UNIVERSITY OF CAPE COAST

EFFECT OF VARYING MALARIA PARASITE MULTIPLICITY OF INFECTION ON THE QUALITY AND QUANTITY OF ANTI-GAMETOCYTE ANTIBODIES IN SCHOOL CHILDREN: A LONGITUDINAL STUDY

BY

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature …… Date ……

Name: Fermin Kwaku Broni

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

The sexual stages (gametocytes) of *Plasmodium falciparum* are critical to malaria transmission and are a target for the development of malaria transmission blocking vaccine candidates. Accelerating development of this vaccine requires understanding the characteristics of naturally-induced antibody responses against gametocyte antigens, including Pfs230, Pfs48/45 that have the potential to prevent parasite transmission. The number of *P. falciparum* clones in an infection can vary over time, which may lead to variation in antibody quality and quantity against gametocytes in a host. This study therefore assessed the impact of varying multiplicity of infection (MOI) on the quantity (level) and quality (avidity) of naturally-induced antibody responses against Pfs230 and Pfs48/45 in asymptomatic children. Venous blood (2.5 ml) was collected from 109 children (6 to 12 years old) every 2-months beginning in November 2017 to May 2018. Plasma samples were used in indirect ELISA test to measure IgG concentrations and avidity against Pfs230 and Pfs48/45 antigens. Msp2 genotyping was done on extracted DNA from dried blood spot to determine MOI. Increased MOI at time points close to peak season positively correlated with IgG against Pfs48/45. Positive correlation existed between IgG against both antigens despite the quality and quantity of IgG against them seems to be inversely correlated from the middle to the end of the dry season. Increased MOI near the peak season positively correlated with Pfs48/45 but not Pfs230 IgG levels suggests that repeated infections in the community preferentially boosts antibodies targeting Pfs48/45 than Pfs230 in children. However, larger studies are needed to affirm this finding.

KEY WORDS

Avidity

Multiplicity of infection (MOI)

Gametocyte antigen

Plasmodium falciparum

Antibody responses

Asymptomatic

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DEDICATION

To my lovely mother, Mrs. Patience Asare Awusah

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LIST OF ACRONYMS

ACD	Acid citrate dextrose
AU	Arbitrary Unit
ddH_2O	Double distilled water
ELISA	Enzyme linked immunosorbent assay
EtBr	Ethidium bromide
g/dL	Gram per decilitre
GES	Ghana education service
HB	Haemoglobin
IgG	Immunoglobulin G
kDa	Kilodalton
Km	kilometre
M	metre
MOI	Multiplicity of infection
Msp2	Merozoite surface protein 2
NMIMR	Noguchi Memorial Institute for Medical Research

^{O}C	Degrees Celsius
OD	Optical density
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
PCR	Polymerase Chain Reaction
RBC	Red blood cell
Rpm	Revolution per minute
SFMA	Standard feeding membrane assay
SGDs	Sustainable development goals
SMS	School of Medical Sciences
SV	Simiw visit
TAE	Tris acetate EDTA
TMB	Tetramethylbenzidine
UCC	University of Cape Coast
WHO	World health organization

CHAPTER ONE

INTRODUCTION

Gametocytes of malaria parasites are the transmissible form of malaria parasites, yet most antimalarial drugs have limited effect on them (Lamptey et al., 2018) and asymptomatic parasite carriage helps in the sustainability of parasites within population (Chotivanich et al., 2006; Okell, Drakeley, Ghani, Bousema, & Sutherland, 2008). Interestingly, natural exposure to gametocyte antigens can trigger the production of antibodies that have the potential to interrupt parasite development in mosquitoes upon blood meal (Acquah et al., 2017; Kengne-Ouafo et al., 2019) suggests parasite transmission cycle can be blocked. However, each parasite clone of multiclonal infections common in endemic residents, is capable of eliciting antibody responses (Murugan et al., 2018; Pava et al., 2017) suggests diverse antibody responses can be elicited against parasites (gametocytes). Hence understanding whether and how varying number of *P. falciparum* clones contained in an infection impact the quantity and quality of anti-gametocyte antibodies may help accelerate malaria transmission-blocking vaccine development.

Background to the study

Globally, malaria is one of the diseases that have received lots of attention for a very long time. Malaria as a disease could be dated far back as 2700 B.C, where it was initially thought to be associated with bad smell coming from swamps and this got it the name malaria from Italian word "*mal'aria*", meaning

bad air (Akande & Musa, 2011) until certain identifications and analysis were done, that led to the realization of the actual cause of the disease (malaria). These identifications included the causative organism (parasite) for avian malaria, followed by identification of *Plasmodium* parasite for human malaria with identification of mosquito as the vector (Akande & Musa, 2011). Further studies that included the finding of single genome amplification analysis of ape *Plasmodium* species, suggested that human *P. falciparum* evolved from a single gorilla-to-human cross-species transmission event (Liu et al., 2011). All the findings paved the way for a better understanding of the epidemiology, life cycle of malaria parasite, possible interventions and parasite proteins that could serve as vaccine candidates.

Malaria still remains a major problem and a burden upon all the discoveries that was made long ago concerning malaria parasites, especially in developing countries including sub-Saharan countries (Beeson et al., 2016; Diallo, Akweongo, Maya, Aikins, & Sarfo, 2017; World Health Organization, 2018). Malaria, though a preventable and treatable disease, yet lots of malaria cases and deaths (Abagna et al., 2018) occur each year. In 2017, the estimated cases and deaths recorded alone for malaria were 219 million and 435,000 respectively, where children under five years among all the age groups are vulnerable to malaria (Chatterjee, 2019). However in the recent time, the number of malaria cases is less than what was previously recorded and this could be due to the effect of the continuous use of the control interventions (Amoah et al., 2019). Although cases of morbity and mortality due to malaria, have reduced than before, there are

still some challenges to malaria infection prevention that still need to be addressed or taken care of. One of these challenges is the silent reservoir (asymptomatic individual). These asymptomatic individuals harbour malaria parasites which have the potential of causing severe malaria upon infecting another person who has never been exposed to this strain of parasite (Botwe et al., 2017; Contreras, 2018). Another challenge is the difficulty in producing highly efficacious and commercial vaccine that can prevent individuals from transmitting the parasites. Aside all these challenges, it is also known that residents of endemic areas including asymptomatic children in higher transmission settings often harbour multiclonal infections. Multiclonal infections can vary seasonally or with time and induce diverse immune responses against parasite. The variation occurring in the number of *P.falciparum* co-infecting parasites can impact the development of the antibody responses in the human host.

There are more than 100 types of *Plasmodium* parasites that can infect a variety of species. Out of these numerous types, only five different malaria parasite species are known to infect humans (Gomes et al., 2016; Loy et al., 2017; Miguel-Oteo et al., 2017). These are *P. falciparum, P. vivax, P. malariae, P. ovale and Plasmodium knowlesi* (a species of zoonotic origin) (WHO, 2017). Out of the five different human malaria parasites, *Plasmodium falciparum* causes most severe malaria disease and malaria death cases (about 99%) especially in children living in Africa (Belachew, 2018). *Plasmodium* parasite has two main forms and these are the asexual and sexual with gametocytes as initial stage of the sexual form that occurs in the human host.

Gametocyte is one of the important stages of the parasite that helps in the continuation of the life cycle of malaria parasites. The fertilization and further development of the gametocyte in the mosquito leads to sporozoite formation that can be transmitted to human to start another cycle (Kengne-Ouafo et al., 2019). Aside pregnant women, younger children are the most vulnerable to malaria, yet gametocyte is more prevalent in children than adult (Chan et al., 2019; Lamptey et al., 2018; Torgby et al., 2016). It is therefore necessary to put in special strategy that can eliminate the parasite without missing out on those harboured by the asymptomatic individuals. One of these strategies that can be very effective in protecting an individual from the parasite is by blocking the transmission of the parasite to the mosquitoes, which further prevent the transmission from mosquito to the human (Gebru et al., 2017).

So amidst the challenges of finding a protective vaccine that can prevent malaria parasite infection, transmission-blocking vaccine (TBV) emerged as important group vaccine as they would prevent an individual from transmitting the parasites. With the help of TBV, immunity against gametocyte can be strategically induced. This can be permanently sustained individually, locally and globally to completely eliminate malaria parasite. This requires that the quantity and quality of elicited antibodies against these antigens on the gametocytes are well assessed to fully understand the characteristic of anti-gametocyte antibody responses. Among the several gametocytes antigens, Pfs230 and Pfs48/45 are some of the transmission-blocking vaccine candidates currently undergoing developmental processes (Acquah et al., 2019; Kengne-Ouafo et al., 2019; Zheng

et al., 2016). Antibody avidity to *P.falciparum* antigens develops overtime (Ademolue & Awandare, 2018; Ssewanyana et al., 2017), however the variation in the complexity of *P.falciparum* infection can impact the development of antibody avidity (Nhabomba et al., 2014). There is limited data available on the development and quality of sexual stage antibody responses to *P.falciparum* infection in asymptomatic children, as compared to the data available on asexual antibody development (Acquah et al., 2019). Some studies observed that there was a significant parasite genetic variation associated with the strength of antibody responses (Murugan et al., 2018; Patel et al., 2017), however there is limited data on the strength of anti-gametocyte antibody responses to parasite genetic variation in asymptomatic children. Aside the data on sexual antibody responses, the knowledge of the estimated multiplicity of *P. falciparum* infections, malaria season and geographical location will also help in designing control interventions (Adjah, Fiadzoe, Ayanful-Torgby, & Amoah, 2018; Noor et al., 2017). Therefore studying the influence of MOI on the quality and quantity of anti-gametocyte antibodies in asymptomatic children over time will provide data on the quality and development of sexual antibody responses that can also help in designing control intervention.

Asymptomatic individual are silent reservoirs of *Plasmodium falciparum* parasites in population as they do not show symptoms clinically to be noticed for treatment (Amoah et al., 2019; Ayanful-Torgby, Quashie, Boampong, Williamson, & Amoah, 2018; Botwe et al., 2017). This research involving the asymptomatic individuals will provide data on changes in antibody responses in

the presence of parasites in asymptomatic individuals. According to some report, asymptomatic individuals are known to be partially immune to malaria; however this immunity is only for a while (Ayanful-Torgby, Quashie, Boampong, Williamson, & Amoah, 2018; Pava et al., 2017).

The cellular as well as the humoral immune responses play major roles in asymptomatic plasmodium infection (de Mendonça & Barral-Netto, 2015; Gomes et al., 2016); T regulatory cell activity (through the production of interleukin-10 and transforming growth factor-β) as well as B-cells (with a broad antibody response) play prominent role in asymptomatic infection (Murugan et al., 2018; Patel et al., 2017). The sexual form or gametocyte antigens such as Pfs230 and Pfs28/45 of *Plasmodium falciparum* parasite are protein components that are essential in the parasite's life cycle, and therefore are candidates for a malaria transmission-blocking vaccine development (Acquah et al., 2019; Kengne-Ouafo et al., 2019; Zheng et al., 2016). In the induction of antibody responses against parasite, it has been observed that current *P. falciparum* infection does not induce IgG responses, but previous exposure does (Mayor et al., 2018). The IgG antibody titre and avidity test can be used to distinguish between acute and past infections. Also levels in IgG avidity can be used to estimate the difference between primary infection and non-primary infection (Abagna et al., 2018; Mayor et al., 2018).

Avidity test carried out can be used to estimate the quality of IgG responses to antigens (Pfs48/45 and Pfs230 each) by utilizing Sodium thiocyanate (chaotropic agent) in an indirect ELISA (Abagna et al., 2018; Naghili et al., 2017; Ssewanyana et al., 2017). Indirect ELISA is a two-step ELISA involving two

binding process of primary antibody and labeled secondary antibody (Abagna et al., 2018; Naghili et al., 2017). The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody. Sodium thiocyanate is a chaotropic agent thus protein-dissociating agent. Sodium thiocyanate (Giannini et al., 2014) will only cause dissociation of complex between anti Pfs230-IgG and also Pfs48/45-IgG protein complex if the overall strength of antigen-antibody protein complex bond is weaker than the dissociating effect of the Sodium thiocyanate occurring in an optimal condition.

Statement of the problem

Gametocytes are necessary for malaria parasite transmission, as they are infectious to the mosquito. Children are the most vulnerable to malaria and also contribute significantly to malaria transmission, as they are more efficient carriers of gametocytes than adults. The worst part is that most malaria control interventions including antimalarial drugs do not target gametocytes. Also, the parasites are becoming resistant to many of the antimalarial drugs just as the vectors are becoming resistant to majority of insecticides and the spread of the resistant parasites can be enhanced and sustained within the population by resistant gametocytes. Aside the large numbers of gametocytes present at the submicroscopic levels, asymptomatic individuals also harbouring gametocytes, are not even identified for elimination and eventually lots of gametocytes in the population remain undetected. By preventing these transmissible parasites (gametocytes) from infecting the vector, could eventually reduce the contribution of these undetected gametocytes to malaria transmission. Natural exposure to

gametocyte antigens Pfs230 and Pfs48/45 are known to induce antibody responses that block transmission and can result in the development of transmission-blocking immunity. But multiclonal infections are common in residents including children of endemic countries with parasite diversity changing with time. This implies that changes in the immune responses are likely to occur overtime. The quality of antibody responses does not always correspond to the quantity of the responses. Some studies have shown that protection against malaria parasite infection is associated with functional antibody rather than the antibody titre (Adu et al., 2016; Dodoo et al., 2008; Mayor et al., 2018). Understanding the mechanism behind the influence of varying parasite MOI on the quantity and quality of anti-gametocyte antibody responses in naturally exposed children, will provide vital information that can help in the development of transmission-blocking vaccine candidates.

Purpose of the Study

To assess whether and how varying MOI impact on the quantity and quality of naturally-induced antibody responses against Pfs230 and Pfs48/45 among asymptomatic children living in the Simiw in the Central Region of Ghana.

Research Objectives

1. To determine parasite prevalence (by microscopy and PCR) and MOI (by msp2 genotyping) among the children.
2. To measure IgG levels and avidity against Pfs48/45 and Pfs230 in the plasma samples collected.

3. To determine the relationship between MOI and IgG responses, and between IgG responses against Pfs230 and Pfs48/45.

Significance of the study

The result of this study will provide data on the quantity and quality of antibody responses against gametocytes in asymptomatic children. Information gained from this study can serve as a baseline data for a larger study to identify and validate the influence parasite multiplicity of infection and diversity has on the development of transmission-blocking immunity. The knowledge to be gained on the MOI, anti-gametocyte antibody responses, malaria season and location can help in designing control intervention. The study can be used to test or predict the efficacy of vaccine candidates and may help accelerate the development of transmission-blocking vaccine candidate.

