immunization of man against sporozoite-induced falciparum malaria. *American journal of medical sciences, 266*(3), 169-177.

Coetzee, M., Hunt, R. H., Wilkerson, R., Della Torre, A., Coulibaly, M. B., & Besansky, N. J. (2013). Anopheles coluzzii and Anopheles amharicus, new members of the Anopheles gambiae complex. *Zootaxa, 3619*(3), 246-274.

Coppi, A., Natarajan, R., Pradel, G., Bennett, B. L., James, E. R., Roggero, M. A., . . . Sinnis, P. (2011). The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *Journal of Experimental Medicine, 208*(2), 341-356.

Cowman, A. F., & Crabb, B. S. (2006). Invasion of red blood cells by malaria parasites. *Cell, 124*(4), 755-766.

Cox-Singh, J., Davis, T. M. E., Lee, K.-S., Shamsul, S. S. G., Matusop, A., Ratnam, S., . . . Singh, B. (2008). Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clinical infectious diseases, 46*(2), 165-171.

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L. S., . . . Pierce, S. K. (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annual review of immunology, 32*, 157-187.

Crompton, P. D., Pierce, S. K., & Miller, L. H. (2010). Advances and challenges in malaria vaccine development. *The Journal of clinical investigation, 120*(12), 4168-4178.

Cuadros, D. F., Branscum, A. J., & Crowley, P. H. (2011). HIV–malaria co-infection: effects of malaria on the prevalence of HIV in East sub-Saharan Africa. *International journal of epidemiology*, *40*(4), 931-939.

Datoo, M. S., Natama, M. H., Somé, A., Traoré, O., Rouamba, T., Bellamy, D., . . . Ouedraogo, F. (2021). Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *The Lancet*, *397*(10287), 1809-1818.

Denny, P. W., Preiser, P. R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., . . . Williamson, D. H. (1996). Complete Gene Map of the Plastid-like DNA of the Malaria ParasitePlasmodium falciparum. *Journal of molecular biology*, *261*(2), 155-172.

Desai, M., Ter Kuile, F. O., Nosten, F., McGready, R., Asamoa, K., Brabin, B., & Newman, R. D. (2007). Epidemiology and burden of malaria in pregnancy. *The Lancet Infectious Diseases*, *7*(2), 93-104.

Dhiman, N., Ovsyannikova, I. G., Cunningham, J. M., Vierkant, R. A., Kennedy, R. B., Pankratz, V. S., . . . Jacobson, R. M. (2007). Associations between Measles Vaccine Immunity and Single-Nucleotide Polymorphisms in Cytokine and Cytokine Receptor Genes. *The Journal of infectious diseases*, *195*(1), 21-29. doi:10.1086/510596

Didierlaurent, A. M., Collignon, C., Bourguignon, P., Wouters, S., Fierens, K., Fochesato, M., . . . Lambrecht, B. N. (2014). Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. *The Journal of Immunology*, *193*(4), 1920-1930.

Dilthey, A. T., Gourraud, P.-A., Mentzer, A. J., Cereb, N., Iqbal, Z., & McVean, G. (2016). High-accuracy HLA type inference from whole-genome sequencing data using population reference graphs. *PLoS computational biology*, *12*(10), e1005151.

Dluzewski, A. R., Mitchell, G. H., Fryer, P. R., Griffiths, S., Wilson, R. J., & Gratzer, W. B. (1992). Origins of the parasitophorous vacuole membrane of the malaria parasite, Plasmodium falciparum, in human red blood cells. *Journal of Cell Science*, *102*(3), 527-532.

Doolan, D. L., Dobaño, C., & Baird, J. K. (2009). Acquired immunity to malaria. *Clinical microbiology reviews*, *22*(1), 13-36.

Doud, M. B., Koksal, A. C., Mi, L.-Z., Song, G., Lu, C., & Springer, T. A. (2012). Unexpected fold in the circumsporozoite protein target of malaria vaccines. *Proceedings of the National Academy of Sciences*, *109*(20), 7817-7822.

Duffy, C. W., Assefa, S. A., Abugri, J., Amoako, N., Owusu-Agyei, S., Anyorigiya, T., . . . Awandare, G. A. (2015). Comparison of genomic signatures of selection on Plasmodium falciparum between different regions of a country with high malaria endemicity. *BMC genomics*, *16*(1), 1-11.

EMA. (2015). European Medicines Agency. Summary of opinion: Mosquirix

. Retrieved from http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/document_listing/document_listing_000395.jsp&mid

Escalante, A. A., Grebert, H. M., Isea, R., Goldman, I. F., Basco, L., Magris, M., . . . Lal, A. A. (2002). A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of Plasmodium falciparum from different transmission areas—XVI. Asembo Bay Cohort Project. *Molecular and Biochemical Parasitology, 125*(1-2), 83-90.

Fenner, F. (1989). Risks and benefits of vaccinia vaccine use in the worldwide smallpox eradication campaign. *Research in virology, 140*(5), 465-466.

Ferguson, D. J. P., Balaban, A. E., Patzewitz, E.-M., Wall, R. J., Hopp, C. S., Poulin, B., . . . Sinnis, P. (2014). The repeat region of the circumsporozoite protein is critical for sporozoite formation and maturation in Plasmodium. *PLoS One, 9*(12).

Galinski, M. R., Arnot, D. E., Cochrane, A. H., Barnwell, J. W., Nussenzweig, R. S., & Enea, V. (1987). The circumsporozoite gene of the Plasmodium cynomolgi complex. *Cell, 48*(2), 311-319.

Gandhi, K., Thera, M. A., Coulibaly, D., Traore, K., Guindo, A. B., Ouattara, A., . . . Plowe, C. V. (2014). Variation in the circumsporozoite protein of Plasmodium falciparum: vaccine development implications. *PLoS One, 9*(7).

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., . . . Bowman, S. (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature, 419*(6906), 498-511.

Gehde, N., Hinrichs, C., Montilla, I., Charpian, S., Lingelbach, K., & Przyborski, J. M. (2009). Protein unfolding is an essential requirement for transport across the parasitophorous vacuolar membrane of Plasmodium falciparum. *Molecular microbiology, 71*(3), 613-628.

Gilson, P. R., & Crabb, B. S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by Plasmodium falciparum merozoites. *International journal for parasitology, 39*(1), 91-96.

Golassa, L., Enweji, N., Erko, B., Aseffa, A., & Swedberg, G. (2013). Detection of a substantial number of sub-microscopic Plasmodium falciparum infections by polymerase chain reaction: a potential threat to malaria control and diagnosis in Ethiopia. *Malaria journal, 12*(1), 1-10.

Good, M. F., Berzofsky, J. A., & Miller, L. H. (1988). The T cell response to the malaria circumsporozoite protein: an immunological approach to vaccine development. *Annual review of immunology, 6*(1), 663-688.

