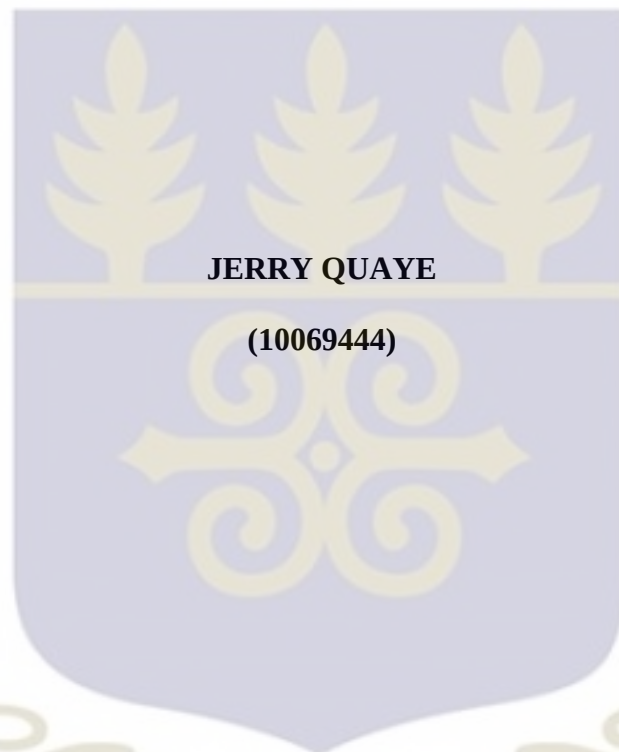


**ANTI-GAMETOCYTE ANTIBODY LEVELS AND SUBMICROSCOPIC
GAMETOCYTAEMIA IN AN AREA OF SEASONAL MALARIA TRANSMISSION**

BY



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DECLARATION

I Jerry Quaye of the Department of Medical Biochemistry, School of Biomedical and Allied Health Sciences, do hereby declare that, with the exception of quoted articles and references, this work was duly carried out by me and the results obtained herein are true reflection of the work done under the supervision of Dr. Bartholomew Dzudzor at the Department of Medical Biochemistry and Dr. Linda Eva Amoah, Research Fellow at Noguchi Memorial Institute for Medical Research.

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DEDICATION

I dedicate this project to my hard working mother Miss Beatrice Adokarley Codjoe for her immense contribution to my education and her ceaseless prayers for me.



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My ultimate gratitude first and foremost goes to the Almighty God for giving me life to do my work.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACT	Artemisinin-based Combination Therapy
AMA-1	Apical Membrane Antigen 1
AU	Arbitrary Unit
BCR	B Cell Receptor
bp	Base Pair
BSC	Biological Safety Cabinet
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary deoxyribonucleic Acid
CI	Confidence Interval
CPM	Complete Parasite Medium
CSP	Circumsporozoite Protein
DDT	Dithiothreitol
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate Mix
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
FcR	Fc Receptor
GHS	Ghana Health Service
GLURP	Glutamate Rich Protein
G-6-PD	Glucose-6-Phosphate Dehydrogenase
HMIS	Health Management Information System
HRP	Horseradish Peroxidase
IE	Infected Erythrocyte

Ig	Immunoglobulin
IgG	Immunoglobulin G
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
ITN	Insecticide-treated Nets
MHC	Major Histocompatibility Complex
MICS	Multiple Indicator Cluster Survey
mRNA	Messenger Ribonucleic Acid
MSP3	Merozoite Surface Protein 3
NAG	N-acetylglucosamine
NAI	Naturally Acquired Immunity
NASBA	Nucleic Acid Sequence-Based Amplification
NK	Natural Killer
NMCP	National Malaria Control Programme
NMIMR	Noguchi Memorial Institute for Medical Research
OD	Optical Density
OPD	Out Patient Department
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween 20
PC	Positive Control
PCR	Polymerase Chain Reaction
<i>Pf</i>	<i>Plasmodium falciparum</i>
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RPM	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SM	Submicroscopic