Delimitation

This study used archived samples from children (6-12 years old), who were pupils of Simiw basic school in Simiw in the Central Region of Ghana. Children were used because they generally tend to harbour gametocyte more efficiently than adults and hence are major contributors to malaria parasite transmission. The subject were asymptomatic and the samples taken during off-peak malaria season. Since data available on sexual stage immunity among asymptomatic children are limited, data obtained from this study will add new insight into antibody responses against sexual stage antigens among

asymptomatic school children residing in Simiw community during off peak malaria season.

Limitations

Only *Plasmodium falciparum* parasites were characterized in this study by utilizing msp2-based PCR. Any extra exposure to *P. falciparum* parasites before or after a sampling time point could be missed. Detecting msp2 genotyping by gel electrophoresis has limited effect in discriminating between two closely sized clones; this can ultimately under estimate MOI (Abukari et al., 2019; Zhong, Koepfli, Cui, & Yan, 2018). Antigenic markers including msp2 can be under immune selective pressure (Vieira, Santini, Diniz, & Munhoz, 2016; Zhong et al., 2018) affecting *P. falciparum* parasite characterization and detection. The archived samples collected only during off-peak malaria season (from November 2017 until May 2018) could reduce result's reliability level than sampling time points that include both peak and off-peak malaria season using fresh samples.

Organisation of the Study

The study was organized into five chapters. Chapter one (introduction) as the first, followed by Chapter two (Literature Review), Chapter three (Methodology), Chapter four (Results and Discussions) and chapter five (Conclusion and Summary) as last. Chapter one introduces the study by describing Background, Problem Statement, Purpose, Research Objectives, Significance, Delimitations and Limitations.

Chapter two reviewed available literature by exploring key areas in malaria epidemiology, lifecycle of parasite, infection, immunity, relationship between infection and immune response. Chapter three describes research methods from ethical clearance and to data analysis. Chapter four is result presentation and discussion. Chapter five outlines conclusions, recommendations and suggestions for further research.

Chapter Summary

Persistence of malaria, which is caused by *P.falciparum,* can be attributed to the availability of asymptomatic parasites (gametocytes) carriage especially by endemic children. The parasite transmission cycle can be blocked by antibodies elicited against parasites' sexual stage (gametocyte) antigen. Gaining knowledge on the characteristics of the responses of these antibodies against gametocyte antigens as well as the number of parasite clones in an infection (single child) can benefit transmission-blocking vaccine development. As the introduction on this study had been dealt in this chapter, various aspect of this study such as the literature review, research methodology, results and discussion, summary, conclusion and recommendations were addressed in subsequent chapters.

CHAPTER TWO

LITERATURE REVIEW

Introduction

One of the major reasons for malaria parasite persistence is the lack of availability of efficacious vaccine that would prevent an individual from malaria parasite infection. Gametocytes are necessary for transmission of *P. falciparum* parasites, and are targets for malaria transmission-blocking vaccine development. Accelerating the development of this vaccine requires understanding the intensity of antibody responses against gametocytes antigens upon natural exposure. Studies observed that natural exposure to Pfs230 and Pfs48/45 induces antibody responses that block parasite transmission. Meanwhile *P.falciparum* infection varies seasonally with multiclonal infection occurring mostly in endemic residents that induces diverse intensity of antibody responses against antigen of the parasite. This research sought to determine impact of MOI on the quantity and quality of anti-gametocyte antibodies in asymptomatic children. This chapter reviews any available literature on malaria epidemiology, parasite life cycle, infection, immunity, relationship between infection and immune response.

Conceptual Base of the Study

Younger children are the most vulnerable to malaria (Chatterjee, 2019) and asymptomatic ones pose challenges to malaria parasite elimination as they sustain the malaria parasite within the population (Botwe et al., 2017; Pava et al., 2017). Gametocytes (sexual forms of malaria parasites) are critical for malaria

parasites transmission and are prevalence in children than adult (Ayanful-Torgby et al., 2018; Lamptey et al., 2018). Since residents of endemic areas are naturally exposed to gametocyte antigens and most time harbour multiclonal infection, wider range of immune response can be elicited (Chan et al., 2019; Kengne-Ouafo et al., 2019). Variation in the coinfecting parasite impacts antibody responses overtime (Klasse, 2016; Patel et al., 2017). Antibody avidity responds overtime with a gradual process (Mayor et al., 2018; Patel et al., 2017; Wang et al., 2016) however genetic complexity or clone composition of the parasite impacts the intensity of the response of the antibody in the course of the developmental process (Murugan et al., 2018; Ssewanyana et al., 2017).

The impact from the clonal complexity can cause the intensity of antibody's response to either decrease, increase or remain same level for a while under the influence of the genetic complexity or clonal composition of the parasite (Abagna et al., 2018; Amoah et al., 2019; Ssewanyana et al., 2017). To further clarify the interpretation of the theories in relation to this study, this chapter explored some of key concepts in certain areas in malaria epidemiology, life cycle of malaria parasite, infection, immunity, relationship between infection and immune response.

Malaria epidemiology

Malaria epidemiology involves the study of distribution pattern, cause and factors that influence spread of malaria disease within the population under study (Akande & Musa, 2011; Ghana Malaria initiative, 2018; Koram et al., 2016;

Piarroux et al., 2016; Zhong et al., 2018). The key concepts explored under the malaria epidemiology were global burden of malaria, malaria transmission/seasonality pattern, malaria control, vaccine production and challenges (strategies for malaria controls, vaccine categories, vaccine development process and specific vaccine candidate examples, some challenges in getting commercial vaccine).

Global malaria burden and trend

Malaria, though a preventable and treatable disease, it still kills many individuals especially in endemic countries. For instance in the year 2016, the number malaria cases estimated and for that matter recorded from 91 countries was 216 million (World Health Organization, 2018). Among the various age categories, it was observed that the age group under 5 years are the most vulnerable group affected by malaria (World Health Organization, 2018). In 2017, report on malaria cases showed that more than 50% of the world's death associated with malaria, were mostly of younger children than any other categories of age groups (Chatterjee, 2019; World Health Organization, 2018).

In recent years, the number of deaths and cases of malaria has reduced as compared to the previous years (World Health Organization, 2018) but this has not lifted off the burden of malaria. Of all the reasons, lack of required capacity, quality research, education and adequate resources in these endemic settings are contributing factors making it difficult to reduce the spread of the malaria (Chatterjee, 2019). Among the children of endemic settings, those of the poor are

at most risk of getting malaria due to lack of education, financial struggle, lack of access to quality health care, issues of transportation to the health centres for proper checkup (Akande & Musa, 2011) etc.

Financial burden is one of the major malaria one that affects the world. This is more in the developing countries as majority of these countries lack health resources including financial capability to extend health coverage (Akande & Musa, 2011). It is very important for health coverage to be extended to reach all in achieving the sustainable development goals (SDGs), as this will ensure that everybody benefit from healthcare services without any financial barrier (World Health Organization, 2018). An estimated amount of US$ 3.1 billion as total funding was used for malaria control and elimination in 2017 (World Health Organization, 2018). Out of this total amount (US $ 3.1 billion), an amount of US$ 900 million (representing 28% of the total) was contributions from various governments of endemic countries (World Health Organization, 2018).

Though most endemic countries in sub-Saharan Africa including Ghana are now considered to be in the control phase (Awine, 2017), this hasn't completely lifted off the malaria burden from these countries. WHO African Region continues to account for higher proportion of malaria cases that is about 90% of cases and deaths globally. On further account, fourteen countries in sub-Saharan Africa account for 80% of the global malaria burden (WHO, 2017). There is still the need for more effective ways and control interventions to continuously minimize malaria burden to a point of no burden. This will mean

that malaria is completely eradicated forever from the globe and no traces of the malaria disease is noticed anywhere.

Malaria transmission pattern

Knowledge of malaria transmission pattern helps in the optimization of control interventions, where the transmission intensity can vary from time to time (Akande & Musa, 2011; Chatterjee, 2019). Malaria transmission/seasonality pattern is usually affected by climatic conditions (Amoah et al., 2019). Perennial malaria transmission usually occurs year round (Amoah et al., 2019).Seasonal one is mostly characterized by peaking in-between May and October coinciding with the raining season, which may be followed by off peak usually occurring between November and May the following year coinciding with the dry season (Adjah et al., 2018).

Number of bites received from infected female anopheles mosquitoes also affect transmission intensity, where the bites are more during wet season and low during the dry season (Ayanful-Torgby et al., 2018; Wang et al., 2016). During the raining season, suitability of the breeding habitat for mosquitoes increases causing an increase in the number, rate of mosquitoes survival and parasite development (Akande & Musa, 2011; Ayanful-Torgby et al., 2018; Ghana Malaria initiative, 2018). The peaking of seasonal malaria occurs mostly from June to October the same year (Adjah et al., 2018; Amoah et al., 2019; Ardiet et al., 2014; Awine, 2017; Ayanful-Torgby et al., 2018; Chatterjee, 2019) and off peak mostly from November to May of the following year (Awine, 2017).

Malaria control, vaccine production and challenges

Studying to know and understand the complexity of malaria parasite infection within an area especially endemic area is very important (Mohammed et al., 2017). Children in endemic countries are often infected with more than one malaria parasite clone, which eventually affects the immune state of these children (Dinko et al., 2017). Most control interventions such as treated nets, mosquitoes spray, repellents, coil, and anti-malarial drugs available are not enough to completely eliminate malaria as most of them are becoming ineffective due to vectors and parasites developing resistance to these control interventions (Ghana Malaria initiative, 2018; Kweku, Takramah, Takase, Tarkang, & Adjuik, 2017; WHO, 2017) . Vaccine in addition to the malaria interventions, will be very helpful in controlling and eliminating the parasites and for that matter malaria (Dinko et al., 2017; Mohammed et al., 2017; Tadesse et al., 2015).

Vector control is one of the malaria interventions that involve measures taken to avoid or reduce contact with the blood-feeding mosquitoes purposely to reduce or avoid malaria parasite transmission (Ghana Malaria initiative, 2018) etc. Indoor residual spraying, use of insecticide-treated bed nets (ITNs), mosquitoes coil and repellent are some of strategies for controlling the (mosquitoes) vectors (Chatterjee, 2019). Other strategies include diagnosis and treatment, social and behavioral change that reduces the spread of malaria parasites (Ghana Malaria initiative, 2018). Of all the strategies, vaccine has the potential of reducing and eliminating the malaria parasites completely (Kang et al., 2018; Roestenberg, 2013). Unfortunately there is no commercial vaccine

available as of now (Kang et al., 2018) although there is for the first time malaria vaccine (RTS,S), accepted by scientific community but has low efficacy (Agency, 2015; Asante, Adjei, Enuameh, & Owusu-Agyei, 2016; Wykes, 2013).

With regards to other diseases apart from malaria, vaccine has since time memorial been one of the effective strategy for controlling in mostly endemic countries. Therefore, vaccine is one of the most powerful control and elimination tools for diseases (Giannini et al., 2014; Klasse, 2016; Valéa et al., 2018; Zheng et al., 2016). There are lot of categories of vaccines from different types of species or organism ranging from bacteria, virus, fungi (Asante et al., 2016; Barclay et al., 2012; Klasse, 2016; Wykes, 2013) and so on. Vaccines can be put into various categories irrespective of the types of organism from which it is derived, example of such vaccines are recombinant, synthetic subunit protein, peptide vaccine either in a yeast or bacterial vector; (attenuated) whole organisms (Klasse, 2016; Lee et al., 2017; Wykes, 2013).

It is very important that what is considered as vaccine should trigger immune response for the immune system to build up memory of the antigen in question and produce this response the next time the same or similar antigens reappear (Valéa et al., 2018). This property displayed by the antigen is termed immunogenicity, however immunogenicity of most deoxyribonucleic acid (DNA) vaccine is not of high quantity (de Mendonça & Barral-Netto, 2015; Mayor et al., 2018) and therefore are enhanced by the help of an adjuvant (Asante et al., 2016; Roestenberg, 2013). There are several vaccine candidates based on the part of the life cycles of malaria parasites they target. Some vaccine candidates target pre-

erythrocytic stage, whiles some target the erythrocytic stage and others target the sexual stage. Those that target the sexual stage prevent the development of the sexual form of the parasite and as such block transmission of parasites to the mosquitoes. These vaccine candidates are classified as malaria transmission-blocking vaccine candidates. Examples of these vaccine candidates include Pfs230, Pfs48/45 and Pfs25 (Acquah et al., 2019; Asante et al., 2016).

The development of malaria vaccine has been on-going for almost 60 years now (Asante et al., 2016) and yet no commercial vaccine has been produced, even the recent malaria vaccine (RTS,S), which is based on the circumsporozoite antigen of the parasite, is also of limited efficacy (Wykes, 2013). The process of vaccine development, starts right from the discovery of target antigen where the target antigen of parasite is identified through scientific research mostly at the basic research level (Draper et al., 2018).The identified antigen is then passed through preclinical trials in animal models. This is done to test for the immunogenicity of the antigen, taking into consideration the safety effects as well. After obtaining satisfactory results from the preclinical research in animal models, the potential vaccine candidate is followed by clinical trials (from phase i-iii) in human beings (Draper et al., 2018).

These clinical trials are done to determine the safety, immunogenicity, and efficacy of potential vaccine candidate before submission for regulatory approval (Draper et al., 2018). Should the vaccine candidates pass all the phases of the clinical trials, it is then sent for approval by scientific community. If vaccine should also pass regulatory approval, the vaccine is now ready and can legally be

distributed and administered for use in accordance to the accepted regulation (WHO, 2019). Out of the several vaccine candidates that have undergone or still undergoing development processes, the RTS, S malaria vaccine is the only currently approved vaccine against malaria. This vaccine is under the trade name of Mosquirix (GlaxoSmithKline plc., Brentwood, UK) (Asante et al., 2016).

Over 20 *Plasmodium falciparum* vaccine candidates are undergoing development processes (Draper et al., 2018). Most of these vaccine candidate are at either preclinical or clinical stages of evaluation (WHO, 2016). Of the various candidates, transmission-blocking vaccine candidate also emerged as a very important group vaccine candidate and currently under development (Doumbo, Niare, Healy, Sagara, & Duffy, 2018). Specific examples of transmission-blocking vaccine candidates are gametocyte/gamete antigens Pfs230, Pfs48/45 (Acquah et al., 2019) and zygote/ookinete antigens Pfs25 and Pfs28 (Roestenberg, 2013).