Gordon, D., McGovern, T., Krzych, U., Cohen, J., Schneider, I., LaChance, R., . . . Slaoui, M. J. J. o. I. D. (1995). Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine. *The Journal of Infectious Diseases, 171*(6), 1576-1585.

Healy, S. A., Anderson, C., Swihart, B. J., Mwakingwe, A., Gabriel, E. E., Decederfelt, H., . . . Muratova, O. (2021). Pfs230 yields higher malaria transmission–blocking vaccine activity than Pfs25 in humans but not mice. *The Journal of clinical investigation, 131*(7).

Hellmann, J. K., Münter, S., Kudryashev, M., Schulz, S., Heiss, K., Müller, A.-K., . . . Frischknecht, F. (2011). Environmental constraints guide migration of malaria parasites during transmission. *PLoS pathogens, 7*(6), e1002080.

Henderson, D. A. (1987). Principles and lessons from the smallpox eradication programme. *Bulletin of the World Health Organization, 65*(4), 535.

Hickey, M. J., Valenzuela, N. M., & Reed, E. F. (2016). Alloantibody generation and effector function following sensitization to human leukocyte antigen. *Frontiers in immunology, 7*, 30.

Hviid, L. (2005). Naturally acquired immunity to Plasmodium falciparum malaria in Africa. *Acta tropica, 95*(3), 270-275.

Jalloh, A., Jalloh, M., & Matsuoka, H. (2009). T-cell epitope polymorphisms of the Plasmodium falciparum circumsporozoite protein among field isolates from Sierra Leone: age-dependent haplotype distribution? *Malaria journal, 8*(1), 120.

Jalloh, A., van Thien, H., Ferreira, M. U., Ohashi, J., Matsuoka, H., Kanbe, T., . . . Kawamoto, F. (2006). Sequence variation in the T-cell epitopes of the Plasmodium falciparum circumsporozoite protein among field isolates is temporally stable: a 5-year longitudinal study in southern Vietnam. *Journal of clinical microbiology, 44*(4), 1229-1235.

Jayabalasingham, B., Bano, N., & Coppens, I. (2010). Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. *Cell research, 20*(9), 1043-1059.

John, J. (2009). Role of injectable and oral polio vaccines in polio eradication. *Expert review of vaccines, 8*(1), 5-8.

Jongo, S. A., Church, L. W. P., Mtoro, A. T., Schindler, T., Chakravarty, S., Ruben, A. J., . . . Tumbo, A.-M. (2020). Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ vaccine in Tanzanian adults. *Clinical infectious diseases, 71*(11), 2849-2857.

Jongwutiwes, S., Tanabe, K., Hughes, M. K., Kanbara, H., & Hughes, A. L. (1994). Allelic variation in the circumsporozoite protein of Plasmodium falciparum from Thai field isolates. *The American journal of tropical medicine and hygiene, 51*(5), 659-668.

Karema, C., Aregawi, M. W., Rukundo, A., Kabayiza, A., Mulindahabi, M., Fall, I. S., . . . Cibulskis, R. (2012). Trends in malaria cases, hospital admissions and deaths following scale-up of anti-malarial interventions, 2000–2010, Rwanda. *Malaria journal*, *11*(1), 236.

Kariu, T., Yuda, M., Yano, K., & Chinzei, Y. (2002). MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. *The Journal of experimental medicine*, *195*(10), 1317-1323.

Kaslow, D. C., & Biernaux, S. (2015). RTS, S: toward a first landmark on the Malaria Vaccine Technology Roadmap. *Vaccine*, *33*(52), 7425-7432.

Kaufman, J. (2000). The simple chicken major histocompatibility complex: life and death in the face of pathogens and vaccines. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1400), 1077-1084.

Kirkman, L. A., & Deitsch, K. W. (2012). Antigenic variation and the generation of diversity in malaria parasites. *Current opinion in microbiology*, *15*(4), 456-462.

Kitchen, A. D., & Chiodini, P. L. (2006). Malaria and blood transfusion. *Vox sanguinis*, *90*(2), 77-84.

Kojin, B. B., Costa-da-Silva, A. L., Maciel, C., Henriques, D. A., Carvalho, D. O., Martin, K., . . . Capurro, M. L. (2016). Endogenously-expressed NH 2-terminus of circumsporozoite protein interferes with sporozoite invasion of mosquito salivary glands. *Malaria journal*, *15*(1), 153.

Kollars Jr, T. M., Phasuk, R., & Sattabongkot, J. (2004). Variation of circumsporozoite 26 and 29 genotypes of Plasmodium falciparum infecting patients and association with HLA-DQA allotypes in western Thailand. *Journal of Parasitology*, *90*(1), 182-184.

Kooij, T. W. A., Carlton, J. M., Bidwell, S. L., Hall, N., Ramesar, J., Janse, C. J., & Waters, A. P. (2005). A Plasmodium whole-genome synteny map: indels and synteny breakpoints as foci for species-specific genes. *PLoS pathogens*, *1*(4), e44.

Kouyate, B., Sie, A., Ye, M., De Allegri, M., & Müller, O. (2007). The great failure of malaria control in Africa: a district perspective from Burkina Faso. *PLoS medicine*, *4*(6).

Kumar, K. A., Sano, G.-i., Boscardin, S., Nussenzweig, R. S., Nussenzweig, M. C., Zavala, F., & Nussenzweig, V. (2006). The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. *Nature*, *444*(7121), 937-940.

Kurtovic, L., Reiling, L., Opi, D. H., & Beeson, J. G. (2021). Recent clinical trials inform the future for malaria vaccines. *Communications Medicine*, *1*(1), 1-5.

Lee, A. H., Symington, L. S., & Fidock, D. A. (2014). DNA repair mechanisms and their biological roles in the malaria parasite Plasmodium falciparum. *Microbiology and Molecular Biology Reviews*, *78*(3), 469-486.

Lee, P.-C., Terasaki, P. I., Takemoto, S. K., Lee, P.-H., Hung, C.-J., Chen, Y.-L., . . . Lei, H.-Y. (2002). All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation, 74*(8), 1192-1194.

Leigh, J. W., & Bryant, D. (2015). popart: full‐feature software for haplotype network construction. *Methods in Ecology and Evolution, 6*(9), 1110-1116.

Leroux-Roels, G., Leroux-Roels, I., Clement, F., Ofori-Anyinam, O., Lievens, M., Jongert, E., . . . Cohen, J. (2014). Evaluation of the immune response to RTS, S/AS01 and RTS, S/AS02 adjuvanted vaccines: randomized, double-blind study in malaria-naive adults. *Human vaccines & immunotherapeutics, 10*(8), 2211-2219.

Li, J., Wirtz, R. A., McConkey, G. A., Sattabongkot, J., & McCutchan, T. F. (1994). Transition of Plasmodium vivax ribosome types corresponds to sporozoite differentiation in the mosquito. *Molecular and Biochemical Parasitology, 65*(2), 283-289.