TBV	Transmission Blocking Vaccine
TCR	T-cell Receptors
Th2	T Helper 2
TMB	3,3', 5,5-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TRAP	Thrombospondin-related Adhesion Protein
VSA	Variable Surface Antigen
WHO	World Health Organization



ABSTRACT

Background: Malaria is one of the leading vector-borne infectious diseases in the world with alarming death rates, which is of public health concern. Only 0.1-1 gametocyte per μL blood is sufficient to spread malaria; however microscopy which is the gold standard for detection has a detection limit of 8 gametocytes per μL . Therefore, some individuals who may be diagnosed by microscopy as negative for gametocyte may be possible reservoirs of malaria transmission.

Main Objective: This study determined sub-microscopic gametocyte prevalence and anti-gametocyte antibody levels to gamete surface antigens *Pfs230*, *Pfs48/45* (transmission blocking vaccine candidates) and crude gametocyte antigen (G. Extract) from collected blood samples in recruited volunteers from the Bongo District in the Upper East Region of Ghana, which is an area of seasonal malaria transmission. Also, a determination of the correlation between the prevalence of submicroscopic gametocyte levels and anti-gametocyte antibody levels was conducted.

Design: Indirect enzyme immunosorbent assay (ELISA) was used to quantify the levels of antigens (*Pfs230*, *Pfs48/45*, crude gametocyte antigen, glutamate rich protein (GLURP) and merozoite surface protein 3 (MSP3)) specific antibodies in archived serum samples. Messenger ribonucleic Acid (mRNA) was extracted from archived blood samples preserved in RNA later (an RNA preservative) at the Noguchi Memorial Institute for Medical Research (NMIMR). Complementary DNA (cDNA) was synthesized from the mRNA using a reverse transcriptase. Nested PCR was carried out to amplify the *Pfs25* gene from the cDNA products and visualized on a 2% agarose gel.

Results: It was established that prevalence of gametocyte carriage at submicroscopic level (2.76 %) was two times higher than gametocyte carriage estimated by microscopy (1.26 %).

Antibodies to all the antigens tested were lowest among the under-five (5) years and seroprevalence (%) frequently increased with age. Linear regression analysis revealed that, gametocyte-specific antibody response was influenced by level of gametocytaemia and other confounding factors.

A moderate but significant correlation was found to exist between *Pfs*230 antibody titres and antibody titres against the crude gametocyte antigen during both the wet and dry seasons (Wet season: Spearman $r = 0.414$, $p < 0.0001$; Dry season: Spearman $r = 0.390$, $p < 0.0001$).

Conclusion:

There were antibody levels to gametocyte antigens *Pfs*48/45, *Pfs*230 and crude gametocyte antigens and generally antibody levels decreased in the dry season. In addition, antibody seroprevalence increased with age for both wet and dry seasons. This is a confirmation that these antigens are potential candidates for transmission blocking vaccines. A submicroscopic level of gametocytes was over two-fold higher compared to microscopic estimates of gametocytes. Also, there was weak but significant correlation between *Pfs*230 and G. Extract. This means that *Pfs*230 can be used in place of crude gametocyte antigen for the estimation of gametocyte exposure. Finally, from this study it was established that the gametocyte-specific antibody response was influenced by level of gametocytaemia and other confounding factors.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Research