The non-availability of commercial malaria vaccines since 1960 could be due to various challenges (Draper et al., 2018). Some of the challenges facing commercial vaccines production are insufficient funding affecting quality of research and production, difficulty in identifying malaria antigen type that can correlates well enough for adequate protection, and development of adjuvant systems among others (Asante et al., 2016) that can enhance immunogenicity. Other issue is with the complex nature of the parasite's structure and variation of antigenic types from time to time, antigenic types keep on changing from time to time and that consequently pose a challenge for efficacious vaccine to be

produced commercially (Wykes, 2013). Aside the vaccine production, the vaccine should be cost effective, easily administered, significant in the prevention of the spread and burden of disease (Gupta & Chaphalkar, 2016).

Life cycle of human malaria parasite

Just as there are various stages in the life cycle of a parasite, so are there are various stages in the life cycle of *P. falciparum*. Malaria is a disease caused by protozoan parasite of the genus *Plasmodium*. There are five known species of human malaria parasites these are *P. falciparum*, *P. malariae*, *P. ovale*, *P.vivax* and *P. Knowlesi* (Chatterjee, 2019). The life cycle of malaria parasite involves two main hosts; vertebrate example human and vector (Anopheles mosquito).

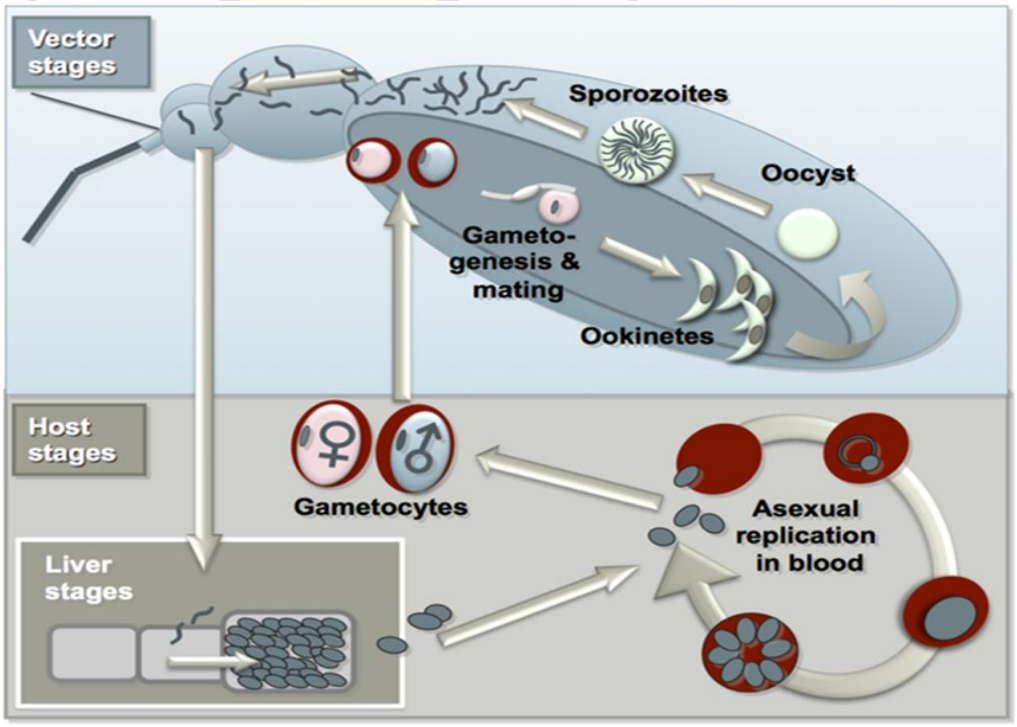

Figure1: **Life cycle of malaria parasite (source: https://reecelab.science>malaria-biology)**

Asexual phase is the stage of the parasite's life cycle, which takes place in human. This phase starts from entering of the parasite into human due to the bite of the vector, to the liver, to the RBC and end just at the formation of gametocyte (sexual form) (Pinkevych et al., 2012). This asexual form of the parasite is characterized into 2 phases. These phases are pre-erythrocytic stage and erythrocytic stage (within the red blood cells) (Pinkevych et al., 2012).

Pre-erythrocytic stage marks the beginning of the asexual life form of the parasite in human. This occurs when the female anopheles mosquito upon taking a blood meal, release saliva containing anticoagulant that prevent the coagulation of the blood as the mosquitoes feed (Kostense et al., 2011). In the releasing of the anticoagulant containing saliva during the blood meal, malaria parasites in the form of sporozoites get released into blood of human through the bite of infected mosquito (Acquah et al., 2019). The sporozoites enter the skin, blood and move to the liver by using the cholesterol pathway and invade the hepatocytes (cells of the liver). The sporozoites inside the hepatocytes, undergo development, multiplication and become a tissue schizont that contains thousands of merozoites (Cohee & Laufer, 2018). The mature schizont ruptures releasing merozoites. The merozoites move in to the blood. In the blood, the merozoites actively invade erythrocytes. Although *P. falciparum* exits the liver to infect the red blood cells however some malaria parasite species like *P.vivax* and *P.ovale*, are able to stay in the liver as "hypnozoites" for number of days, months or even years that are eventually responsible of the phenomenon of relapse (Cohee & Laufer, 2018).

The blood stage is another stage of the asexual life form of the parasite after the pre-erythrocytic stage. It is the blood stage that most signs and symptoms of malaria are experienced (Gomes et al., 2016). Two typical examples of malaria symptoms that can occur during asexual life of parasite in the blood are anaemia (reduction in the number of red blood cells or haemoglobin level) and significant rise in temperature (≥37.5 ^{o}C) clinically. This blood stage in the parasites life cycle occurs when merozoites upon release from the hepatocytes invade the erythrocytes. In two to three days of erythrocyte infection, the parasite develops from young trophozoites (ring stage) to matured trophozoites and then later on develops into schizonts (Chan et al., 2019). When parasite raptures and lots of multiple forms of the merozoites are out of the red blood cells, the merozoites reinfect other red blood cells. When this occurs as the merozoites evade uninfected erythrocytes, the whole process of multiplication and development from one form into another are repeated and later rapturing of red blood cells occurs as well (Patel et al., 2017). In about 8-12 hours after the rupture of the schizonts, the appearance of the malaria febrile paroxysm occurs. This situation of malaria febrile appearance is associated with three stages. These stages are the cold stage marked by the rapid rise of the temperature associated with chills. The next stage is the hot stage with the temperature peak, skin vasodilation, headache and myalgia. The third stage is the sweat stage with effervescence (Cohee & Laufer, 2018).

The sexual stage of the life cycle of the parasite is very important for the continuation of parasite's life cycle and transmission of the parasite to the vector

and back to man (Kengne-Ouafo et al., 2019). Although the sexual form is very important for continuation of parasite, the asexual form is critical to the initiation of the sexual form (gametocyte). During the process of asexual cycle, small portion of the asexual form (merozoites) differentiate into sexual forms; female (macrogametocytes) and male (microgametocytes) (Oue et al., 2011). According to some research, the ratio of asexual to sexual form is less than the ratio of 10:1, where another research specifically reported that the ratio is 156:1 (Henry et al., 2019; Neal, 2011). This further implies that the lower number representing gametocyte proportion in the ratio will require higher number of asexual form to increase number of gametocyte formation. This also implies that higher transmission intensity increasing the number of asexual forms can eventually increase the sexual forms (gametocytes) and vice versa in lower transmission. Upon conversion of small proportion of the asexual form into gametocytes, the gametocytes undergo sequestration (moving out of the peripheral blood circulation into deep tissue) and further development from phase one to four in deep tissue such as the gut, spleen, bone marrow, heart and so on occur (Ayanful-Torgby et al., 2018; Kengne-Ouafo et al., 2019).

Upon maturation (developing into phase five), gametocytes move back into the circulation and are ready to be picked up by anopheles mosquitoes. The continuous conversion of the asexual form into the gametocytes maintains the availability of the gametocytes in the circulation and increases chances of the gametocytes to be picked up by the female anopheles mosquito (Acquah et al., 2019). The phase five development and movement of gametocytes into the

peripheral blood circulation marks attainment of matured gametocytes that are ready to be picked up by the vector. Intraerythrocytic gametocytes remain intact in the red blood cell until they are inside the midgut of the mosquito. Female anopheles mosquitoes upon blood meal pick up the intraerythrocytic gametocytes. The ingestion of the intraerythrocytic gametocyte for further development and completion of the sexual development in the mosquito requires a biological suitable female anopheles mosquito (Antinori, Galimberti, Milazzo, Corbellino, & Asia, 2012).

When the intraerythrocytic gametocytes are in the midgut of biological suitable female anopheles mosquito, the environment conditions, such change in pH, temperature and other chemicals in the midgut of the mosquitoes cause the release of the gametocytes out of the red blood cells. The macrogametocyte (female gametocyte) becomes a macrogamete whereas the microgametocyte (male counterpart) undergoes three more slowly replications to form eight flagellated microgametes, where this increases the chances of microgametocytes to be able to attach to macrogametocytes during fertilization (Neal, 2011). Microgamete and macrogamete fuse to form the zygote by the fusion of the two nuclei (fusion of nucleus of microgamete and the nucleus of macrogamete) (Neal, 2011). After the fertilization between the macrogamete and the microgamete into a zygote, the zygote develops gradually into an ookinete (a motile one) (Arredondo et al., 2012). Oocyst develops out from the penetration of the membrane and epithelium of the mid gut by the ookinete (Zheng et al., 2016). The

nucleus of the oocyst continuously divides to form mature oocyst (Miura et al., 2016).

The matured oocyst contains numerous sporozoites generally thousands of them. The sporozoites infect the salivary gland of the mosquito, where sporozoites present in the salivary ducts are carried away by the saliva produced and passed (generally hundreds to thousands sporozoites) on into the human host during skin probing by the mosquito (Wykes, 2013). Once the sporozoites moved away from the salivary gland and are released into the human host, they are unable to reverse this process of going back to infect the salivary gland as they have totally lost the ability to do so and in addition too, they are formed in a manner that they cannot reverse the process of going back to infect the salivary gland but to continue with next stage of the process (Antinori et al., 2012).

Infection

The key concepts explored under infection are co-infection; multiplicity of infection; gametocyte (Pfs230 and Pfs48/45) antigens; msp2 genotyping and nature's effect on parasite (natural selection, mutations, migration and gene drift).

Co-infection

When two or more different parasites infect a single host, such infection qualifies to be described as a co-infection (Chen et al., 2018; Njunda et al., 2015; Zhong et al., 2018). Co-infection can include co-existence of organisms ranging from bacteria, virus, parasites or fungi in an individual (Njunda et al., 2015). Examples are infection consisting of *Plasmodium falciparum* and *Mycobacterium*

tuberculosis, Plasmodium falciparum and HIV virus co-infection, *Plasmodium falciparum* and *Schistosoma haematobium* (Njunda et al., 2015). Co-infection occurs often and some of this co-infection may have adverse effect on the host including making the host more vulnerable to other infections (Njunda et al., 2015) where under normal circumstances those infections separately may not have severe effect in the host relatively.

Most often it is very difficult to identify and differentiate several *Plasmodium falciparum* parasites that are of distinct clones under the microscope than to differentiate *Plasmodium falciparum* from other *Plasmodium* species such as *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* under the microscope. This implies that before the advent of molecular method for diagnosis, nearly all infections that were diagnosed as mono-infection using microscope, could be co-infection. This was a result of the co-existence of the organisms from the same species that were morphologically similar but genetically distinct which require molecular method for identification and differentiation (Zhong et al., 2018). Since co-infection can and do involve parasites of the same species (such as *Plasmodium falciparum* parasites) just as with parasites of different species, then it is very important to have means of estimating dynamics of parasites involved in the co-infection.

Multiplicity of infection

Multiplicity of infection (MOI) is the number of parasite species or strains that varied genetically but co-exist in an individual (Adjah et al., 2018). Limiting

this definition to the involvement of only *Plasmodium falciparum* infection in children as the host this study, then MOI is the number of *Plasmodium falciparum* strains that are of varied clone genetically but co-exist in a child. When the parasite strains infecting the single host (one child) are more than one strain (clone), the infection is considered multiple clone infections (Chen et al., 2018). But when the infecting strain is of only one distinct strain (clone) in that single host (single child) (Rundi et al., 2016), the infection is considered single clone infection.

Multiple clone infection can occur as a result of deletion, insertion, repetition of nucleotide base pairs among the infecting strains (Somé et al., 2018). Chances of an individual to be infected with multiple clones can be location dependent for instance individuals residing in endemic countries have higher chances of getting infected with multiple clones (Zhong et al., 2018). MOI is related to transmission intensity and malaria season according to some studies (Abukari et al., 2019; Adjah et al., 2018; Zhong et al., 2018). Also chances of individual to be infected with multiple clones can be time or season dependent, for instance in certain season individuals are infected with multiple clones than in other seasons, where the seasons are identified as peak and off-peak malaria seasons (Adjah et al., 2018; Ardiet et al., 2014; Hajison, Mwakikunga, Mathanga, & Feresu, 2017; Mueller et al., 2018). MOI increases when individuals are infected with multiple clones and vice versa. Some studies report that in the peak malaria season MOI increases and decreases in the off peak malaria season

(Hajison et al., 2017). MOI can vary over time; this is manifested more particularly when there are changes in the malaria season, locality or both.

MOI is considered as one of the parameters that can be used in describing the dynamics of the parasite population over time (Lerch, Koepfl, Hofmann, & Kattenberg, 2019). Since MOI for one time point may not be the same as MOI for another time point which is also due to the transmission intensity at one time point varying from another time point, MOI is seasonal (Adjah et al., 2018; Botwe et al., 2017; Karl et al., 2016). During the wet season (raining season), the breeding ground or habitat becomes suitable for the mosquito to increase breeding (Mobegi et al., 2012) vice versa occurs during the dry season. So in Ghana, peaking of malaria season coincide with raining season, which mostly occurs from June to October. The off-peak malaria season most time occur from November to May the following year. The estimated MOI of a malaria season and the locality can help in making informed decision on how to better mount malaria parasite control intervention in that locality during the various seasons.

Gametocyte antigens (Pfs230 and Pfs48/45)

For the life cycle of malaria parasite to continue, sporozoite from mosquito is injected into man and gametocyte in man is taken up by the mosquito (Gomes et al., 2016; Paul et al., 2017). In a situation where gametocyte is transferred from human to another human, could be through transfusion of gametocyte infected blood, organ transplant or needle prick from infected to another person. However the chances of this situation to occur is very minimal

(Kengne-Ouafo et al., 2019). The most frequent and very high chances of transmission of malaria parasites are between man and mosquitoes.