Li, X., Fasano, R., Wang, E., Yao, K.-T., & Marincola, F. (2009). HLA Associations with Nasopharyngeal Carcinoma. *Current molecular medicine, 9*, 751-765. doi:10.2174/156652409788970698

Li, Z.-K., Nie, J.-J., Li, J., & Zhuang, H. (2013). The effect of HLA on immunological response to hepatitis B vaccine in healthy people: a meta-analysis. *Vaccine, 31*(40), 4355-4361.

Lim, W. H., Chapman, J. R., Coates, P. T., Lewis, J. R., Russ, G. R., Watson, N., . . . Wong, G. (2016). HLA-DQ mismatches and rejection in kidney transplant recipients. *Clinical Journal of the American society of Nephrology, 11*(5), 875-883.

Lockyer, M. J., Marsh, K., & Newbold, C. I. (1989). Wild isolates of Plasmodium falciparum show extensive polymorphism in T cell epitopes of the circumsporozoite protein. *Molecular and Biochemical Parasitology, 37*(2), 275-280.

Madden, D. R. (1995). The three-dimensional structure of peptide-MHC complexes. *Annual review of immunology, 13*(1), 587-622.

Malik, A., Egan, J. E., Houghten, R. A., Sadoff, J. C., & Hoffman, S. L. (1991). Human cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein. *Proceedings of the National Academy of Sciences, 88*(8), 3300-3304.

Marsh, S. G. E., Albert, E. D., Bodmer, W. F., Bontrop, R. E., Dupont, B., Erlich, H. A., . . . Hurley, C. K. (2010). Nomenclature for factors of the HLA system, 2010. *Tissue antigens, 75*(4), 291.

Mejia, G. A., Alvarez, C. A., Pulido, H. H., Ramírez, B., Cardozo, C., Suarez, Y., . . . Uribe, A. M. (2006). *Malaria in a liver transplant recipient: a case report*.

Ménard, R., Sultan, A. A., Cortes, C., Altszuler, R., Van Dijk, M. R., Janse, C. J., . . . Nussenzweig, V. (1997). Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. *Nature, 385*(6614), 336-340.

Ménard, R., Tavares, J., Cockburn, I., Markus, M., Zavala, F., & Amino, R. (2013). Looking under the skin: the first steps in malarial infection and immunity. *Nature Reviews Microbiology*, *11*(10), 701-712.

Menendez, C., & Mayor, A. (2007). *Congenital malaria: the least known consequence of malaria in pregnancy*.

Miller, L. H., Ackerman, H. C., Su, X.-z., & Wellems, T. E. (2013). Malaria biology and disease pathogenesis: insights for new treatments. *Nature medicine*, *19*(2), 156-167.

Minassian, A. M., Silk, S. E., Barrett, J. R., Nielsen, C. M., Miura, K., Diouf, A., . . . White, M. T. (2021). Reduced blood-stage malaria growth and immune correlates in humans following RH5 vaccination. *Med*, *2*(6), 701-719.

Miura, K., Jongert, E., Deng, B., Zhou, L., Lusingu, J. P., Drakeley, C. J., . . . Vekemans, J. (2014). Effect of ingested human antibodies induced by RTS, S/AS01 malaria vaccination in children on Plasmodium falciparum oocyst formation and sporogony in mosquitoes. *Malaria journal*, *13*(1), 263.

Moorthy, V. S., & Ballou, W. R. (2009). Immunological mechanisms underlying protection mediated by RTS, S: a review of the available data. *Malaria journal*, *8*(1), 312.

Moorthy, V. S., Newman, R. D., & Okwo-Bele, J.-M. (2013). Malaria vaccine technology roadmap. *The Lancet*, *382*(9906), 1700-1701.

Mordmüller, B., Sulyok, M., Egger-Adam, D., Resende, M., de Jongh, W. A., Jensen, M. H., . . . Poulsen, L. (2019). First-in-human, randomized, double-blind clinical trial of differentially adjuvanted PAMVAC, a vaccine candidate to prevent pregnancy-associated malaria. *Clinical infectious diseases*, *69*(9), 1509-1516.

Moss, W. J., & Griffin, D. E. (2006). Global measles elimination. *Nature Reviews Microbiology*, *4*(12), 900-908.

Mukonka, V. M., Chanda, E., Haque, U., Kamuliwo, M., Mushinge, G., Chileshe, J., . . . Chaponda, M. (2014). High burden of malaria following scale-up of control interventions in Nchelenge District, Luapula Province, Zambia. *Malaria journal*, *13*(1), 153.

Mwakingwe-Omari, A., Healy, S. A., Lane, J., Cook, D. M., Kalhori, S., Wyatt, C., . . . Nason, M. (2021). Two chemoattenuated PfSPZ malaria vaccines induce sterile hepatic immunity. *Nature*, *595*(7866), 289-294.

Nájera, J. A., González-Silva, M., & Alonso, P. L. (2011). Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). *PLoS medicine*, *8*(1), e1000412.

Natarajan, K., Li, H., Mariuzza, R. A., & Margulies, D. H. (1999). MHC class I molecules, structure and function. *Reviews in immunogenetics*, *1*(1), 32-46.

Neafsey, D. E., Juraska, M., Bedford, T., Benkeser, D., Valim, C., Griggs, A., . . . Agbenyega, T. (2015). Genetic diversity and protective efficacy of the RTS, S/AS01 malaria vaccine. *New England Journal of Medicine, 373*(21), 2025-2037.

Nielsen, C. M., Vekemans, J., Lievens, M., Kester, K. E., Regules, J. A., & Ockenhouse, C. F. (2018). RTS, S malaria vaccine efficacy and immunogenicity during Plasmodium falciparum challenge is associated with HLA genotype. *Vaccine, 36*(12), 1637-1642.

Nonvignon, J., Aryeetey, G. C., Malm, K. L., Agyemang, S. A., Aubyn, V. N. A., Peprah, N. Y., . . . Aikins, M. (2016). Economic burden of malaria on businesses in Ghana: a case for private sector investment in malaria control. *Malaria journal, 15*(1), 1-10.

Ofori, M. F., Kploanyi, E. E., Mensah, B. A., Dickson, E. K., Kyei-Baafour, E., Gyabaa, S., . . . Ghansah, A. (2021). Ex vivo sensitivity profile of Plasmodium falciparum clinical isolates to a panel of antimalarial drugs in Ghana 13 years after national policy change. *Infection and Drug Resistance, 14*, 267.

Pasternak, N. D., & Dzikowski, R. (2009). PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum. *The international journal of biochemistry & cell biology, 41*(7), 1463-1466.