Malaria is one of the leading vector-borne infectious diseases in the world with an estimated 3.2 billion people at risk of being infected and developing the disease and an expected 214 million cases of malaria occurred globally in 2015 and the disease led to 438,000 deaths. The burden is heaviest in African countries, where an estimated 90% of all malaria deaths occur, and the disease was the cause of 10 % of all deaths in children aged below 5 years (World malaria report, 2015). All Ghana's population of 24.2 million is at risk of malaria infection, but children under five years of age and pregnant women are at higher risk of severe illness due to lowered immunity (The 2011 Ghana Multiple Indicator Cluster Survey (MICS) Report). According to the Ghana Health Service (GHS) health facility data, malaria is the number one cause of morbidity, accounting for about 38 percent of all outpatient illnesses, 35 percent of all hospital admissions, and about 34 percent of all deaths in children below 5 years (NMCP 2010 Annual Report). Between 3.1 and 3.5 million cases of clinical malaria are reported in public health facilities each year from 2008, of which 900,000 cases are in children under five years (Ghana National Malaria Control Strategic Plan, 2008-2015).

Reports from health facilities captured in the routine health information system (HMIS) indicate that, the total Out Patient Department (OPD) malaria cases was 300 cases per 1000 population in 2012 (National Malaria Control Programme, 2013). The direct and indirect costs of malaria in Africa exceed US\$2 billion a year. In addition, the annual economic growth rate reduction due to malaria can be 1.3%, which contributes significantly to the

ongoing poverty in endemic countries (Gallup and Sachs, 2001). The most vulnerable groups remain children under five years, pregnant women and non-immune individuals (Gallup and Sachs, 2001). In acute malaria, gametocytes could occur at a much lower density making microscopic detection limited (Kuamsab, *et al* 2012).

The malaria parasite displays a complex life cycle which involves an insect vector (mosquito) and a vertebrate host (human) (Talman *et al.*, 2004). The life cycle of malaria parasite is divided into several stages and the asexual blood stage is responsible for the clinical symptoms associated with malaria. Intermittent fever paroxysm that arises from malaria infection are due to the synchronous lysis of the infected erythrocytes (Miller *et al.*, 2002; Tuteja, 2007). As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes (Carter *et al.*, 2008). Man to mosquito transmission of malaria depends on the presence of the sexual stage parasites, gametocytes. Gametocyte densities below the microscopic threshold of detection, may be sufficient to infect mosquitoes (Ouédraogo *et al.*, 2009). Gametocyte carriage may be common in asymptomatic individuals (Bousema *et al.*, 2004), in addition, majority of gametocytes are not ingested by mosquitoes, but are instead removed by the human immune system. Immune recognition of gametocytes causes infected erythrocytes to break down, exposing gametocytes and their surface proteins. This then can result in the production of anti-gametocyte immune responses (Bousema and Drakeley 2011).

Gametocyte carriage is common in an area of seasonal malaria transmission and contributes substantially to the human infectious reservoir. The gametocytes have been found to express various proteins, notably, *Pfs230* and *Pfs48/45*, which are of scientific interest as

transmission blocking vaccine candidates. This study was interested in investigating the correlation between gametocyte prevalence and the gametocyte-specific antibody levels.

1.2 Problem Statement

Globally, an estimated 3.2 billion are at risk of being infected with malaria and developing the disease and an estimated 214 million cases of malaria occurred globally in 2015 and the disease led to 438,000 deaths (World malaria report, 2015). Although much effort has been done in various control and preventive measures, the effect of this disturbing disease cannot be under estimated. Even though there is treatment for both the mild and severe forms, the cost is still beyond the reach of many.

The intensity of malaria transmission is determined by the prevalence of gametocytes in endemic populations and the number of *Anopheles* mosquitoes in that area (Bousema and Drakeley, 2011). It has been shown that gametocytes are not effectively cleared by common anti-malarial drugs and this allows for their transmission to mosquitoes despite clearance of asexual parasites (Eksi *et al.*, 2012). Several epidemiological surveys have shown that only a subset of malaria-infected individuals carried gametocytes upon microscopic examination of blood smears (Snounou *et al.*, 1993). More importantly, these gametocyte-negative blood samples remain infective to *Anopheles* vectors akin to those that have patent gametocytaemia (Bousema and Drakeley, 2011). Often gametocytes occur at much lower densities than asexual stages (Kuamsab *et al.*, 2012).