Sporozoites from mosquito to man undergo various asexual development and multiplication, where small percentage turning into sexual form (gametocyte) also get into the mosquito and undergo development and multiplication (Chan et al., 2019; Essangui et al., 2019). On the surface of the intracellular gametocyte, there are various antigens. Some of these antigens are Pfs48/45 and Pfs230, which are vaccine candidates currently under development (Doumbo et al., 2018; Lee et al., 2017). Though these antigens are on the surface of the intracellular gametocyte, they are also retained on the gamete in the mosquitoes as well. Both Pfs230 and Pfs48/45 are part of family of proteins precisely the 6-cys s48/45 (Acquah et al., 2017; Stone et al., 2018).

Briefly, Pfs230 is an antigen located on the surface of intracellular gametocyte and is connected into the plasma membrane of the gametocyte by Pfs48/45 and is made up of 3135 amino acid protein expressed from a 9.4 kb gene (Acquah et al., 2017; Nguyen et al., 2016; Singh et al., 2019). Pfs230 is classified as a member of the 6-cys s48/45 family and contains 14 s48/45 6-cys domains, the maximum number of 6-cys domains known within the family (Lee et al., 2017; Nguyen et al., 2016). Pfs230 is important for male fertility and is a transmission-blocking vaccine candidate (Acquah et al., 2017). Antibodies elicited against some portions of Pfs230 such as Pfs230 amino acid 443 to 1132, Pfs230 amino acid 443 to 588 have shown to cause reduction in parasite transmission to mosquito with Pfs230 amino acid 443 to 730 being part of the top

developed transmission-blocking vaccine candidates and currently in clinical trials (phase1) (Acquah et al., 2017; Kengne-Ouafo et al., 2019; White et al., 2018).

Pfs48/45 is a protein that connects Pfs230 into the plasma membrane of the gametocyte through a glycosylphosphatidylinositol (GPI) and has been known to be a factor for male fertility of the micro-gametocyte/gamete. Just like Pfs230 antigen, Pfs48/45 antigen is also located on the surface of the intracellular gametocyte. The Pfs48/45 protein consists of 448 amino acids expressed from a 1.3 kb gene and this protein also contains three s48/45 domains in addition to 3 main transmission-blocking epitopes (Khan et al., 2010; Outchkourov et al., 2008). The transmission-blocking activity of Epitope I is far higher than epitopes II and III (Sauerwein et al., 2007).

Msp2 genotyping

The genome of *Plasmodium falciparum* is made up of a 23 Mega base nuclear genome containing 14 chromosomes in addition to a mitochondrial genome. The genome is made of about 5500 genes (Bruske, Otto, & Frank, 2018). The msp2 gene is found on chromosome 2 (Chaorattanakawee et al., 2018; Chen et al., 2018; Somé et al., 2018). According to WHO, antigenic markers used for genotyping of *Plasmodium falciparum* are msp1, msp2 and glurp genes (Somé et al., 2018). Out of these antigenic markers for genotyping, msp2 is the most informative (Chaorattanakawee et al., 2018; Mohammed et al., 2017; Zhong et al., 2018). The msp2 is an acronym for merozoite surface protein-two. Merozoite

surface protein 2 is a type of protein (specifically glycoprotein) found on the surface of the merozoites (asexual blood form of the parasites), size of 45kDA formed from the expression of msp2 gene. Msp2 gene is characterized into 5 blocks that include a central block (Kang et al., 2010).

The central block specifically the block 3 is the most polymorphic (Kang et al., 2010). The block 3 of msp2 gene being most polymorphic implies that the DNA sequences at the block 3 of msp2 vary greatly among strains of *Plasmodium falciparum* than do other blocks of the msp2, where these variations are mostly due to one or more base pairs change in the DNA sequences at that locus (Mohammed et al., 2017; Salwa, 2018). Presence of this change in DNA sequences among *P. falciparum* parasites in an infection occurs as distinct clones of *Plasmodium falciparum* msp2 (*Pf*-msp2 positives) parasites visualized as different sizes of DNA band on gel electrophoresis. Although gel electrophoresis might miss the detection of some minority clones due to discrimination difficulties when compared to capillary electrophoresis, it is able to show the presence of the distinct parasite clones in an infection on the gel to some extent.

Immunity

Immunity is a broad term that can be used to describe the level of protection against a pathogen and/or its disease causing effect, which is attributed to wide range of several components of the immune system programmed to function together in various ways for the sole purpose of defending the body against what it (immune systems) perceives as foreign intruders (Gomes et al.,

2016; Kengne-Ouafo et al., 2019; Mayor et al., 2018; Wamae et al., 2019) . Immunity of an individual against malaria can be attributed to the level of protection against *Plasmodium* parasites and malaria disease (Ademolue & Awandare, 2018). Transmission-blocking immunity is said to be attained when antibodies produced (vaccine or naturally-induced) against gametocytes, are able to interrupt the sexual development of malaria parasite in the mosquito and eventually blocking the transmission of the parasite to the mosquito as evidence by standard membrane feeding assay (SMFA) (Stone et al., 2018).

Asymptomatic children

Asymptomatic children harbour malaria parasite yet show no symptoms of malaria over certain period of time. Asymptomatic individuals help in sustaining parasite within a population as they are not noticed for treatment and therefore serve as silent reservoir in transmitting the parasite into a population (Botwe et al., 2017). This poses a challenge to malaria parasite eradication. Asymptomatics are known to be immune to some extent against the malaria but the condition most of the time does not last as malaria symptoms may show later when new parasite or higher number and variation of multiclonal parasites infect the individuals (Wamae et al., 2019). If asymptomatic children in high transmission settings are mostly infected with multiclonal parasites inducing wider range of immunity to malaria than children in low transmission settings (de Mendonça & Barral-Netto, 2015), then it could also be possible that these individuals exposed to multiple clones of gametocyte can also induce wider range of immunity to gametocyte than individuals in the low transmission settings (Arévalo-herrera et

al., 2016; Ouédraogo & Bousema, 2018) . Children compared to adult, have short-lived plasma cells according to a report, which then makes it obvious why antibody levels decline rapidly in the younger than older children (Dobbs, 2018). Understanding the development and quality of immune (antibody) responses to gametocyte antigens in children is very necessary for control interventions.

Immune response

The immune responses occur through two main means. The innate and adaptive immune response of which adaptive include humoral and the cellular response (Kengne-Ouafo et al., 2019). The humoral immune response is mainly antibody (immunoglobulin) response. Cellular-mediated immunity usually targets infected cells whiles antibody-mediated immunity targets antigens occurring freely in the circulation or external to the infected cells (Jäschke, Coulibaly, Remarque, Bujard, & Epp, 2017). Aside helping in the cellular destruction of parasites, responses of antibody may occur alone (Jäschke et al., 2017).

Various stages of the life cycle of malaria parasites can trigger diverse immune responses. At the pre-erythrocytic stage (inside liver cells), cytotoxic cells and other cells capable of secreting IFN-γ can promote the elimination of intracellular parasites. At the erythrocytic stage, rupturing of infected RBC releases parasite antigens that trigger the release of tumour necrosis factor α (TNFα) and other factors. Macrophages are activated by IFN-γ and CD4+ T cells to phagocytize intraerythrocytic parasites and free merozoites (Jäschke et al., 2017).

Parasite antigens can trigger antibody responses that may prevent merozoites invasion into RBC as well as infected RBC from attaching to adhesion molecules on the vascular endothelium that may undergo sequestration. Antibody development against pre-erythrocytic, erythrocytic, and sexual-stage malaria antigens upon repeated exposure may reduce susceptibility to malaria infection and disease (Branch et al., 1998; Dodoo et al., 1999).

Antibodies (immunoglobulins) are glycoproteins produced by B lymphocytes in response to an exposed parasite antigens. . An antibody consists of fragment antigen binding (Fab) and fragment crystallization (Fc), and heavy and light chains (Mayor et al., 2018; Murugan et al., 2018). The heavy chain with it antigenic determinant specify the isotype of immunoglobulin it belongs as such γ, α, μ, δ, ε corresponding to IgG, IgA, IgM, IgD, IgE respectively (Crosnier et al., 2016; Nasir et al., 2016). There are subclasses of IgG which are IgG1, 2, 3 and 4 (Patel et al., 2017). Production of IgG antibodies to some extent can be used to estimate the differences between primary and non-primary infections (Nhabomba et al., 2014).

Specific antibody such as IgG production occurs after B cells develop in the bone marrow and move through the blood to germinal centre (a site within secondary lymphoid organs such as lymph nodes and the spleen). B cells receive a constant supply of antigen through circulating lymph, where the mature B cells become activated, proliferate, differentiate or reorder their DNA fragment of the immunoglobulin (Ig) genes to produce functional genes that has the ability of switching the class of their antibodies (such as switching from IgM to IgG).

Affinity is increased for antigen through somatic hypermutation purposely for attaining higher affinity (Klasse, 2016; Murugan et al., 2018). The antibody titre in the blood reach maximum at about two weeks (Ssewanyana et al., 2017).

Quality of antibody is another important parameter that is characterized by the production of functional (avid) antibodies (Abagna et al., 2018). The quality of the antibody is its ability to function well by binding strongly to the antigen in order to neutralize the effect of the antigen. Studies have shown that protection against *P.falciparum* parasite in an individuals is associated with antibody avidity than merely antibody titre (Acquah et al., 2019; Kengne-Ouafo et al., 2019).

Avidity test can be used to estimate quality of antibodies, where avidity can be measured through an indirect ELISA with thiocyanate treatment incorporation (Abagna et al., 2018; Amoah et al., 2019; Klasse, 2016; Ssewanyana et al., 2017). Antigen-antibody interactions are non-covalent and reversible (Beeson et al., 2016; Chaorattanakawee et al., 2018; Crosnier et al., 2016; Paul et al., 2017). Thiocyanate ion interferes with the antibody-antigen binding primarily by disrupting the hydrophobic interaction; those with weaker bond dissociate, the ones with stronger bond remain intact (Abagna et al., 2018). Avidity relies on three main factors such as the intrinsic affinity of the antibody for epitope, the valency of the antibody and antigen, the geometric arrangement of the interacting components (Essen et al., 2012; Kanayama et al., 2002; Klasse, 2016). Therefore anything that can affect the development of any of these 3 factors, will automatically impact avidity (Klasse, 2016). Variations in the

number of co-infecting parasite species can impacts antibody affinity and ultimately avidity.

Although antibody avidity (quality) is very important, quantity of antibody is also important because is a reflection of the presence of an inducible antibodies and the level produced (Fairlie-Clarke et al., 2013). Quantity of antibody is necessary for availability of inducible antibody for the manifestation of a quality of antibody response to occur. Therefore the measurement of the quantity of antibody responses can aid in the understanding of changing level of antibody over time (Abagna et al., 2018). Enzyme linked immunosorbent assay (ELISA) is one of the means that can be used to measure the concentration of anti-gametocyte antibody in the serum or plasma of an individual. Indirect ELISA will be used to measure the level of anti-Pfs230 and anti-Pfs48/45 in the asymptomatic school children (Abagna et al., 2018; Acquah et al., 2017).

Both the asexual and sexual (gametocyte) antigens are known to naturally-induce antibody responses (Abagna et al., 2018; Acquah et al., 2017). Antibodies produced against the gametocyte are considered anti-gametocyte antibodies. During the asexual parasite multiplication, small portion of the asexual parasite convert into sexual form (gametocyte). Intraerythrocytic gametocytes undergo sequestration and mature gametocytes occur in the peripheral blood circulation. Aside the gametocytes being taken up by mosquitoes, the intraerythrocytic gametocytes remain and die off in the circulation (Kengne-Ouafo et al., 2019). In the course of dying off, antigens on the gametocyte get exposed, where antibodies induced against these antigens and are produced into circulation (Kengne-Ouafo

et al., 2019). The production of these naturally-induced antibodies against the gametocyte antigens is available for a while and responds against subsequent gametocyte antigens upon exposure (Kengne-Ouafo et al., 2019; Wang et al., 2016). Anti-Pfs230 antibodies and anti-Pfs48/45 antibodies respond upon Pfs230 and Pfs48/45 antigen exposure respectively. The binding of these antibodies to their antigens on the gametocytes in the mosquitoes interrupt the fertilization and further development of the gametes (Kengne-Ouafo et al., 2019) .

Relationship between infection and immunity

The key areas explored were relationship between *Plasmodium falciparum* clone complexity and avidity maturation or level. As the process of antibody avidity develops over time, parasite genetic/clone complexity impacts the developmental processes.

According to studies elsewhere, it was observed that individuals with higher level of antibody avidity to *Plasmodium falciparum* parasites were less likely to suffer severe malaria (Akpogheneta, Dunyo, Pinder, & Conway, 2010; Ssewanyana et al., 2017). This implies that antibody avidity response to *Plasmodium falciparum* antigens can decrease the severity of malaria disease. This partly demonstrates that quality and/or quantity of antibody induced is impacted upon by the *Plasmodium falciparum* infection. Then it is important to know the relationship between the complexity of the *Plasmodium falciparum* infections and the avidity/concentration of the antibody induced. It is expected that antigen exposure to the immune system will increase antibody avidity with

time but this is not always the case. Antibody avidity does not always correlate with antibody level in an individual. A study reported that *P.falciparum* parasites in children above the age of 5 years in a location with highest transmission intensity, recorded low avidity even though the antibody level was high (Ssewanyana et al., 2017). The observation from the report implies that antibody avidity is inversely proportional to high transmission intensity but not so for the concentration of the antibody.

Ssewanyana et al.(2017) in their study suggested that employing longitudinal study design into broad analysis of the B cell and antibody responses to *P. falciparum* exposure, will help fully understand the mechanism behind humoral immune response upon parasite exposure. On this note, longitudinal study design was used to assess the effect of varying MOI on the quantity and quality of anti-gametocyte antibodies in asymptomatic children.

Chapter Summary

Despite increase in control interventions, malaria still kills. The currently available malaria vaccine (RTS, S) is also of limited efficacy. One of the new areas is the transmission-blocking vaccine type, which targets the parasites' sexual stages (gametocytes), and the antigens include Pfs230 and Pfs48/45. Since multiple parasite infection is common in endemic residents, assessing the usefulness of these antigens requires gaining an in-depth knowledge on the effect of varying *P.falciparum* MOI on not just the quantity (level or titre) but the quality (avidity) of inducible antibodies against these antigens (Pfs230 and Pfs48/45).