Poland, G. A., Ovsyannikova, I. G., Jacobson, R. M., & Smith, D. I. (2007). Heterogeneity in vaccine immune response: the role of immunogenetics and the emerging field of vaccinomics. *Clinical Pharmacology & Therapeutics, 82*(6), 653-664.

Posteraro, B., Pastorino, R., Di Giannantonio, P., Ianuale, C., Amore, R., Ricciardi, W., & Boccia, S. (2014). The link between genetic variation and variability in vaccine responses: systematic review and meta-analyses. *Vaccine, 32*(15), 1661-1669.

Pradel, G., & Frevert, U. (2001). Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. *Hepatology, 33*(5), 1154-1165.

Pringle, J. C., Carpi, G., Almagro-Garcia, J., Zhu, S. J., Kobayashi, T., Mulenga, M., . . . Norris, D. E. (2018). RTS, S/AS01 malaria vaccine mismatch observed among Plasmodium falciparum isolates from southern and central Africa and globally. *Scientific reports, 8*(1), 1-8.

Prothero, R. M. (1977). Disease and mobility: a neglected factor in epidemiology. *International journal of epidemiology, 6*(3), 259-267.

Rajagopalan, P. K., Jambulingam, P., Sabesan, S., Krishnamoorthy, K., Rajendran, S., Gunasekaran, K., . . . Prothero, R. M. (1986). Population movement and malaria persistence in Rameswaram Island: Foreword. *Social science & medicine, 22*(8), 879-886.

RCTP. (2011). First results of phase 3 trial of RTS, S/AS01 malaria vaccine in African children. *New England Journal of Medicine, 365*(20), 1863-1875.

RCTP. (2012). A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants. *New England Journal of Medicine, 367*(24), 2284-2295.

RCTP. (2015). 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, 386*(9988), 31-45.

Regules, J. A., Cicatelli, S. B., Bennett, J. W., Paolino, K. M., Twomey, P. S., Moon, J. E., . . . Qabar, A. N. (2016). Fractional third and fourth dose of RTS, S/AS01 malaria candidate vaccine: a phase 2a controlled human malaria parasite infection and immunogenicity study. *The Journal of infectious diseases, 214*(5), 762-771.

Richter, J., Franken, G., Mehlhorn, H., Labisch, A., & Häussinger, D. (2010). What is the evidence for the existence of Plasmodium ovale hypnozoites? *Parasitology research, 107*(6), 1285-1290.

Rivière, M., Tretiak, R., Levinton, C., Fitzsimon, C., & Leclerc, C. (1997). Economic benefits of a routine second dose of combined measles, mumps and rubella vaccine in Canada. *Canadian Journal of Infectious Diseases, 8*(5), 257-264.

Rodey, G. E., & Fuller, T. C. (1987). Public epitopes and the antigenic structure of the HLA molecules. *Critical reviews in immunology, 7*(3), 229-267.

Sachs, J., & Malaney, P. (2002). The economic and social burden of malaria. *Nature, 415*(6872), 680-685.

Semakula, H. M., Song, G., Achuu, S. P., & Zhang, S. (2016). A Bayesian belief network modelling of household factors influencing the risk of malaria: A study of parasitaemia in children under five years of age in sub-Saharan Africa. *Environmental Modelling & Software, 75*, 59-67.

Service., G. S. (2010). Ghana population and housing census 2010. Retrieved from http://www.webdeploy.statsghana.gov.gh/nada/index.php/ catalog/51

Sidjanski, S., & Vanderberg, J. P. (1997). Delayed migration of Plasmodium sporozoites from the mosquito bite site to the blood. *The American journal of tropical medicine and hygiene, 57*(4), 426-429.

Singh, A. P., Buscaglia, C. A., Wang, Q., Levay, A., Nussenzweig, D. R., Walker, J. R., . . . Nussenzweig, V. (2007). Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. *Cell, 131*(3), 492-504.

Singh, B., Sung, L. K., Matusop, A., Radhakrishnan, A., Shamsul, S. S. G., Cox-Singh, J., . . . Conway, D. J. (2004). A large focus of naturally acquired Plasmodium knowlesi infections in human beings. *The Lancet, 363*(9414), 1017-1024.

Sinka, M. E., Bangs, M. J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., . . . Temperley, W. H. (2012). A global map of dominant malaria vectors. *Parasites & vectors, 5*(1), 1-11.

Sirima, S. B., Richert, L., Chêne, A., Konate, A. T., Campion, C., Dechavanne, S., . . . Loulergue, P. (2020). PRIMVAC vaccine adjuvanted with Alhydrogel or GLA-SE to prevent placental malaria: a first-in-human, randomised, double-blind, placebo-controlled study. *The Lancet Infectious Diseases*, *20*(5), 585-597.

Sjogren, M. H. (2005). Prevention of hepatitis B in nonresponders to initial hepatitis B virus vaccination. *The American journal of medicine*, *118*(10), 34-39.

Steinhardt, L. C., Richie, T. L., Yego, R., Akach, D., Hamel, M. J., Gutman, J. R., . . . Kc, N. (2020). Safety, tolerability, and immunogenicity of Plasmodium falciparum sporozoite vaccine administered by direct venous inoculation to infants and young children: findings from an age de-escalation, dose-escalation, double-blind, randomized controlled study in Western Kenya. *Clinical infectious diseases*, *71*(4), 1063-1071.

Storti-Melo, L. M., Da Costa, D. R., Souza-Neiras, W. C., Cassiano, G. C., Couto, V. S. C. D. A., Póvoa, M. M., . . . Herrera, S. (2012). Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with Plasmodium vivax. *Acta tropica*, *121*(2), 152-155.

Sturm, A., Amino, R., Van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., . . . Heussler, V. T. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*, *313*(5791), 1287-1290.

Sutcliffe, C. G., Kobayashi, T., Hamapumbu, H., Shields, T., Mharakurwa, S., Thuma, P. E., . . . Moss, W. J. (2012). Reduced risk of malaria parasitemia following household screening and treatment: a cross-sectional and longitudinal cohort study. *PLoS One*, *7*(2), e31396.

Tanabe, K., Sakihama, N., & Kaneko, A. (2004). Stable SNPs in malaria antigen genes in isolated populations. *Science*, *303*(5657), 493-493.

Tebbens, R. J. D., Pallansch, M. A., Cochi, S. L., Wassilak, S. G. F., Linkins, J., Sutter, R. W., . . . Thompson, K. M. (2010). Economic analysis of the global polio eradication initiative. *Vaccine*, *29*(2), 334-343.

Tewari, R., Spaccapelo, R., Bistoni, F., Holder, A. A., & Crisanti, A. (2002). Function of Region I and II Adhesive Motifs ofPlasmodium falciparum Circumsporozoite Protein in Sporozoite Motility and Infectivity. *Journal of Biological Chemistry*, *277*(49), 47613-47618.