Antibodies to gametocytes antigens could be used as proxy estimate of gametocyte density and vice versa. Therefore, this study aimed at comparing anti-gametocyte antibody levels and submicroscopic gametocytaemia, since there is not much study in this area.

1.3 Justification

The determination of submicroscopic gametocytaemia through this study will enable identification of infectious parasite reservoirs and assist in assessing the impact of malaria control interventions within the community. Submicroscopic gametocyte carriage is common in an area of seasonal malaria transmission and contributes substantially to the human infectious reservoir. Gametocytes have been found to express various proteins, notably, *Pfs230*, *Pfs48/45* which are of scientific interest as potential for developing transmission blocking vaccine. The search for efficacious malaria vaccines are still in progress. Since these proteins expressed by the gametocytes evoke antibody response, it is worthwhile to investigate the correlation between gametocyte prevalence and gametocyte-specific antibody production.

1.4 Research Hypothesis

Anti-gametocyte antibody levels correlate with submicroscopic gametocyte prevalence.

1.5 Overall Objective

To assess the correlation that exists between gametocyte prevalence and the levels of gametocyte-specific antibodies during seasonal malaria transmission.

1.6 Specific Objectives

1. To measure antibody levels to the gametocyte antigens *Pfs230*, *Pfs48/45* and crude gametocyte antigen from plasma samples obtained from the blood of volunteers in the Bongo District of the Upper East Region of Ghana.

2. To detect the presence of submicroscopic gametocytes in archived blood samples from volunteers in the Bongo District of the Upper East Region of Ghana and estimate gametocyte prevalence from secondary microscopy data.
3. To assess the correlation between the prevalence of gametocytes and the levels of gametocyte-specific antibodies.



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Global Burden of Malaria

Globally, an estimated 3.2 billion people in 97 countries and territories are at risk of being infected with malaria and developing the disease (fig. 1), and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year). According to the latest estimates, 198 million cases of malaria occurred globally in 2013 (uncertainty range 124 – 283 million) and the disease led to 584,000 deaths (uncertainty range 367,000 – 755,000), representing a decrease in malaria case incidence and mortality rates of 30 % and 47 % since 2000, respectively. The burden is heaviest in the World Health Organization (WHO) African Region, where an estimated 90 % of all malaria deaths occur, and in children aged below 5 years, who account for 78 % of all deaths (World malaria report, 2014).