CHAPTER THREE

RESEARCH METHODS

Introduction

Transmission-blocking vaccines are important group vaccines as they would prevent individuals from transmitting the malaria parasite. Gametocyte antigens such as Pfs230 and Pfs48/45 are some of the transmission-blocking vaccine candidates. Though individuals in endemic areas are naturally exposed to these antigens, they also harbour multiple clone infections, which can trigger diverse immune response against parasite. Understanding the impact of variation in the multiclonal infection on the anti-gametocyte antibody responses may help accelerate malaria transmission-blocking vaccine candidate development. This study therefore determined the influence of varying MOI on the quantity and quality of anti-gametocyte antibody responses in asymptomatic children.

This chapter describes the various methods employed in this study where asymptomatic children where followed up in two-month interval for period of 6 months. The samples used for the study, are archived samples which had been properly stored in appropriate conditions until use. These samples were collected at every two months interval. After polymerase chain reaction (PCR) speciation for *Plasmodium falciparum*, those that tested positive were genotyped using polymorphic marker such as msp2. Indirect enzyme linked immunosorbent assay (ELISA) test with chaotropic treatment test was used to measure the IgG concentration and avidity response to gametocyte antigens (Pfs230 and Pfs48/45).

Details of each method used have further been discussed in this chapter and how the data obtained from the various methods were analyzed.

Ethical consideration

This study used archived samples collected from participants; of which ethical clearance was sought from the Institutional Review Board of Noguchi Memorial Institute for Medical Research.

Research Design

This study utilized archived samples. The research design used in the original study was a longitudinal study that involved sampling of asymptomatic school children every two months for a period of 6 months (from November 2017 to May 2018).

Study Area

The study area was Simiw, a village within the Komenda-Edina-Eguafo-Abrem Municipal Assembly in Central Region of Ghana, in the southern part of Ghana. The area is situated on coordinates 05.16949 ºN and 001.33772 ºW and has an elevation of approximately 36 m above sea level. The village is about 11 km away from Elmina, the district capital and 2 km away from the Ankaful Maximum Prison. Most places in Central Region of Ghana including Simiw community have been observed to have low transmission and seasonal malaria, which can peak during wet season (Adjah et al., 2018)

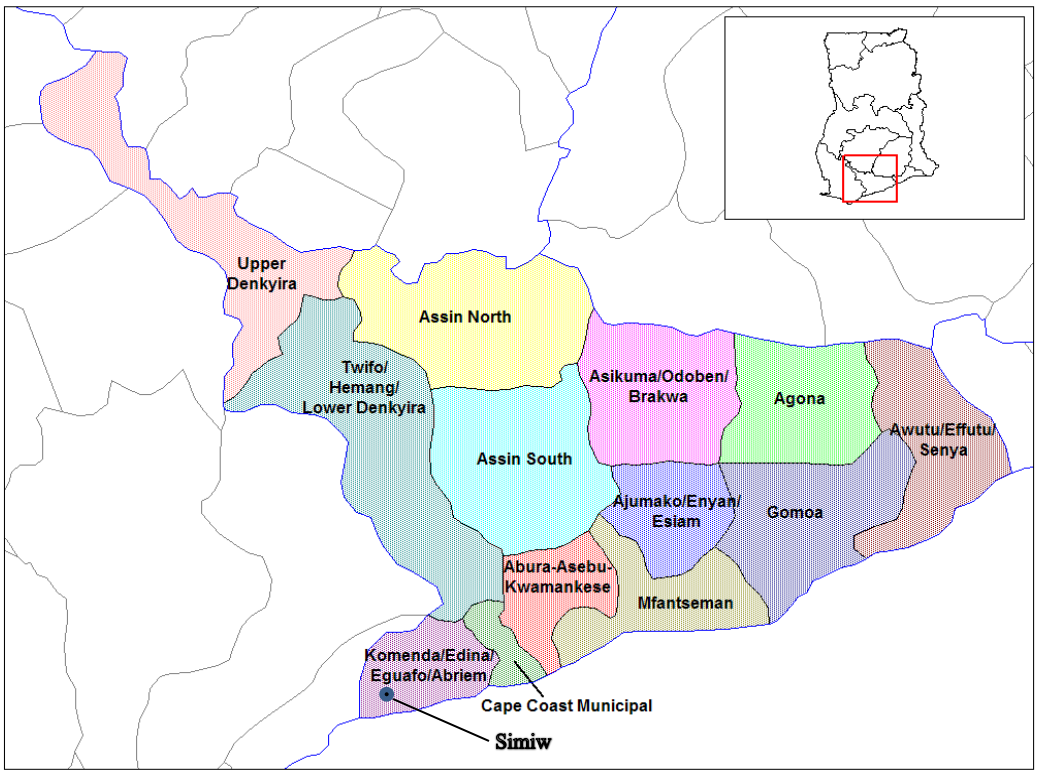

Figure 2: **Map of central region of Ghana showing geographical location of Simiw (source https://maps-ghana.com/map-of-central-region-of-ghana)**

Population

The village has a population of about 2,012 comprising 996 males and 1,016 females. The main occupation of the inhabitants is farming, predominantly cocoa. The target population in this study had been already sampled and archived. Archived samples obtained from pupils of Simiw Basic School were age range of 6-12 years. Initial 109 archived samples comprising of 57 boys and 52 girls represented the participants in this study, who were progressively followed.

Sample Collection Procedure

The study had no field work, it was solely laboratory based, making used of archival samples collected from asymptomatic children (6-12 years) on every 2

months starting from November, 2017 until May, 2018. These archived samples were plasma, blood (thick and thin) smears on slide and filter paper dried blood spots (DBS). The data available and used were PCR *P.falciparum*, parasite density (PD), HB level and axillary temperature.

Laboratory Procedures

DNA Extraction: Saponin/Chelex method

DNA was extracted using the Saponin/Chelex protocol as previously described (Abagna et al., 2018; Abukari et al., 2019). Briefly, dried blood spots (DBS) that were stored at -20 ^{o}C, were brought to room temperature. Punches were made into DBS filter paper at two different places of the filter paper to get two filter paper discs. These two filter paper discs of diameter 2.5 mm each were put into 1.5 ml microfuge tubes and labeled properly.

A 1 mL solution of 1× Phosphate buffer saline (PBS) and 50 µl of 10% saponin was added to the tubes containing the punched DBS, vortexed and stored at 4ºC overnight. The tubes were spin for 1 minute and the reddish PBS/Saponin supernatant aspirated. One ml of ice cold PBS was added to the tubes, vortexed and incubated at 4 ºC for 30 minutes. The tubes were vortexed, centrifuged for 1 minute and the fluid (supernatant) was aspirated. After this, 30 µl of 20 % chelex (Sigma-Aldrich, USA) was added as well as 70 µl of dd H_2O and incubated at 95 ºC for 10 minutes with intermittent vortexing at 2 minutes intervals. The tubes were span for 6 minutes at 13000 rpm and as much supernatant containing the DNA as possible was transferred into a sterile 0.5 ml microfuge tube.

Detection of *P. falciparum* by polymerase chain reaction

A nested PCR procedure similar to previously reported protocols (Abagna et al., 2018; Adjah et al., 2018; Mohammed et al., 2017) was used to detect *P. falciparum* at the molecular level, was done with slight modification. The nested PCR targeted the small subunit ribosomal RNA (18S rRNA) gene. The total volume for both the primary reaction and the secondary reaction mixtures was 15 µL each. The nest 1 (primary reaction) consisted of 200 nM primer set (rPLU5 and rPLU6), 5 µL of DNA, 2.5 mM $MgCl_2$, 200 nM dNTP mix and 1 U OneTaq DNA polymerase. The thermal cycling condition consisted of first denaturation for 2 minutes at 94 °C followed by 35 cycles at 94 °C for 30 s, 54 °C for 1 min and 68 °C for 1 min, with a final 5 min extension at 68 °C. The DNA product from the nest 1 reaction (primary reaction) was used as a template for nest 2 reaction (secondary reaction).

The secondary reaction mixture was made up of 200 nM primer set (rFal1 and rFal2), 0.5 µL of DNA, 2.5 mM $MgCl_2$, 200 nM dNTP mix, 1 U OneTaq DNA polymerase. The secondary reaction mixture and the primary mixture were almost the same except that some of the constituents were different such as volume of DNA template used was 0.5 µL. The sequence and the name of all the primers used in this part of the method (PCR speciation of *Plasmodium falciparum*) were presented in Table 1 below.

The thermal cycling condition for the secondary PCR was similar to that of the primary PCR. The controls used were 3D7 as positive control and double

distilled water (ddH_2O) as the negative control. These controls were run alongside the samples in the primary reaction.

Table 1-Primary and secondary PCR primers for *Plasmodium falciparum* speciation

PCR round	Primer name	Primer sequence (5'-3')
1° PCR	rPLU5	TTAAAATTGTTGCAGTTAAAACG
	rPLU6	CCTGTTGTTGCCTTAAACTTC
2° PCR	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC

Abbreviations:1° primary, 2° secondary (Adjah et al., 2018; Somé et al., 2018)

Agarose gel electrophoresis

The products from the secondary reaction were run on a 2% agarose gel using a previously described procedure (Abagna et al., 2018; Adjah et al., 2018; Amoah et al., 2019). The gel was prepared by dissolving 2 g of agarose powder in 100 ml of 1X TAE (Tris-acetate EDTA) buffer using a microwave oven. The molten gel was then allowed to stand for some few minutes to cool. About 4 µl of ethidium bromide was added to the molten gel and the bottle containing the molten gel with the ethidium bromide was whirled to mix uniformly. The gel was cast to set in an appropriate size chamber with combs to make the wells. The cast gel was allowed to solidify and then placed in a gel tank containing buffer (1X TAE buffer), to be fully submerged in the buffer. The PCR product was mixed with loading dye and transferred into the well. Each sample ID including 3D7 as

positive control and double distilled water as negative control were loaded corresponding to particular wells according to the arrangement of the amplicons number. Loading of 100 bp DNA ladder was done at each row of the wells.

The gel tank was covered completely with it lid. The voltage of the power pack was set at 120 volts, the current 100 amps and the running time was at 50 minute. The electrophoretic gel was taken and photographed under ultraviolet (UV) visualization gel documentation system (Vilber, Germany). The gel picture was labeled and scored. From the scored results, those that were positive for *Plasmodium falciparum* were further genotyped utilizing msp2 antigenic markers.

Merozoite surface protein 2 genotyping

For the msp2 genotyping, the polymorphic region (block 3 as the central block) of merozoite surface protein 2 was amplified from the extracted DNA using similar process described in previous studies (Abagna et al., 2018; Adjah et al., 2018; Torgby et al., 2016). The most polymorphic central block of msp2 was amplified using nested polymerase chain reaction. The msp2 genotyping consisted of two main steps, these are the nest1 (primary PCR reaction) and nest 2 (secondary PCR reaction). The total volume of the primary PCR reaction was 15 µl. The reaction contained 4.0 µl of extracted DNA, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 0.2 mM each of a combination of forward (m_1-oF) and reverse (m_1-oR) primers for msp2 and 0.5 units of OneTaq polymerase. The thermal cycling profile for the nest 1 PCR consisted of first denaturation for 2 minutes at 94 °C

followed by 35 cycles of 94 °C for 30 s, 54 °C for 1 min and 68 °C for 1 min, with a final 5 min extension at 68 °C.

The nested 2 PCR reactions (secondary PCR reactions) mixture was similar to the primary PCR reactions (nested 1 PCR reactions) except that 2 µl of primary PCR reactions was used as template for the secondary PCR reactions. The family allelic primers for the msp2 secondary reactions for 3D7 and FC27 were used. These were S1fw (forward primer) and N5rev (backward primer) for 3D7 allelic marker and S1fw (forward primer) and M5rev (backward primer) for FC27 allelic marker. The sequence and the name of all the primers used in this part of the method (msp2 genotyping) were presented in the Table 2 below.

Double distilled water (ddH_2O) was used as negative control, (MRA-102G) 3D7 as positive control for msp2 3D7 and (MRA-159G) K1 as positive control for msp2 FC27 allelic family were included in the PCR reactions (Somé et al., 2018). The thermal cycling conditions for the nest 2 PCR was similar to that of nest 1 PCR.

Table 2- Primary and secondary PCR primers for msp2 genotyping

PCR round	Primer name	Primer sequence (5'-3')
Primary PCR	M_2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA
	M_2-OR	CTTTGTTACCATCGGTACATTCTT
Secondary PCR	S1fw	GCTTATAATATGAGTATAAGGAGAA
	N5rev	GCATTGCCAGAACTTGAA
	M5rev	CTGAAGAGGTACTGGTAGA

(Adjah et al., 2018; Somé et al., 2018)

Agarose gels preparation and gel electrophoresis

After the nest 2 PCR reaction of the msp2 genotyping, gel electrophoresis was performed. The gel preparation and electrophoresis were done in the same way as for the *Plasmodium falciparum* described previously. After the gel electrophoresis, visualization, scoring of gel and subsequently, MOI was determined.

Estimation of Parasite Prevalence by Microscopy

The archived blood (thick and thin) smears were stained in fresh 10% Giemsa stain and allowed to dry after fixing the thin smear in methanol. The 100x oil immersion objective was used to examine the feathered edge of the smear. The total number of WBCs and parasites in the grid area were counted under the Zeiss light microscope using laboratory cell counter. Each slide was read by two

independent microscopists. The number of parasites counted out of 200 WBCs counted was used to estimate the parasite density.

Measuring IgG antibody level and avidity to Pfs48/45 and Pfs230 antigens

The measuring of IgG level and avidity were done by indirect ELISA where thiocyanate (chaotropic agent) treatment was incorporated into the procedure for the avidity ELISA. This procedure was done as described by Abagna et al., (2018) and Amoah et al., (2019) with slight modification.

An optimization process was carried out first to determine the optimal concentration of antigen to coat as well as the optimal dilution of samples to use. The samples were diluted at 1/50 dilutions in 1% skimmed milk with 0.05% sodium azide. The sample dilution was prepared in the deep well plate (Appendix A), where each well corresponded to a sample identity (ID). It was ensured that all equipment, reagents and dilution buffers were ready before each stage of the ELISA test.

The antigens, Pfs230 and Pfs48/45, were obtained from Dr. Linda Eva Amoah, which were cloned and expressed by one of her student, Festus K. Acquah, at Immunology department of NMIMR. For both antigens, 96-well ELISA plates (NUNC Maxisorp; Thermo Fisher) were coated with 100 µl of 1 µg/ml and 0.5 µg/ml of Pfs230 (Pfs230 dilution - see Appendix A) and Pfs48/45 diluted antigens (Pfs48/45 dilution - see Appendix A) respectively in carbonate buffer (Carbonate buffer preparation - see Appendix A). The plates were covered with a plastic sealer and incubated over night at 4 ºC. The ELISA plates were

washed four times with washing buffer (1X PBS and 0.05 % Tween 20) (Washer buffer preparation - see Appendix A) using a plate washer (Biotek ELx 405, Virginia, USA). The washing was done to remove any unbound antigens. The plates were padded dry to remove excess moisture after the washing.