Tham, W.-H., Healer, J., & Cowman, A. F. (2012). Erythrocyte and reticulocyte binding-like proteins of Plasmodium falciparum. *Trends in parasitology*, *28*(1), 23-30.

Thathy, V., Fujioka, H., Gantt, S., Nussenzweig, R., Nussenzweig, V., & Ménard, R. (2002). Levels of circumsporozoite protein in the Plasmodium oocyst determine sporozoite morphology. *The EMBO journal*, *21*(7), 1586-1596.

Theisen, M., Roeffen, W., Singh, S. K., Andersen, G., Amoah, L., van de Vegte-Bolmer, M., . . . Bousema, T. (2014). A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages. *Vaccine, 32*(22), 2623-2630.

Thomsen, E. K., Koimbu, G., Pulford, J., Jamea-Maiasa, S., Ura, Y., Keven, J. B., . . . Reimer, L. J. (2017). Mosquito behavior change after distribution of bednets results in decreased protection against malaria exposure. *The Journal of infectious diseases, 215*(5), 790-797.

Thorsby, E., & Lie, B. A. (2005). HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. *Transplant immunology, 14*(3-4), 175-182.

Vaidya, A. B., Akella, R., & Suplick, K. (1989). Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. *Molecular and Biochemical Parasitology, 35*(2), 97-107.

Van De Sand, C., Horstmann, S., Schmidt, A., Sturm, A., Bolte, S., Krueger, A., . . . Heussler, V. T. (2005). The liver stage of Plasmodium berghei inhibits host cell apoptosis. *Molecular microbiology, 58*(3), 731-742.

Wang, L.-Y., Chen, C.-F., Wu, T.-W., Lai, S.-K., Chu, C.-C., & Lin, H. H. (2019). Response to hepatitis B vaccination is co-determined by HLA-DPA1 and-DPB1. *Vaccine, 37*(43), 6435-6440.

Wang, N., Qian, R., Liu, T., Wu, T., & Wang, T. (2019). Nanoparticulate carriers used as vaccine adjuvant delivery systems. *Critical Reviews™ in Therapeutic Drug Carrier Systems, 36*(5).

Wang, Q., Fujioka, H., & Nussenzweig, V. (2005a). Exit of Plasmodium sporozoites from oocysts is an active process that involves the circumsporozoite protein. *PLoS pathogens, 1*(1).

Wang, Q., Fujioka, H., & Nussenzweig, V. (2005b). Mutational analysis of the GPI‐anchor addition sequence from the circumsporozoite protein of Plasmodium. *Cellular microbiology, 7*(11), 1616-1626.

Warrell, D. A., & Gilles, H. M. (2017). *Essential malariology*: CRC Press.

Waters, A. P. (1994). The ribosomal RNA genes of Plasmodium. *Advances in parasitology, 34*, 33-79.

White, N. J. (2011). Determinants of relapse periodicity in Plasmodium vivax malaria. *Malaria journal, 10*(1), 1-36.

World Health Organization. Executive, B. (1973). *Handbook of Resolutions and Decisions of the World Health Assembly and the Executive Board: V. 1, 1948-1972* (Vol. 1): World Health Organization.

WHO. (2015). *Guidelines for the treatment of malaria*: World Health Organization.

WHO. 2019. World Health Organization (2019). World Malaria Report 2019.

WHO. 2021. World Health Organization (2021). World Malaria Report 2021

Yuan, L., Zhao, H., Wu, L., Li, X., Parker, D., Xu, S., . . . Yan, G. (2013). Plasmodium falciparum populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci. *Acta tropica, 125*(1), 53-59.

Zavala, F., Cochrane, A. H., Nardin, E. H., Nussenzweig, R. S., & Nussenzweig, V. (1983). Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. *Journal of experimental medicine, 157*(6), 1947-1957.

Zeeshan, M., Alam, M. T., Vinayak, S., Bora, H., Tyagi, R. K., Alam, M. S., . . . Bharti, P. K. (2012). Genetic variation in the Plasmodium falciparum circumsporozoite protein in India and its relevance to RTS, S malaria vaccine. *PLoS One, 7*(8).

APPENDICES

Appendix 1: Capture Master Mix preparation per reaction

Component	Volume (µl)
Water	2.84
Ampligase 10X Reaction Buffer	0.96
dNTP (10 mM diluted 1:50 in 1X Ampligase Reaction Buffer)	0.2
Phosphorylated MIP pool (at determined concentration)	0.4
Ampligase DNA Ligase (u/µl)	0.4
Q5 DNA Polymerase (diluted 1:50 in 1X Ampligase Reaction Buffer)	0.2

Appendix 2: Exonuclease Master Mix preparation per reaction

Component	Volume (µl)
Water	0.8
Ampligase 10X Reaction Buffer	0.2
Exonuclease I	0.5
Exonuclease III	0.5

Appendix 3: Master mix components per PCR reaction

Component	Volume (µl)
Phusion High-Fidelity 5X Reaction Buffer	5
5X MMC	5
10 mM dNTP Solution Mix	0.5
Phusion High-Fidelity DNA Polymerase	0.25

Appendix 4: Capture Master Mix preparation per reaction for HLA

Component	Volume (µl)
Water	0.84
Ampligase 10X Reaction Buffer	0.96
5X Q Solution	2
dNTP (10 mM diluted 1:50 in 1X Ampligase Reaction Buffer)	0.2
Phosphorylated MIP pool (at determined concentration)	0.4
Ampligase DNA Ligase (u/µl)	0.4
Q5 DNA Polymerase (diluted 1:50 in 1X Ampligase Reaction Buffer)	0.2

Appendix 5: PCR Master Mix preparation per reaction for HLA

Component	Volume (µl)
5X Q5 Reaction Buffer	5
5X MMC	5
10 mM dNTP	0.5
Q5 DNA Polymerase	0.25

Appendix 6: Th2R haplotype frequency (hap freq) and Amino acid Difference with reference to the 3D7 (AA diff) for Begoro and Cape Coast.