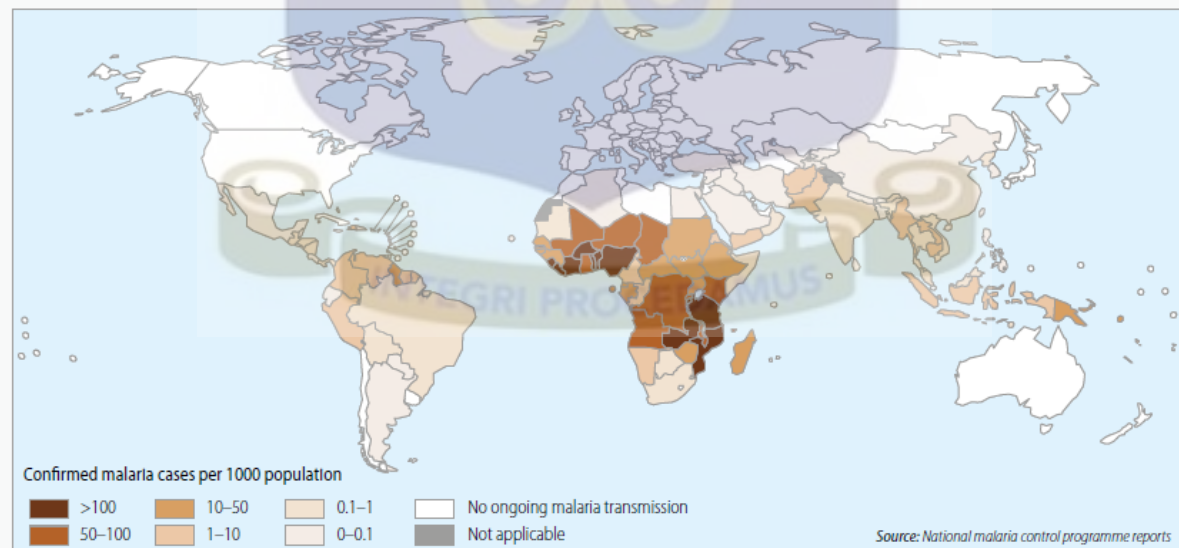


Figure 1: Countries with on-going malaria transmission in 2013. Figure directly taken from World malaria report, 2015.

2.1.1 Malaria Situation in Ghana

Malaria is endemic and perennial in all parts of the country, with seasonal variations that are more pronounced in the north. All Ghana's population of 24.2 million is at risk of malaria infection, but children under five years of age and pregnant women are at higher risk of severe illness due to lowered immunity (The 2011 Ghana Multiple Indicator Cluster Survey (MICS) Report).

According to the Ghana Health Service (GHS) health facility data, malaria is the number one cause of morbidity, accounting for about 38 percent of all outpatient illnesses, 35 percent of all hospital admissions, and about 34 percent of all deaths in children below 5 years (NMCP 2010 Annual Report). Between 3.1 and 3.5 million cases of clinical malaria are reported in public health facilities each year, of which 900,000 cases are in children under five years (Ghana National Malaria Control Strategic Plan, 2008-2015). Malaria is also a significant cause of adult morbidity, and the leading cause of workdays lost due to illnesses (The 2011 Ghana Multiple Indicator Cluster Survey (MICS) Report). Transmission tends to be less intense in large urban centres.

2.2 Strategies to Control and Eliminate Malaria

Cost-effective intervention approaches exist for malaria prevention and treatment. Among these are: vector control, which reduces transmission of parasites from humans to mosquitoes and then back to humans, which is achieved largely through use of insecticide-treated mosquito nets (ITNs) or indoor residual spraying (IRS); chemoprevention, which suppresses blood-stage infection in humans; and case management, which includes prompt diagnosis and treatment of infections (World Malaria Report, 2015).

When the ITNs are used by pregnant women, they are also efficacious in reducing maternal anaemia, placental infection and low birth weight. Historical and programme documentation has established a similar impact for IRS, although randomized trial data are limited (MEASURE DHS, Roll Back Malaria 2013). In a few specific settings and circumstances, the core interventions of ITNs and IRS can be supplemented by larval source management or other environmental modifications (World Malaria Report, 2015).

Chemoprevention is particularly effective in pregnant women and young children. Intermittent preventive treatment in pregnancy (IPTp) involves administration of sulfadoxine-pyrimethamine (SP) during antenatal clinic visits in the second and third trimesters of pregnancy. It has been shown to reduce severe maternal anaemia (Cibulskis *et al.*, 2009), low birth weight (Kim *et al.*, 2000) and perinatal mortality. By maintaining therapeutic antimalarial drug concentrations in the blood during periods of greatest malaria risk, seasonal malaria chemoprevention (SMC) with amodiaquine plus SP (AQ+SP) for children aged 3–59 months has the potential to avert millions of cases and thousands of deaths in children living in areas of highly seasonal malaria transmission in the Sahel subregion (Liu *et al.*, 2000; (World Malaria Report 2015).