The ELISA plates with unbound regions in the wells were blocked with 150 µL of 3 % blocking buffer (Blocking buffer preparation – see Appendix A) containing skimmed milk (Marvel, UK) and kept at room temperature for 1 hour. This was done to ensure reduction in the non-specific binding. The plates were washed again four times with the washing buffer (1X PBS and 0.0 5 % Tween 20) using a plate washer (Biotek ELx 405, Virginia, USA). The ELISA plate was padded dry. This was done to remove excess moisture plate after washing.

The test samples were added in quadruplicate (samples added into four different wells) that corresponded to only one participant (meaning same sample in four wells of the ELISA plate, where two wells were used for IgG avidity test and the other two wells were used for Normal ELISA test to determine IgG titre). As the test samples were already prepared at 1/50 dilution (Sample dilution preparation – see Appendix B) into the deep well plate, the test samples were added to the ELISA plate at dilution of 1/200 in 1XPBS containing 1 % skimmed milk. This was achieved by pipetting 25 µL of the test sample from the deep well plate, where the 25 µL pipetted was added to 75 µL of 1XPBS containing 1 % skimmed milk into the well and mixed gently to obtain uniform mixture.

Negative controls comprising of plasma samples pooled together from malaria naïve Danish blood donors (never exposed to the malaria) and therefore tested negative to the gametocyte antigens (Pfs230 and Pfs48/45) in previous ELISA test, as well as positive controls comprising of plasma pooled together from malaria exposed individuals who have previously been found tested positive to the gametocyte antigens in a previous ELISA, were both included in the ELISA plate set up.

The positive and negative controls were prepared at a dilution of 1:200 in 1x PBS containing 1 % skimmed milk. This was achieved by pipetting 1 µl and adding it to 199 µl of IXPBS containing 1 % skimmed milk, mixed well to obtain uniform mixture. The controls were added in duplicate to the wells assigned for only controls on the ELISA plate.

Addition of the standard was done by putting standard into the first well of the ELISA plate, where the first well contained standard with dilution at 1/50. The standard was then further diluted 2 fold over eight wells. A blank was set on the plate to check for background interference that may have occurred during the procedure or from the reagents. For blank, only 1XPBS containing 1% skimmed milk was used.

After the samples, controls, standard were added and the blank was set, ELISA plates were then incubated for 1 hour at room temperature. The plates were washed four times with the washing buffer and padded dry.

To two wells of the quadruplicate of each sample, 100 µl of thiocyanate salt solution (Thiocyanate salt solution preparation - see Appendix A) was added and then 1 % skimmed milk was added to the other remaining two wells of that same sample. The remaining wells such as wells for the standard, controls and blank, were filled with 1% milk. This was incubated for 15 minutes at room temperature. Thiocyanate is a chaotropic agent that can cause dissociation of the antigen-antibody complex that is not tightly bound. The ELISA plates were washed four times with the washing buffer and padded dry.

To each well, 100 µL of goat anti-human IgG-HRP secondary antibodies (Invitrogen, USA) was added at a concentration of 1:3000 (IgG conjugate antibody preparation - see Appendix A). This was followed by incubation for 1 hour at room temperature. The plates were washed four times with the washing buffer and padded dry. To each well, 100 µL of Tetramethylbenzidine (TMB) substrate (++) was added followed by incubation at room temperature for 10 minutes in the dark. It was noted that there was blue colour formation. The reaction was stopped with 100 µL 0.2 M H_2SO_4 and a colour changed from blue to yellow was observed. The ELISA plates were read at absorbance of 450 nm on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The results of the reading were recorded in optical density (OD).

Data Processing and Analysis

After the reading the ELISA result, the optical density (OD) was exported unto the excel sheet and converted into comma delimited separated value (CSV)

format. ADAMSEL (version1.1 © EJ Remarque 2007) software, was used to convert OD into concentrations in arbitrary unit (AU). The relative avidity for IgG antibody was calculated as the ratio of the IgG concentration of the sodium thiocyanate-treated sample to the IgG concentration of the untreated sample multiplied by 100 ([antibodies following Sodium thiocyanate (NaSCN) treatment/antibodies without NaSCN treatment] × 100). Graph pad prism 5 was used to plot graph of IgG relative avidity and concentration level in the various time point. Statistical significance was considered when p value is ≤ 0.05 (95% confidence interval).

Data processing and analysis for msp2 genotyping was done, by first scoring of DNA bands on agarose gel electrophoresis after visualization of gel as positive or negative. The scoring results of the gel was for 3D7 (N5 specific primer) allele and the other for FC27 (M5 specific primer) allele. The number of clones was recorded. A clone was identified as a distinct amplified fragment observed on the gel plate in any of the specific allelic marker used for the genotyping. Those samples ID having more than one clone were considered as multiple clones and sample ID with only one clone was considered as single clone. Geometric mean multiplicity of infection was calculated for the various time points. Average multiplicity of infection is the number of distinct clones for a particular marker (msp2 marker) divided by number of samples positive for that particular marker.

$$\text{Mean MOI} = \frac{\text{Number of distinct clones for msp2 marker}}{\text{Number of sample positive for the marker}}$$

Parasite density (PD) was obtained from the conversion of number of parasites counted out of 200 WBCs counted multiplied 40 based on the fact that 1μL of blood is predicted to contain 8000 WBCs.

Therefore the PD estimated as: $PD = \frac{\text{Number of parasite counted}}{\text{Number of WBC counted}} \times 8000$

Analysis was done with GraphPad Prism version 5 software, where non-parametric test with Kruskal-Wallis and post test done with Dunn's multiple comparisons test. Statistical significance was considered when p value was less than or equal to 0.05 ($p \leq 0.05$). Differences between the variations in the ratio of male and female across various time points were analysed by Chi-Square test. Relationship among anti-Pfs230, anti-Pfs48/45 concentration (level), avidity, MOI was determined using Spearman's rank correlation.

Chapter Summary

Briefly, data available and obtained from the larger study was on temperature, HB level, parasitaemia, PCR *P.falciparum* result of the participants. However, msp2 genotyping was done on extracted DNA of participants who tested and confirmed positive for PCR *P.falciparum* and subsequently MOI was determined. Relative avidity and normal ELISA were determined using plasma samples of the participants. The ELISA optical data obtained was converted into concentration using ADAMSEL software. Data analysis was done using ANOVA, Chi Square test, and Spearman's rank correlation in GraphPad Prism version 5.

CHAPTER FOUR

RESULTS AND DISCUSSION

Introduction

This study sought to determine the impact of varying MOI on the quantity and quality of anti-gametocyte antibody in asymptomatic children living in Simiw, Ghana during an off peak malaria season. A longitudinal study design was employed that randomly selected a total of 109 archived samples from study participants and were followed progressively at every 2 months for period of 6 months. Antibody (IgG) level and avidity against Pfs230 and Pfs48/45 were measured using indirect ELISA. ADAMSEL was used to convert ELISA data into concentration. Parasite density and prevalence was determined by microscopy and PCR. Using nested PCR, msp2 genotyping was done on extracted DNA of participants who tested positive for PCR *P.falciparum* and subsequently MOI was determined. Data was analysed and graphs were plotted using GraphPad Prism 5.

Results

Description of the study participants

Samples from a total of 109 participants were followed over the four time points. However, a few participants missed a few visits as such only 106, 101, 93, 108 archived samples were available during the 1st, 2nd, 3rd and 4th time point respectively. There was no significant difference between the number of females and that of males throughout the four time points (Mann-Whitney test; U=2.00, *P*=0.1143) as shown in Table 3. The age range of the study participants was

between 6 to 12 years (Table 3). Apart from the significant differences (*P*=0.0294) that occurred between median haemoglobin (Hb) level of 1st and 2nd time point (see Appendix C), the median Hb level was within normal range and did not vary across the time points with (Table 3). All the median temperature at the various time points were less than the temperature epidemiologically considered as fever (≥ 37.5 ^{O}C) as well did not vary significantly (*P*=0.0600) across the time points (Table 3).

Table 3. Characteristics of the study participants

	Nov. 2017	Jan. 2018	Mar. 2018	May 2018	*P*-value
Total individuals	106	101	93	108	
Sex ratio (M/F)	51/55	48/53	43/50	51/57	0.1143
Age (years)					
Median	10	10	9	10	0.9994
IQR	6-12	6-12	6-12	6-12	
Haemoglobin (g/dL)					
Median	11.00	11.50	11.70	11.70	0.0294
IQR	10.5-12.05	10.70-12.20	10.70-12.38	11.0-12.5	
Temperature (^{o}C)					
Median	36.70	36.40	36.40	36.50	0.0600
IQR	36.2-36.9	36.3-36.8	36.10-36.7	36.1-36.78	

IQR, interquartile range; M/F, ratio of male to female

Parasite density, prevalence and MOI among participants

Table 4 shows parasite density (PD), prevalence and MOI among the study participants across the various time points. No gametocyte parasite was microscopically observed but asexual parasites observed with significant variations in PD between Nov.2017 and Jan.2018; Nov.2017 and May.2018;

Jan.2018 and Mar.2018; Mar.2018 and May 2018 (P<0.0001, Dunn's multiple comparison test). Relatively, the mean PCR parasite prevalence (34.4%) was higher than that of microscopy (24.8%). The distinct bands at positive control (3D7 and K1) with no band present at double distilled water (dd H_2O) being no-template control (or negative control), and presence of varied number of distinct bands observed at test samples on the msp2 electrophoregram (see Appendix B) represented clones and varied number thereof. However, the differences in MOI across the time points did not vary significantly (P = 0.5671) as shown in Table 4.

Table 4. Parasite density and prevalence (by microscopy and PCR) and MOI (msp2 genotyping by PCR) throughout the four time points

	Nov.2017	Jan.2018	Mar.2018	May.2018	Mean	*P*-value
MICROSCOPY						
Mean PD/ µL of blood	974.1 (132.3 - 1816)	48.44 (3.4- 100.3)	1149 (673.4-1625)	124.4 (13.44-262.3)		<0.0001
Asexual Prevalence (%)	33.03 (36/109)	11.01 (12/109)	49.54 (54/109)	5.5 (6/109)	24.8	
Gametocyte Prevalence (%)	0 (0/109)	0 (0/109)	0 (0/109)	0 (0/109)		
PCR						
Asexual Prevalence (%)	28.3 (30/106)	49.5 (50/101)	37.6 (35/93)	22.2 (24/108)	34.4	0.0242
Total number of msp2 positives	13	22	17	9		
MOI						
Geometric mean	1.48 (22/13)	1.47 (36/22)	1.41 (29/17)	1.96 (25/9)		0.5671

PD, parasite density; PCR, polymerase chain reaction; MOI, multiplicity of infection

Antibody (IgG) level and avidity to Pfs48/45 and Pfs230 over time

Despite median IgG level against Pfs230 seems to be relatively higher than IgG level against Pfs48/45 across the four time points, this relative difference may not hold unless otherwise affirmed on the account that same controls were utilized during the measurement of both IgG levels in the ELISA methods. Median IgG level against Pfs48/45 varied significantly (P<0.0001) across the time points specifically between Nov.2017 and May 2018, Jan.2018 and May 2018, and Mar. 2018 and May 2018 (Appendix C) with the highest (85.73 AU) and lowest (56.26 AU) median IgG recorded in May 2018 and Mar. 2018 respectively (Table 5). Median IgG against Pfs230 also varied significantly (P<0.0001) across the time points specifically between Nov.2017 and May 2018, Jan.2018 and Mar. 2018, and Jan. 2018 and May 2018 (see Appendix C) with the highest (128.8 AU) and lowest (72.67 AU) median IgG recorded in May 2018 and Jan. 2018 respectively (Table 5).

Relatively, median IgG avidity was higher against Pfs48/45 than Pfs230 throughout the study. The IgG avidity against Pfs48/45 in the plasma samples of the participants varied significantly (P<0.0001) across the time points specifically between Nov.2017 and Mar.2018, Nov.2017 and May 2018, Jan.2018 and Mar.2018, Jan.2018 and May 2018, and Mar.2018 and May 2018 (see Appendix C), with the highest (45.74 %) and lowest (26. 35 %) median IgG avidity recorded in Mar.2018 and Jan.2018 respectively (Table 5). However, the IgG avidity against Pfs230 did not vary significantly (P=0.1078) across the time points

despite the highest (30.71 %) and lowest (23.12 %) median Pfs48/45 IgG avidity was recorded in Mar.2018 and May 2018 respectively (Table 5).

Table 5. Antibody levels and avidities to Pfs48/45 and Pfs230 in the study participants throughout four time points

		Nov.2017	Jan.2018	Mar.2018	May.2018	*P*-value
Antibody Level (AU)	**Pfs48/45 IgG**					
	Median	68.35	61.49	56.26	85.73	<0.0001
	IQR	(44.92-101.9)	(28.94-101.8)	(39.87-70.89)	(58.73-125.9)	
	Pfs230 IgG					
	Median	113.6	72.67	128.8	145.5	<0.0001
	IQR	(45.53-184.1)	(24.04-162.5)	(82.10-179.1)	(104.0-222.6)	
Antibody Avidity (%)	**Pfs48/45 IgG**					
	Median	33.17	26.35	45.74	38.84	<0.0001
	IQR	(18.24-44.24)	(13.74-40.37)	(34.84-55.15)	(30.61-46.24)	
	Pfs230 IgG					
	Median	27.36	23.94	30.71	23.12	0.1078
	IQR	(18.05-38.60)	(13.41-38.79)	(14.85-46.44)	(14.17-37.27)	

IQR, Interquartile range; AU, Arbitrary unit

Association between MOI and IgG responses

Spearman's rank correlation was used to determine the association between MOI and various IgG responses (levels and avidities to Pfs230, Pfs48/45) as shown in Table 6. The correlation coefficient (r) showed strength and direction of the association, where the P value less than 0.05, that association was considered significant (Table 6). None of the correlations between MOI and IgG responses was significant except the correlation between MOI and Pfs48/45 IgG level in May 2018 (time point close to peak malaria season, where r = 0.7625,

P = 0.0246) as shown in Table 6. This positive correlation between MOI and Pfs48/45 IgG level in May was a strong one. Although at that same time point (May, 2018) the correlation between MOI and Pfs230 IgG level was a positive one, the association was not significant (r = 0.5320, *P* = 0.1397) as shown in Table 6.