Begoro haplotype (Th2R)	Hap freq	AA diff		Cape Coast haplotype (Th2R)	Hap freq	AA diff
DKHIKEYLNKIQNSL(3D7)	12	0		DKHIKEYLNKIQNSL(3D7)	27	0
DKHIEQYLKTIKNSL	13	5		DKHIEQYLKTIKNSL	20	5
DQHIEKYLKTIKNSL	9	6		DQHIEKYLKTIKNSL	22	6
DQHIEKYLKIIKNSL	2	6		DQHIEKYLKIIKNSL	8	6
DKHIEQYLKKIKNSI	12	5		DKHIEQYLKKIKNSI	11	5
DQHIEKYLNKIKNSL	7	4		DQHIEKYLNKIKNSL	11	4
DQHIEKYLKRIQNSL	4	5		DKHITEYLKRIQNSL	12	3
DKHITEYLKRIQNSL	5	3		DQHIEKYLKIIQNSL	16	5
DQHIEKYLKIIQNSL	13	5		DKHIEQYLNKIKNSI	16	4
DKHIEQYLNKIKNSI	6	4		DKHIEQYLKTIQNSL	35	4
DKHIEQYLKTIQNSL	15	4		DQHIEKYLKTIQNSL	13	5
DQHIEKYLKTIQNSL	8	5		DQHIEKYLQKIKNSL	4	5
DQHIEKYLQKIKNSL	1	5		DKHIEKYLKEIQNSL	3	4
DKHIEKYLKEIQNSL	4	4		DQHIEKYLKRIQNSL	10	5
DKHIKEYLNKIQNSI	1	1		DKHIKEYLNKIQNSI	6	1
DKHIEQYLKRIQNSL	4	4		DKHIEQYLKRIQNSL	8	4
DQHIEKYLQKIQNSL	2	4		DQHIEKYLQKIQNSL	9	4
DKHIEQYLKKIQNSL	1	3		DQHIEKYLKKIQNSL	5	4
DQHIEKYLKKIQNSL	3	4		DKHITEYLKKIKNSI	2	4
DKHITEYLKKIKNSI	2	4		DQHIEKYLKKIKNSI	2	6
DKHIKEYLKIIQNSL	2	2		DKHIKEYLTKIQNSL	1	1
DQHIEKYLKKIKNSI	5	6		DQHIEKYLQKIRNSL	3	5
DKHIEQYLNKIQNSL	1	2		DKHITEYLKKIQNSL	3	2
DKHIKEYLTKIQNSL	1	1		DKHIEQYLKIIRNSL	6	5
DKHITEYLKKIQNSL	3	2		DQHIEKYLNKIKNSI	2	5
DKHIKKYLKEIQNSL	1	3		DKHIEEYLKKIKNSI	4	4
DKHIEQYLKIIRNSL	2	5		DKHIKEYLKIIQNSL	2	2
DKHITEYLKKIKNSL	1	3		DKHIEQYLKKIQNSL	2	3
DQHIEKYLKLIQNSL	1	5		DKHIEQYLKIIKNSL	2	5
DKHIEEYLKKIKNSI	1	4		DQHIEKYIKKIQNSL	3	5
DKHITDYLKKIQNSI	1	4				

Appendix 7: Shared Th2R haplotypes, their frequency (hap freq), and amino acid difference with reference to the 3D7 (AA diff) in Begoro and Cape Coast

Shared haplotype (Th2R)	Begoro Hap freq	Cape Coast Hap freq	AA diff
DKHIKEYLNKIQNSL(3D7)	12	27	0
DKHIEQYLKTIKNSL	13	20	5
DQHIEKYLKTIKNSL	9	22	6
DQHIEKYLKIIKNSL	2	8	6
DKHIEQYLKKIKNSI	12	11	5
DQHIEKYLNKIKNSL	7	11	4
DQHIEKYLKRIQNSL	4	10	5
DKHITEYLKRIQNSL	5	12	3
DQHIEKYLKIIQNSL	13	16	5
DKHIEQYLNKIKNSI	6	16	4
DKHIEQYLKTIQNSL	15	35	4
DQHIEKYLKTIQNSL	8	13	5
DQHIEKYLQKIKNSL	1	4	5
DKHIEKYLKEIQNSL	4	3	4
DKHIKEYLNKIQNSI	1	6	1
DKHIEQYLKRIQNSL	4	8	4
DQHIEKYLQKIQNSL	2	9	4
DKHIEQYLKKIQNSL	1	2	3
DQHIEKYLKKIQNSL	3	5	4
DKHITEYLKKIKNSI	2	2	4
DKHIKEYLKIIQNSL	2	2	2
DQHIEKYLKKIKNSI	5	2	6
DKHIKEYLTKIQNSL	1	1	1
DKHITEYLKKIQNSL	3	3	2
DKHIEQYLKIIRNSL	2	6	5
DKHIEEYLKKIKNSI	1	4	4

Appendix 8: Th3R haplotype frequency (hap freq) and Amino acid Difference with reference to 3D7 (AA diff) for Begoro and Cape Coast.

Begoro haplotype (Th3R)	Hap freq	AA diff		Cape Coast haplotype (Th3R)	Hap freq	AA diff
NKPKDELDYAND(3D7)	19	0		NKPKDELDYAND(3D7)	38	0
NKPKDQLDYEND	17	2		NKPKDQLDYEND	39	2
NKPKDQLDYAND	48	1		NKPKDQLDYAND	93	1
NKPKDELDYEND	20	1		NKPKDELDYEND	16	1
NKPKDELNYEND	11	2		NKPKDELNYEND	34	2
DKPKDQLDYAND	5	2		NKPKEELNYEND	2	3
NKPKEELNYEND	2	3		NKPKDQLNYEND	11	3
NKPKDQLNYEND	2	3		GKPKNELDYEND	2	3
GKPKNELDYEND	4	3		DKPKDQLDYIND	3	3
DKPKDQLDYIND	1	3		GKSKNELDYEND	14	4
GKSKNELDYEND	8	4		DKPKDQLDYAND	12	2
GKPKEELNYEND	2	4		NKPKEELDYEND	2	2
DKPKNELDYEND	1	3		GKSKDELDYEND	2	3
GKSRDELDYEND	2	4				
NKPKEELDYEND	1	2				

Appendix 9: Shared Th3R haplotypes, their frequency (hap freq), and amino acid difference from 3D7 (AA diff) in Begoro and Cape Coast.

Shared haplotype (Th3R)	Begoro Hap freq	Cape Coast Hap freq	AA diff
NKPKDELDYAND(3D7)	19	38	0
NKPKDQLDYEND	17	39	2
NKPKDQLDYAND	48	93	1
NKPKDELDYEND	20	16	1
NKPKDELNYEND	11	34	2
DKPKDQLDYAND	5	12	2
NKPKEELNYEND	2	2	3
NKPKDQLNYEND	2	11	3
GKPKNELDYEND	4	2	3
DKPKDQLDYIND	1	3	3
GKSKNELDYEND	8	14	4
NKPKEELDYEND	1	2	2

Appendix 10: Th2R haplotype frequency (hap freq) and amino acid difference with reference to 3D7 (AA diff) for Begoro and Cape Coast in 2014, 2015, and 2016.