Parasitological confirmation of malaria ensures treatment is given only to those infected with malaria parasites; current medicines against malaria are highly effective. In most malaria endemic areas, less than half of patients with suspected malaria infection are truly infected with a malaria parasite. Therefore, parasitological confirmation by light microscopy or rapid diagnostic tests (RDTs) is recommended in all patients before antimalarial treatment is started. Artemisinin-based combination therapy (ACT) of uncomplicated *P. falciparum* malaria has been estimated to reduce malaria mortality in children aged 1–23 months by 99%

(range: 94–100%), and in children aged 24–59 months by 97% (range: 86–99%) (World Malaria Report 2015).

The control tools enumerated above are continuously affected by the emergence of insecticide-resistant mosquitoes and drug-resistant parasites. Therefore, it is important that other tools such as vaccine are made available in order to reduce the clinical cases and mortality caused by malaria (World Malaria Report 2015).

From the above information, prompt parasite detection requires more sensitive and reliable methods. In this study, nested RT-PCR which is a sensitive tool was used to detect the presence of gametocytes and ELISA another sensitive and specific tool was used to quantify anti-gametocyte antibody levels to some sexual-stage specific antigens.

2.3 Pathogenesis of Malaria

Generally, malaria is classified as uncomplicated or severe in which various disease-related complications are observed. The complicated manifestations are directly related to the infection and the host immune factors as well as the environmental factors. Some of the major complications include severe anaemia, cerebral malaria, respiratory distress and pregnancy associated malaria. Severe anaemia is mainly found in the patients with chronic infections and those with acute infections and high parasitaemia (Phillips *et al.*, 1986; Dondorp *et al.*, 1999; Kurtzhals *et al.*, 1997).

Symptoms associated with malaria include recurrent fevers, nausea, muscle pain and headache. The recurrent fever is associated with the cyclic development of the parasite in the erythrocytic phase in which the rupture of the infected erythrocyte (IE) release hemozoin and

other proteins that induce proinflammatory cytokine response including TNF- α which lead to fever development (Kwiatkowski *et al.*, 1989).

2.4 Types of Malaria Parasites

Five species of *Plasmodium* are known. The species that cause malaria in human are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and a primate malaria parasite, *P. knowlesi* that also can infect human host. *P. falciparum* is the most common and responsible for the majority of the severe cases and mortality in Africa (Tuteja, 2007).

2.5 Life Cycle of *Plasmodium falciparum*

The malaria parasite displays a complex life cycle which involves an insect vector (mosquito) and a vertebrate host (human) (Talman *et al.*, 2004). The life cycle of malaria parasite is divided into several stages. Infected female *Anopheles* mosquitoes inject sporozoites into human blood stream when taking a blood meal (fig. 2). The sporozoites are carried by the circulatory system to the liver and invade hepatocytes (Tuteja, 2007). The intracellular parasite goes through an asexual replication known as exoerythrocytic schizogony within the hepatocyte. Exoerythrocytic schizogony culminates in the production of merozoites which are released into the bloodstream (Tuteja, 2007).