Table 6: Spearman's rank correlation coefficient of MOI and IgG responses

		IgG avidity against		IgG level against	
		Pfs48/45	**Pfs230**	**Pfs48/45**	**Pfs230**
Nov.2017	**MOI**(1.48)	r = 0.2969 *P* =0.3291	r = 0.4082 *P* = 0.1709	r = 0.1857 *P* = 0.5512	r = -0.1670 *P* = 0.3382
Jan.2018	**MOI**(1.47)	r = -0.1907 *P* = 0.4075	r = -0.1152 *P* = 0.6190	r = 0.1279 *P* = 0.5806	r = 0.0329 *P* = 0.8873
Mar.2018	**MOI**(1.41)	r = 0.0682 *P* = 0.6249	r = 0.2658 *P* = 0.3203	r = 0.0539 *P* = 0.5872	r = -0.1724 *P* = 0.1555
May.2018	**MOI**(1.96)	r = -0.0532 *P* = 0.7706	r = -0.2660 *P* = 0.3921	r = 0.7625 *P* = 0.0246	r = 0.5320 *P* = 0.1397

MOI, multiplicity of infection; r, correlation coefficient; P, p-value (significant when <0.05)

Association between IgG responses against Pfs48/45 and Pfs230

The Table 7 shows strength and type of association between IgG levels and avidities against Pfs48/45 using Spearman's rank correlation matrix. Significant positive correlations were observed between IgG levels against Pfs230 and Pfs48/45 in November 2017 (0.6298, *P*<0.0001) and January 2018 (0.5941, *P*<0.0001). However, no significant correlation was observed between IgG

avidities against Pfs48/45 and Pfs230 avidity throughout the study period (Table 7).

Also, the strength and type of association between the each antigen's naturally-induced IgG level and avidity were assessed by Spearman's rank correlation as shown in Table 7. Apart from the positive correlation between Pfs48/45 IgG level and avidity in first (r = 0.0251, *P* = -0.7999) and second time point (r = 0.4175, *P* < 0.0001), negative correlations were observed between the IgG level and avidity against each antigen (Table 7) throughout the four time points. In addition to this, all the associations were significant except in first time (r = 0.0251, *P* = -0.7999) and third time point (r = -0.2177, *P* = 0.0360).

Table 7: Spearman's rank correlation between various antibody (IgG) responses

	Pfs48/45 IgG avidity and Pfs230 IgG avidity	**Pfs48/45 IgG avidity and Pfs230 IgG avidity**	**Pfs48/45 IgG level and avidity**	**Pfs230 IgG level and avidity**
Nov.2017	r = 0.1673, *P* = 0.0865	r = 0.6298, *P*< 0.0001	r = 0.0251, *P* = 0.7999	r = -0.5287, *P* < 0.0001
Jan.2018	r = -0.1460, *P* = 0.1450	r = 0.5941, *P*< 0.0001	r = 0.4175, *P* < 0.0001	r = -0.6911, *P* < 0.0001
Mar.2018	r = 0.0998, *P* = 0.0998	r = 0.1820, *P*= 0.0808	r = -0.2177, *P* = 0.0360	r = -0.1824, *P* = 0.0800
May.2018	r = -0.0275, *P* = 0.7774	r = -0.1038, *P* = 0.2850	r = -0.2609, *P* = 0.0081	r = -0.2405, *P* = 0.0149

r, correlation coefficient; *P*, p-value (considered significant when <0.05)

Discussion

Children are one of the most vulnerable groups to malaria yet they are a major contributor to malaria parasite transmission as they are more efficient carriers of gametocytes than adult (Lamptey et al., 2018). Gametocytes are critical to malaria parasite transmission, yet the effects of most antimalarial drugs are not directed against the gametocytes (Lamptey et al., 2018). Also asymptomatic individuals are not identified for treatments as they do not show symptoms of malaria for a while, implies that children who are asymptomatic, pose greater challenge to the prevention of malaria parasite transmission. The challenge of finding a protective vaccine has been overdue, especially as the currently available malaria vaccine (RTS, S), which is based on the circumsporozoite antigen of the parasite, is of limited efficacy (Asante et al., 2016; Draper et al., 2018). Amidst the challenges of seeking for highly efficacious and protective vaccine that can block transmission of parasite, transmission-blocking vaccine were identified as very important group vaccines as they would prevent an individual from transmitting the malaria parasite to another.

Blocking infection and further development of parasites (gametocytes/gametes) in mosquitoes can help eliminate malaria parasites from the population. Antibodies induced against gametocyte antigens upon natural exposure can interrupt transmission process in the mosquito. It has been observed that residents including children of endemic settings are naturally exposed to the gametocyte antigens (Pfs230 and Pfs48/45). However children residing in higher transmission intensity areas most often harbour multiple clone infections than

those in low transmission intensity areas, implying that diverse immune responses can be elicited against these parasites. Understanding whether and how varying MOI impact antibody responses to gametocytes can help accelerate the development of malaria transmission-blocking vaccine candidates. This study sought to determine the impact of varying MOI on the quality (avidity) and quantity (level) of antibody responses to gametocyte antigens Pfs230, Pfs48/45 in asymptomatic children residing in Simiw, a community in the southern part of Ghana during the off-peak malaria season.

Research design employed was longitudinal one of which archived samples were collected from asymptomatic children during the off-peak malaria season on every 2 months for a period of 6 months. Although there can be several symptoms and complications of malaria, the two main symptoms of malaria are anaemia (i.e. low haemoglobin concentration) and fever (i.e. rise in temperature usually $\leq 37.5\ ^{O}C$) (Okell et al., 2008). Being asymptomatic requires that one does not have a fever although infected with malaria parasites. The normal range of haemoglobin level for children (5-12 years old) is $\geq$ 11.50 g/dL (Awad, Bashir, Osman, & Ibrahim, 2019; Dinko et al., 2017). The median haemoglobin of the study participants were all at or above 11.50 g/dL (Table 3) except that of November 2017 (11.0 g/dL) although relatively lower than 11.50 g/dL, it was relatively above the haemoglobin of symptomatic malaria children recorded less than 8 g/dL in one study (Dinko et al., 2017). Also the median temperature of the study participants ranged between 36.40 ^{o}C and 37.0 ^{o}C throughout the various time points, this range of temperature was below the temperature (37.5 ^{o}C) of

symptomatic individuals (>37.50 ^{O}C) according to research (Imai, Nakade, Tsuboyama, & Takimoto, 2016). These results implied that the median temperature and haemoglobin recorded in this study showed that the study participants were not symptomatic for malaria.

The mean *P.falciparum* parasite prevalence detected by PCR (34.41 %) was higher than mean prevalence estimated by microscopy (23.27 %) in Table 4. The difference in the mean prevalence by both methods is not surprising as the sensitivity and specificity of PCR are higher than that of microscopy as PCR can detect submicroscopic parasites (Zhong et al., 2018), which the differences in submicroscopic *P. falciparum* carriage across the time point was significant (X^2 = 9.424, df = 3, *P* = 0.0242). The mean PCR prevalence in this study was similar to PCR parasite prevalence (35%) reported by previous study involving children from nearby community in Cape Coast (Ayanful-Torgby et al., 2018). However the microscopy prevalence was higher than what was recorded (6%) by Ayanful-Torgby et al (2018). This could be due to variation in the suitability of an area for mosquito breeding, as Simiw (rural area) relatively has higher suitable breeding habitat for mosquitoes than Cape Coast (urban).

Gaining more information on the multiplicity of *Plasmodium falciparum* infection by utilization of msp2 marker was useful for determining and understanding the changes in the number of coinfecting *P. falciparum* clones among the study participants over time. Appearance of bands of different sizes on the msp2 gel electrophoresis (Appendix B) implies that varied clones of *Plasmodium falciparum* infections existed among study participants. Those

positively tested for PCR *P.falciparum* but negatively tested for msp2 observed, could be the fact that they low density minority clones (Lerch et al., 2019), occurrence of mutation at detection site rendering msp2 genotype less detected or could be due to effect of the host immune selective pressure on the msp2 antigenic determinant of parasite affecting the msp2 detection of some *P. falciparum* clones (Zhong et al., 2018). Genotyping with other markers should be included in this similar study to ascertain this reason. Microsatellite marker, a marker known to be selectively neutral (Abukari et al., 2019) could be added to the markers for the genotyping.

Although mean MOI was highest in May 2018 and lowest in March (Table 4), PCR prevalence of *P. falciparum* carrier on the other hand was lowest in May 2018 but highest in January 2018 (Table 4). This implies that MOI of *Plasmodium falciparum* at a time point does not always depend on the prevalence of *Plasmodium falciparum* at that time point but rather associated mostly with transmission intensity and malaria season. The mean MOI not varying significantly throughout the four time points in this study could be due to collection of samples from the same malaria season and site.

The presence of IgG responses to Pfs48/45, Pfs230 throughout the four times, implies that these children have been exposed naturally to these gametocyte antigens (Pfs230 and Pfs48/45) and which support what is known in literature that residents of endemic areas are naturally exposed to gametocyte as Kumar (as cited in Acquah et al., 2019) observed. Although there was antibody responses to both antigens (Pfs230 and Pfs48/45), the level of Pfs230 IgG seemed relatively higher

than the level of Pfs48/45 IgG throughout the four time points (Table 5) whereas the avidity of Pfs230 IgG was relatively lower than avidity of Pfs48/45 IgG (Table 5). The differences in avidity could be due to IgG being more efficiently avid processed against Pfs48/45 than Pfs230.

Spearman's rank correlation was used to determine the strength and nature of association between MOI and IgG responses (avidity and level) to Pfs230 and Pfs48/45. None of the association between MOI and avidity correlated significantly throughout the four time points could implied that avidity maturation processes might have been compromised throughout the four time points where the plasma cells including the antibodies produced were only short-lived.

According to Kumar (as cited in Acquah et al., 2019), Pfs230 antigen is anchored to the plasma membrane of gametocyte by Pfs48/45 antigen through glycosylphosphatidylinositol (GPI) anchor, suggested possibility of IgG antibodies elicited against both antigens as they known to be closely located on the surface of the plasma membrane of gametocytes. Using Spearman's rank correlation, positive correlations were observed in November 2017 ($r = 0.6298$, $P < 0.0001$) and January ($r = 0.5941$, $P < 0.0001$) and this could be that as both antigens are closely located on the gametocytes, they both elicited IgG concurrently in the same direction. A previous study also observed significant positive correlation (0.378, P=0.0002) between the concentrations of IgG against Pfs230 and Pfs48/45 (Acquah et al., 2017), and it is not surprising that another study being done observed that the fusion of both antigens elicited additive IgG responses against transmission of *P.falciparum* (Singh et al., 2019). The lack of

significant correlation in the last two time points could be due to constant decay of IgG against both antigens as antibodies to these antigens are known have short half-life of about 3 months (Bousema et al., 2010). However, none of the association between IgG avidities against both antigens was significant (Table 7), could be due to interruption in avidity maturation processes and constant decay of IgG.

Having determined the association between IgG responses against both antigens, it was also relevant to assess the strength and type of association that might occurred between the level and avidity of IgG elicited against the each antigen. This would help gain in-depth knowledge on the strength and nature of association existed between the quality and quantity of naturally-induced IgG against either of the two antigens (Pfs230 and Pfs48/45). It was observed that from the middle to the end of the dry season (March 2018 to May 2018), IgG level correlated negatively to its avidity elicited against each antigen (Table 7). A previous study done by Amoah et al.,(2018) observed that IgG level was relatively lower against Pfs230 in one site (relatively, a lower transmission setting) than another site (relatively, a higher transmission setting) although the IgG avidity was relatively higher (Amoah et al., 2019). So from this previous study as well as the existence of negative correlation between level and avidity from March to May (Table 7) further suggest that the negative association could be exploited by deliberately attempting to reduce IgG level by reducing parasite transmission using vector control and chemoprevention (Jagannathan et al., 2016) to see if IgG avidity could be enhanced among children in the community.

Chapter Summary

In summary, parasite prevalence of PCR higher than microscopic could be due to higher sensitive level of PCR. Although MOI did not vary significantly, MOI positively correlated with Pfs48/45 IgG level only in May (0.7625, $P<0.0246$). Despite IgG level seemed relatively higher against Pfs230, IgG avidity was relatively higher against Pfs48/45 with negative correlation existed between each antigen's naturally-induced IgG level and avidity from March to May, a positive correlation was observed between their IgG levels against both antigens.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Introduction

Although plasmodium and mosquito have been identified respectively as pathogen and vector long ago, malariologists can still not boast of high efficacious and commercial vaccine. The only available malaria vaccine (RTS,S), is of limited efficacy. In seeking for protective vaccine with the aim of targeting the gametocyte, transmission blocking vaccine emerged as important group of vaccines as they would prevent individuals from malaria parasite infection. Studies show that natural exposure to *P.falciparum* gametocyte antigens (Pfs230 and Pfs48/45) can cause immunity against gametocytes and prevent transmission of parasites. However residents of endemic areas mostly harbour multiclonal infections that can trigger diverse immune response against the parasites. Understanding the impact of varying MOI will help accelerate transmission-blocking vaccine development. The research determined the effect of varying multiplicity of infection (MOI) on the quantity and quality of anti-gametocyte antibodies in school children.

Summary

The method employed to achieve the purpose of the research was carried out by using archived plasma samples collected from asymptomatic school children (age range of 6-12 years). Genomic DNA extracted from dried blood spot on filter paper, was genotyped utilizing PCR-based msp2 marker and MOI

subsequently determined. Plasma sample was used in an indirect ELISA test to measure IgG level and avidity against Pfs230 and Pfs48/45 antigens. Optical density was converted into concentrations using ADAMSEL version1.1. Data analyzed using nonparametric ANOVA Kruskal Wallis, Dunn's multiple comparison, Chi Square test and association between parameters determined by Spearman's rank correlation in Graph Pad Prism 5 (significant, if P <0.05).

The results obtained from this study showed that Pfs230 IgG and Pfs48/45 IgG levels correlated significantly (0.6298, p<0.0001) in Nov.2017 and (0.5941, p<0.0001) in Jan.2018, despite the IgG level and avidity against Pfs230 and Pfs48/45 differed. This could be attributed to the fact that as both Pfs230 and Pfs48/45 are closely located on the surface of the plasma membrane of gametocyte, upon exposure both antigens elicited antibodies concurrently in the same direction. None of the IgG except Pfs48/45IgG level correlated significantly (0.7625, *P*<0.0246) with MOI in May. The level and avidity of IgG against each antigen correlated negatively from the middle to the end of the dry season.