Haplotypes (Th2R)	2014 Hap freq	2015 Hap freq	2016 Hap freq	AA diff
DKHIKEYLNKIQNSL(3D7)	15	10	7	0
DQHIEKYLNKIKNSL	6	2	8	4
DKHITEYLKRIQNSL	6	4	3	3
DQHIEKYLKIIQNSL	11	7	3	5
DQHIEKYLKTIKNSL	13	7	5	6
DKHIEQYLNKIKNSI	7	4	6	4
DQHIEKYLKTIQNSL	6	4	2	5
DKHIEQYLKTIQNSL	13	11	9	4
DKHIKEYLNKIQNSI	4	1	0	1
DKHIEQYLKKIQNSL	2	0	0	3
DKHITEYLKKIKNSI	2	0	1	4
DKHIKEYLKIIQNSL	1	0	0	2
DKHIEQYLKKIKNSI	4	6	6	5
DQHIEKYLKKIKNSI	3	1	0	6
DKHIEQYLNKIQNSL	1	0	0	2
DQHIEKYLKRIQNSL	5	2	4	5
DKHIKEYLTKIQNSL	2	0	0	1
DKHIEQYLKTIKNSL	8	1	8	5
DKHITEYLKKIKNSL	1	0	0	3
DQHIEKYLKIIKNSL	3	1	4	6
DKHIEQYLKIIRNSL	3	2	0	5
DQHIEKYLQKIQNSL	1	1	4	4
DKHITEYLKKIQNSL	2	0	0	2
DKHIEQYLKRIQNSL	0	4	0	4
DQHIEKYLQKIKNSL	0	1	3	5
DKHIEEYLKKIKNSI	0	1	0	4
DQHIEKYLKKIQNSL	0	1	4	4
DQHIEKYIKKIQNSL	0	0	2	5
DKHIEKYLKEIQNSL	0	0	2	4

DQHIEKYLQKIRNSL	0	0	1	5
DQHIEKYLNKIKNSI	0	0	1	5

Appendix 11: Th3R haplotypes frequency (hap freq) for Begoro and Cape Coast in 2014, 2015, and 2016 and their amino acid difference in reference to the 3D7 (AA diff).

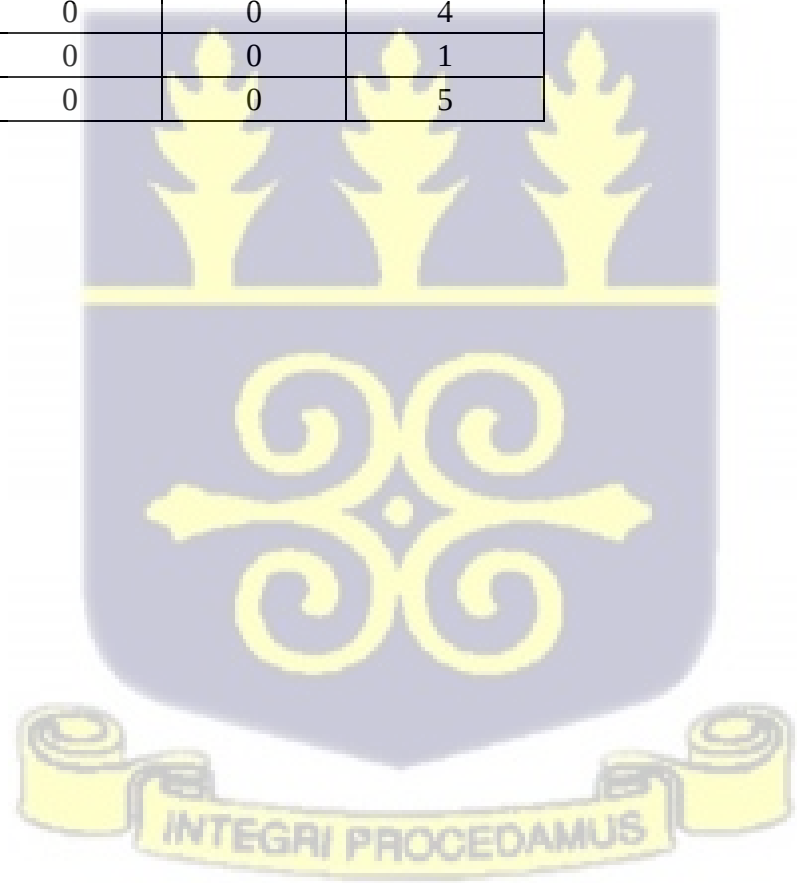
Haplotypes (Th3R)	**2014 Hap freq**	**2015 Hap freq**	**2016 Hap freq**	**AA diff**
NKPKDELDYAND(3D7)	20	11	8	0
NKPKDQLDYAND	46	24	32	1
NKPKEELNYEND	2	1	1	3
NKPKDELNYEND	12	5	12	2
NKPKDQLDYEND	14	13	10	2
DKPKDQLDYIND	2	1	0	3
GKSKNELDYEND	8	3	2	4
NKPKDELDYEND	9	7	7	1
GKPKEELNYEND	1	0	0	4
DKPKDQLDYAND	3	4	2	2
GKSKDELDYEND	1	0	0	3
NKPKEELDYEND	1	0	1	2
NKPKDQLNYEND	0	2	6	3
GKPKNELDYEND	0	0	1	3

Appendix 12: CSP haplotypes frequency for Ghana, Kenya and Malawi.

Haplotype	Ghana (freq)	Kenya (freq)	Malawi (freq)
3D7	38	20	9
H2	3	0	0
H3	26	0	0
H4	10	0	0
H5	23	0	0
H6	17	0	0
H7	11	0	0
H8	2	0	0
H9	4	0	0
H10	29	0	0
H11	22	0	0
H12	50	0	0
H13	21	0	0
H14	5	0	0
H15	6	0	0
H16	12	0	0
H17	4	0	0
H18	4	0	0
H19	13	0	0
H20	4	0	0
H21	1	0	0
H22	4	0	0
H23	4	0	0
H24	2	0	0
H25	7	0	0
H26	1	0	0
H27	2	0	0
H28	2	0	0
H29	1	0	0
H30	4	0	0
H31	1	0	0
H32	9	0	0
H33	8	0	0
H34	2	0	0
H35	1	0	0
H36	4	0	0
H37	1	0	0
H38	7	0	0

H39	3	0	0
H40	2	0	0
H41	1	0	0
H42	4	0	0
H43	3	0	0
H44	1	0	0
H45	4	0	0
H46	2	0	0
H47	5	0	0
H48	2	0	0
H49	1	0	0
H50	2	0	0
H51	2	0	0
H52	2	0	0
H53	1	0	0
H54	1	0	0
H55	1	0	0
H56	3	0	0
H57	2	0	0
H58	1	0	0
H59	2	0	0
H60	1	0	0
H61	0	16	0
H62	0	2	0
H63	0	3	0
H64	0	4	0
H65	0	1	0
H66	0	3	0
H67	0	5	0
H68	0	7	0
H69	0	4	0
H70	0	1	0
H71	0	1	0
H72	0	4	0
H73	0	1	0
H74	0	0	14
H75	0	0	50
H76	0	0	2
H77	0	0	10
H78	0	0	22
H79	0	0	5
H80	0	0	5