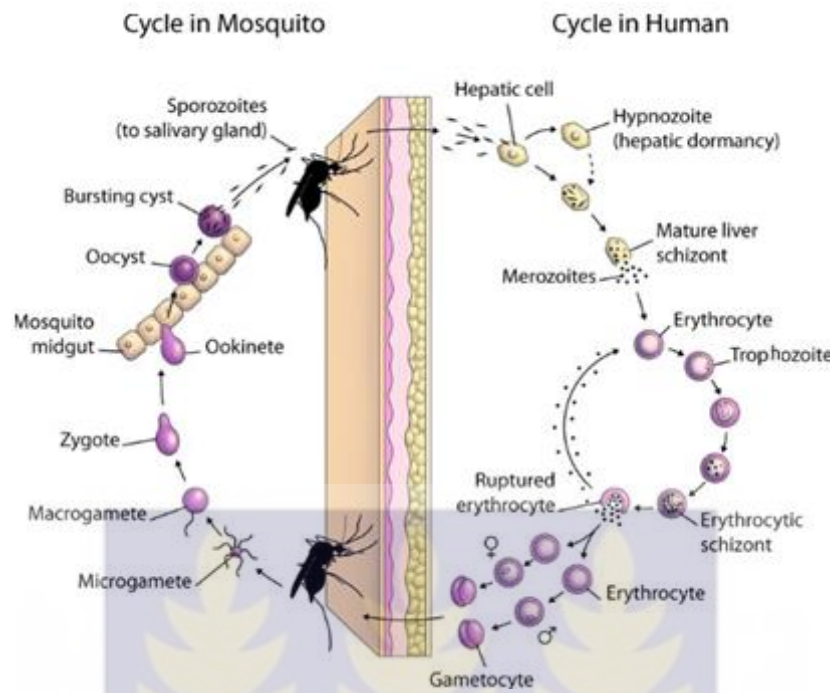


Figure 2: Life Cycle of Malaria Parasite. Figure directly taken from reference source ([http://www.malariasite.com/life cycle/](http://www.malariasite.com/life%20cycle/))

The merozoites invade erythrocytes and undergo a trophic period in which the parasite enlarges (Miller *et al.*, 2002). The early trophozoite is often referred to as 'ring form' because of its morphology. Trophozoite enlargement is followed by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into amino acids (Tuteja, 2007). The end of the trophic period is marked by multiple rounds of nuclear division without cytokinesis resulting in a schizont (Miller *et al.*, 2002). The merozoites bud from the mature schizont, also called a segmenter, and the merozoites are released following rupture of the infected erythrocyte. Invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle (Miller *et al.*, 2002).

The asexual blood stage is accountable for the clinical symptoms associated with malaria. The intermittent fever paroxysms are due to the synchronous lysis of the infected erythrocytes. *P. malariae* exhibits a 72 hour periodicity, whereas the other four species exhibit 48 hour

cycles (Miller *et al.*, 2002; Tuteja, 2007). However, *P. falciparum* often displays a continuous fever rather than the periodic paroxysms. *P. falciparum* also is responsible for more morbidity and mortality than the other species. This increased virulence is due in part to the higher levels of parasitaemia associated with *P. falciparum* infections (Tuteja, 2007). Also, more complications are associated with *P. falciparum* because of the sequestration of the trophozoite- and schizont-infected erythrocytes in the deep tissues (Tuteja, 2007).

As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes (Carter *et al.*, 2008). The gametocytes are large parasites which fill up the erythrocyte, but only contain one nucleus. The newly formed gametocyte will then undergo five different maturation stages namely stage I to V (fig. 3) (Hawking, Wilson, & Gammage, 1971) before being transmitted into mosquito. All five stages can be observed in parasites from in vitro culture (Ponnudurai *et al.*, 1986), but in the circulation only stage V gametocytes can be observed, since the immature stages I-IV sequester from the bloodstream (Sinden, 1982). The first gametocyte stage cannot be distinguished from young asexual stages (Sinden, 1982). The presence of a subpellicular membrane underneath the parasitophorous membrane, which develops from the late stage I onwards differentiate gametocyte from asexual stage (Sinden, 1983). Morphological changes start from the second gametocyte stage and morphological differences between male and female gametocytes are clearly visible from stage III gametocytes onwards (Sinden, 1982).