Conclusion

Positive correlation existed between IgG against both antigens despite the quality and quantity of IgG against them seems to be inversely correlated from the middle to the end of the dry season. Increased MOI near the peak season positively correlated with Pfs48/45 but not Pfs230 IgG levels suggests that repeated infections in the community preferentially boosts antibodies targeting Pfs48/45 than Pfs230 in children. However, larger studies are needed to affirm this.

Recommendations

Capillary electrophoresis-based msp2 genotyping should be used to overcome the limitations of slab gel electrophoresis-based msp2 genotyping in discrimination between two closely sized distinct parasites clones.

Comparison between utilizing gametocyte marker (example Pfg377) and asexual marker (such as msp2) for genotyping should be made in order to establish which marker form (or stage of malaria parasite) will give adequate information about the effect of varying MOI on the quality of anti-gametocyte antibodies.

The intervals between any two sampling time points should be reduced to minimize the chances of missing out on any extra parasite exposure. The duration of the longitudinal study can be extended further to include both peak and off peak malaria season to give adequate information about whether and how varying malaria season impact the responses of anti-gametocyte antibodies.

Since a positive correlation was observed between IgG against both antigens, larger studies can be undertaken to affirm the relationship between antibody responses to these two antigens.

The attachment between the Pfs230 and Pfs48/45 on the gametocyte should be further researched for the possibility of finding antigen that might consist of both Pfs230 and Pfs48/45. Where this antigen can be tested for it transmission reducing activities by the standard membrane feeding assay (SMFA) that can be considered as a potential transmission-blocking vaccine candidate.

Similar research should be carried out in symptomatic individuals to determine which of the gametocyte antigens and/or individuals (either symptomatic or asymptomatic) are likely to induce or record higher anti-gametocyte antibody responses. This study should be done involving wide range of age groups to see how age, for that matter age differences and varying MOI will impact the quality and quantity of anti-gametocyte antibody responses.

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APPENDIX A

REAGENTS, DILUENTS AND OTHER SOLUTION PREPARATION

Carbonate buffer preparation

To prepare 500ml of carbonated buffer, the following recipes were weighed or measured

NaHCO3 - 1.46g

Na2CO3 - 0.795

1% Phenol red indicator - 500µl

Distilled water - 499.5ml (499,500µl)

After mixing the recipes together to attain uniform mixture, the Ph of the prepared buffer was measured. The Ph measured and it was 9.2

Sample dilution preparation in deep well

Samples were diluted at 1/50 to make 1ml volume in the deep well plate. This was prepared in the biosafety cabinet. Since 1 ml equals 1000µl, to prepare 1/50 dilution of samples to make up 1ml volume, below are the calculation for the preparation of sample

If 50µl is 1µl of sample

1000µl will be (1000x1)/50 = 20µl

Thus 20µl of the sample (plasma) needed was added to 980 (1000µl-20µl) of the diluent containing 1% milk with 0.05% sodium azide. This was mixed well in the deep plate to obtain uniform dilution mixture. The

sodium azide was added to preserve the sample dilution for longer time (about two weeks)

1XPBS (Phosphate buffer saline) as dilution buffer

To prepare 2L of 1xPBS (dilution buffer), 4 big PBS tablets (1 big tablet is dissolved in 500ml of distilled water) were dissolved in 2L of distilled water.

Gametocyte antigen dilution

a. Pfs230 antigen

To prepare the dilution of Pfs230 antigen to be used for the ELISA, the initial (Stock) concentration (C1) is 1mg/ml, working concentration (C2) to be used is 0.5µg/ml, the working volume (V2) to be used is 250ml (this figure was based on the 25 ELISA plates used where 10ml of antigen was used per each plate, with 100µl per each well) but the volume (V1) to be aliquoted from the stock was unknown and need to be calculated for.

Using C1V1 = C2V2, V1 = (C2V2)/C1= (1X250) / ($1.367X10^{3}$) = 0.1828ml

249.817ml of carbonated buffer was mixed with 0.1828ml of stockPfs230 antigen.

b. Pfs48/45 antigen

To prepare the dilution of Pfs48/45 antigen for the ELISA test, the same procedure and calculation was used except that the stock

concentration (C1) was 0.5mg/ml that was different from the stock concentration of Pfs48/45. So using the formula above

C1V1=C2V2, V1= (C2V2)/C1 = (0.5X250) /($1.367X10^3$) = 0.09144ml

249.9085ml of carbonated buffer was mixed with 0.09144ml of the stock Pfs48/45 antigen

Blocking buffer preparation

To preparation for 3% blocking buffer for the ELISA test, since two antigens were used and two set of 25 plates (50 ELISA plates) were to be blocked, 750ml (50 ELISA plates used, where 15ml of blocking buffer per plate) of blocking buffer was prepared that contained 3% skimmed milk.

To prepare 3% skimmed milk in a total volume of 750,

100 ml contain 3g of skimmed milk

750ml is (750x3)/100 = 22.5g. Thus 22.5g of skimmed powder was weighed into 750ml of 1XPBS, and mixed well to obtain uniform mixture.

IgG conjugate antibody preparation

The IgG conjugate used was Goat anti-Human IgG (IgG conjugated to Horseradish Peroxidase). The dilution prepared was1/3000 conjugate dilution. This was prepared by first calculating the volume of the conjugate antibody aliquoted, thus if 3000µl dilution contains 1µl of conjugate, 500ml (500,000µl) will contain (500,000x1)/3000 = 166.667µl

The stock volume of IgG conjugate antibodies aliquoted was 166.667µl and mixed well with 499833.34µl of diluent buffer (1% skimmed milk in 1xPBS buffer).

PBST as washing buffer

Preparation of 4L PBST using crude (salt) and adding Tween (0.05%)

Constituent	1x	10x	10x
NaCL	8g	80g	320g
KCL	0.2g	2g	8g
Na_2HPO_4	1.44g	14.4g	57.6g
KH_2PO_4	0.24g	2.4g	9.6g
		for 1L	for 4L

To add 0.05% Tween to the preparation (4L),

If 100 = 0.05 of Tween, 4L (4000ml) will contain (4000x0.05)/100=2ml

0.002L (2ml) of Tween was well mixed with 3.998L of the PBS.

Thiocyanate salt solution

For thiocyanate solution, molecular weight (Mw) = 81.07g/mol, Concentration (C) = 2.4mol/dm^3, Volume (V) = 0.4L, C = m/ (MxV)

m=CMV = 2.4X81.07X0.4=77.8g. Hence 77.8g of sodium thiocyanate salt dissolve in 400ml of distilled water.

Preparation of 2M H_2SO_4

To prepare 500ml 2M of H_2SO_4, 5.55ml of H_2SO_4 was aliquoted and mixed with 494.45ml of distilled water.

APPENDIX B

PCR REACTION FOR *P. falciparum* DETECTION AND MSP2 GENOTYPING

Primary reaction (Nest 1) for *P. falciparum* detection

Reagents	Concentration	volume
ddH_2O	5.99	
5X PCR Buffer	1X	3
$MgCl_2$(25mM)	2.5 mM	0.42
dNTP mix (10mM)	167 nmol/L	0.25
PLU5	80 nmol/L	0.12
PLU6	80 nmol/L	0.12
One Taq	1 unit	0.1
Template	5	

Cycling conditions

94- 4m

94- 30s
55- 1m } 35 cycles
68- 1m

68- 5m

4- ∞

Secondary reaction (Nest 2)

Reagents	Concentration	volume
ddH_2O	5.99	
5X PCR Buffer	1X	3
$MgCl_2$(25mM)	2.5mM	0.42
dNTP mix (10mM)	167nmol/L	0.25
Fal 1	80 nmol/L	0.12
Fal 2	80 nmol/L	0.12
One Taq	1unit	0.1
Template	0.5	

Cycling conditions

94- 4m

94- 30s ┐
58- 1m ├ 35 cycles
68- 1m ┘

68- 5m

4- ∞

Nested PCR reaction for msp2 genotyping

Primary reaction (Nest 1)

Reagents	Concentration	volume
ddH_2O	5.99	7.03
5X PCR Buffer	1X	3.0
$MgCl_2$(25mM)	2.5mM	0.12
dNTP mix (10mM)	167nmol/L	0.30
M2-OR	80 nmol/L	0.20
M2-OF	80 nmol/L	0.20
One Taq	1unit	0.15
Template		4.0
		15 uL

Cycling conditions

94- 2m

94- 30s
54- 1m
68- 1m
} 35 cycles

68- 5m

4- ∞

Secondary reaction (Nest 2)

Reagents	Concentration	volume
ddH_2O	5.99	8.95
5X PCR Buffer	1X	3.0
dNTP mix (10mM)	167nmol/L	0.30
S1fw	80 nmol/L	0.30
N5/M5	80 nmol/L	0.30/0.30
One Taq	1unit	0.15
Template		2.0
		15 uL

Cycling conditions

94- 2 m

94- 30s
58- 1m
68- 1m
} 35 cycles

68- 5m

4- ∞

1X TAE: 20ml of 50X TAE into 980ml of distilled water

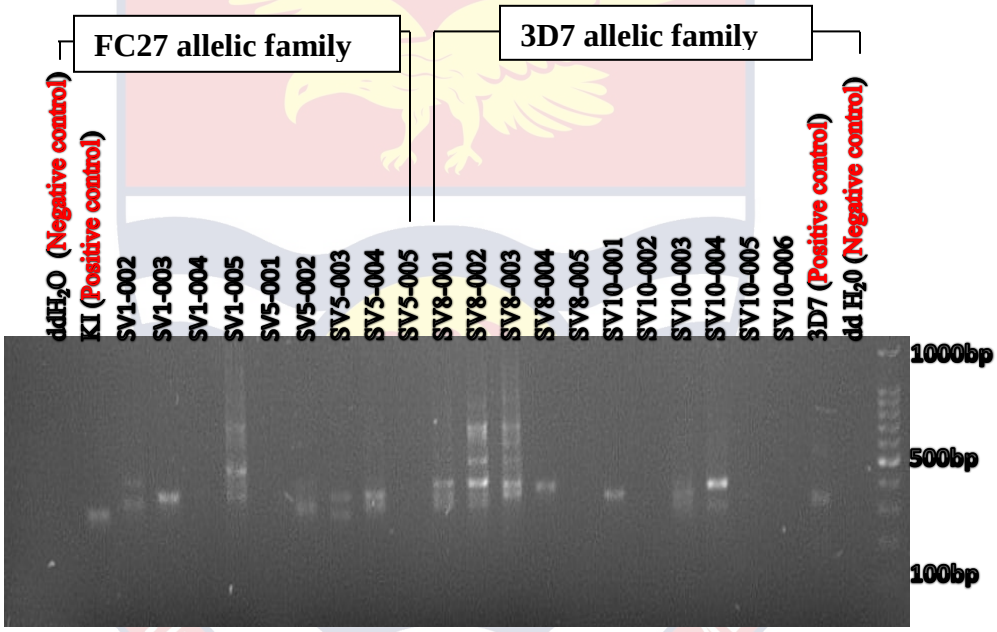
Casting of 2% Agarose gel

% Concentration = [Proportion of agarose (g)/ Proportion of TAE (ml)] x 100 %

2 % gel = [mass of agarose (g)/100 ml] x 100 %.

Therefore, mass of agarose (g) required = 0.02 x 100 g = 2 g.

Dissolve 2 g of agarose in 100 ml of 1X TAE in a microwave. Add 4µl of ethidium bromide for visualization after vapour ceases and pour into gel cast tray to solidify.



Agarose Gel Electrophoresis image of msp2 PCR products

APPENDIX C

SUMMARY OF STATISTICAL ANALYSIS

Table 3: Temperature of participants –Kruskal Wallis test

P value	0.0600
Do the medians vary significantly (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	7.406

Table 3: Hb level of participants - Kruskal-Wallis test and Dunn's Multiple comparison test

P value	0.0294
Do the medians vary significantly (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	8.990
	Is P <0.05?
Nov.2017 vs Jan.2018	No
Nov.2017 vs Mar.2018	No
Nov.2017 vs May.2018	Yes
Jan.2018 vs Mar.2018	No
Jan.2018 vs May.2018	No
Mar.2018 vs May.2018	No

Table 4: mean MOI throughout the study – Kruskal Wallis test

P value	0.5671
Do the means vary significantly (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	2.026

Table 4: Parasite density – Kruskal-Wallis and Dunn's multiple comparison test

P value	< 0.0001
Do the means vary significantly (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	76.89
	Is P<0.05?
Nov.2017 vs Jan.2018	Yes
Nov.2017 vs Mar.2018	Yes
Nov.2017 vs May.2018	Yes
Jan.2018 vs Mar.2018	Yes
Jan.2018 vs May.2018	No
Mar.2018 vs May.2018	Yes

Table 5: Change in Pfs48/45 level overtime -Kruskal-Wallis and Dunn's multiple comparison test

P value	<0.0001
Do the medians vary significantly (P<0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	38.48
	Is P < 0.05?
Nov.2017 vs Jan.2018	No
Nov.2017 vs Mar.2018	No
Nov.2017 vs May2018	Yes
Jan.2018 vs Mar.2018	No
Jan.2018 vs May2018	Yes
Mar.2018 vs May2018	Yes

Table 5: Change in Pfs230 level overtime - Kruskal-Wallis and Dunn's multiple comparison test

P value	< 0.0001
Do the medians vary significantly (P<0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	30.78
	Significant? P < 0.05?
Nov.2017 vs Jan.2018	No
Nov.2017 vs Mar.2018	No
Nov.2017 vs May2018	Yes
Jan.2018 vs Mar.2018	Yes
Jan.2018 vs May2018	Yes
Mar.2018 vs May2018	No

Table 5: Pfs48/45 IgG avidity overtime -Kruskal-Wallis and Dunn's multiple comparison

P value		< 0.0001
Do the medians vary significantly (P<0.05)?		Yes
Number of groups		4
Kruskal-Wallis statistic		55.09
	Is P < 0.05?	
Nov.2017 vs Jan.2018	No	
Nov.2017 vs Mar.2018	Yes	
Nov.2017 vs May2018	Yes	
Jan.2018 vs Mar.2018	Yes	
Jan.2018 vs May2018	Yes	
Mar.2018 vs May2018	Yes	

Table 5: Change in Pfs230 IgG avidity overtime - Kruskal-Wallis test

P value		0.1078
Do the medians vary significantly (P<0.05)?		No
Number of groups		4
Kruskal-Wallis statistic		6.080