H81	0	0	8
H82	0	0	12
H83	0	0	10
H84	0	0	2
H85	0	0	3
H86	0	0	1
H87	0	0	1
H88	0	0	3
H89	0	0	1
H90	0	0	1
H91	0	0	1
H92	0	0	6
H93	0	0	4
H94	0	0	1
H95	0	0	2
H96	0	0	2
H97	0	0	4
H98	0	0	1
H99	0	0	5



Appendix 13: HLA class II haplotype for Begoro and Cape Coast

DQA1	Begoro	Cape Coast
01:01/01:01	5	1
01:01/01:02	10	4
01:01/02:01	4	0
01:01/03:01	1	2
01:01/04:01	7	2
01:01/05:01	11	2
01:02/01:02	6	2
01:02/01:03	5	2
01:02/02:01	5	7
01:02/03:01	12	6
01:02/04:01	12	7
01:02/05:01	12	9
01:02/08:04	0	1
01:03/04:01	3	1
01:03/05:01	3	2
02:01/01:01	0	1
02:01/01:02	1	1
02:01/02:01	1	0
02:01/03:01	2	1
02:01/04:01	8	2
02:01/05:01	4	0
03:01/03:01	1	0
03:01/04:01	5	0
03:01/05:01	5	3
04:01/01:01	0	1
04:01/01:02	0	3
04:01/04:01	7	3
04:01/05:01	18	4
05:01/01:02	0	1
05:01/03:01	0	1
05:01/05:01	4	0
01:03/05:01	1	0
04:01/05:01	1	0
05:01/05:01	1	0

DQB1	Begoro	Cape Coast
05:01/05:01	20	5
05:01/05:02	0	1
05:02/05:02	5	2
05:02/05:03	0	1
05:03/05:03	2	1
Total per site	27	10

DRB3	Begoro	Cape Coast
01:01/01:01	15	1
01:01/02:02	2	1
01:01/03:01	1	0
01:01/17:01	1	0
02:02/02:02	4	1
02:02/03:01	2	1
03:01/01:01	1	1
03:01/02:02	1	0
03:01/03:01	7	7
Total per site	34	12

DRB4	Begoro	Cape Coast
01:01/01:01	19	4
01:01/03:01	10	2
03:01/01:01	0	1
03:01/03:01	4	7
Total per site	33	14

Total per site	155	69

DRB1	Begoro	Cape Coast
01:01/01:01	1	0
01:01/02:01	1	0
01:01/07:01	2	0
01:01/08:04	1	0
01:01/15:03	2	0
01:02/01:02	3	2
01:02/03:01	3	0
01:02/03:02	1	1
01:02/08:04	4	0
01:02/11:02	2	0
01:02/13:02	2	2
01:02/13:03	1	0
01:02/13:04	0	0
01:02/15:03	2	0
03:01/03:01	1	1
03:01/03:02	1	0
03:01/08:04	2	1
03:01/13:02	1	1
03:01/14:01	0	1
03:01/15:03	3	1
03:01/16:02	0	1
03:02/03:02	4	2
03:02/04:02	0	1
03:02/07:01	5	2
03:02/08:04	3	3
03:02/08:06	1	0
03:02/09:01	1	0
03:02/11:02	2	1
03:02/13:01	2	1
03:02/13:02	0	1
03:02/13:03	4	0
03:02/14:01	1	0
03:02/15:03	4	1
03:02/16:02	1	1
05:01/05:01	1	0
07:01/07:01	2	1

07:01/08:04	1	1
07:01/11:01	0	0
07:01/13:02	0	2
07:01/15:03	3	3
08:04/08:04	10	2
08:04/08:06	1	0
08:04/10:01	0	1
08:04/11:01	4	0
08:04/11:02	1	1
08:04/12:01	1	1
08:04/13:01	3	1
08:04/13:03	7	0
08:04/14:01	1	0
08:04/15:01	0	0
08:04/15:03	0	1
08:04/16:02	2	0
08:06/10:01	0	1
08:06/11:17	1	0
08:06/15:03	1	0
10:01/10:01	0	1
10:01/11:01	2	0
10:01/13:02	1	0
10:01/14:01	1	0
11:01/07:01	1	0
11:01/11:01	2	0
11:01/11:02	1	0
11:01/13:01	1	0
11:01/13:02	1	0
11:01/13:03	0	2
11:01/15:03	2	0
11:02/11:02	2	1
11:02/13:02	1	0
11:02/13:03	1	1
11:02/14:01	0	1
11:02/15:03	1	0
12:01/12:01	1	0
12:01/13:03	1	0
13:01/13:02	2	2
13:01/13:03	1	0
13:01/15:03	1	0
13:02/01:02	1	0
13:02/03:02	0	1
13:02/07:01	0	1
13:02/13:02	1	0
13:02/13:03	1	0

13:02/14:01	0	1
13:02/15:03	1	0
13:02/16:02	1	1
13:03/03:02	0	1
13:03/13:03	0	1
13:03/13:04	0	0
13:03/15:03	4	0
13:04/13:04	1	0
15:03/03:02	0	1
15:03/08:04	1	0
15:03/15:03	6	1
16:02/09:01	1	0
16:02/11:02	0	1
16:02/16:02	2	0
Total per site	143	56

DPB1	Begoro	Cape Coast
01:01/01:01	31	11
01:01/02:01	7	4
01:01/03:01	9	0
01:01/04:01	0	1
01:01/04:02	27	12
01:01/11:01	8	1
01:01/13:01	4	1
01:01/17:01	16	5
01:01/26:01	2	0
01:01/30:01	1	0
01:01/333:0	1	0
01:01/39:01	1	2
01:01/411:0	0	1
01:01/59:01	1	0
01:01/85:01	4	3
01:02/08:06	1	0
02:01/01:01	1	0
02:01/04:02	1	1
02:01/10:01	0	0
02:01/11:01	1	0
02:01/13:01	0	1
02:01/17:01	4	2
02:01/18:01	1	0

02:01/39:01	1	0
02:01/85:01	0	1
04:01/01:01	0	1
04:01/04:01	1	0
04:01/17:01	1	0
04:01/39:01	1	0
04:02/01:01	1	2
04:02/04:02	0	1
04:02/11:01	1	0
04:02/13:01	1	0
04:02/158:0	1	0
04:02/17:01	4	1
04:02/39:01	1	0
04:02/85:01	1	0
11:01/18:01	0	1
11:01/85:01	1	0
13:01/11:01	0	0
13:01/13:01	1	0
13:01/17:01	1	0
15:01/85:01	1	0
17:01/01:01	0	1
17:01/04:02	0	1
17:01/17:01	3	1
17:01/30:01	1	0
17:01/39:01	1	1
17:01/85:01	1	0
18:01/01:01	0	1
85:01/01:01	1	0
85:01/39:01	0	1
Total per site	146	58