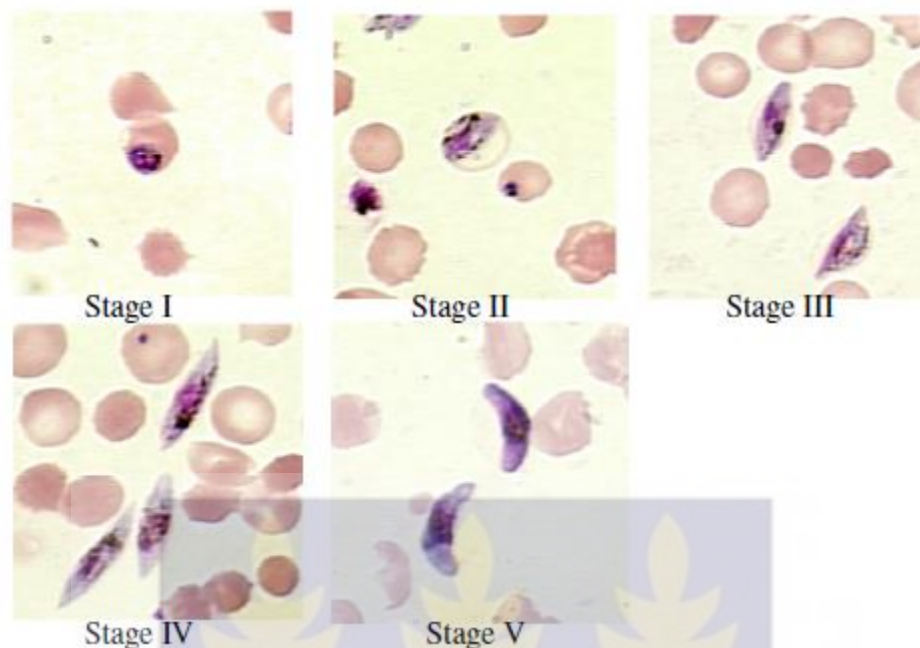


Figure 3: Giemsa Smears of Gametocyte Stages I-V. Figure directly taken from referenced publication (Tuteja, 2007).

The drastic changes in morphology are preceded by an array of genes expressed in a sexual stage-specific way at the RNA or the protein level (Alano, 1991). Sexual stage development starts in the asexual cycle preceding morphological changes of the gametocyte with the expression of *Pfs16* mRNA as the earliest marker of parasite commitment to sexual stage development (Bruce *et al.*, 1990). Schizonts that are committed to develop into sexual stage parasites will produce either all male or all female gametocytes (Silvestrini *et al.*, 2000).

Studies based on standard procedures have shown that *P. falciparum* gametocytes are present in a relatively low proportion of infected individuals and gametocyte densities are in general low (Shute & Maryon, 1951). The ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and escape from the host erythrocyte. These gametocytes are essential for transmitting infection to new hosts through female *Anopheles* mosquitoes (Carter *et al.*, 2008).

Microgametes, formed by a process known as exflagellation, are flagellated forms which will fertilize the macrogamete leading to a zygote (Tuteja, 2007). The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst goes through multiple rounds of asexual replication resulting in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito. The sporozoites migrate to and invade the salivary glands, thus completing the life cycle (Tuteja, 2007).

2.6 Gametocytaemia and Submicroscopic Gametocytaemia

Gametocytaemia implies the presence of gametocytes in the peripheral blood. Submicroscopic gametocytaemia refers to the presence of gametocyte (s) in the peripheral blood below the detectable threshold of the light microscope. Transmission of *P. falciparum* is successfully done from human host to mosquito host through the specialized cells referred to as gametocytes. Gametocytes are either male or female and are programmed to mature into male or female gametes in the mosquito midgut before fertilization to a zygote that establishes infection in the insect vector. Gametocytes appear in the peripheral blood circulation 7-15 days after the initial asexual wave (Eichner *et al.*, 2001; Day *et al.*, 1998).

Majority of gametocytes are not ingested by mosquitoes, but are instead removed by the human immune system which breaks down infected erythrocytes to expose gametocytes and their surface proteins which can result in the production of anti-gametocyte immune responses (Bousema and Drakeley 2011). This anti-gametocyte immunity has been suggested to function in a number of different ways by clearing gametocytes from the site of sequestration or from blood circulation or by blocking transmission to the mosquito (Bousema and Drakeley 2011). Evidence suggests gametocyte specific antibodies recognize

molecules on the gametocyte infected erythrocyte, and serum reacting to this has been associated with reduced gametocyte prevalence (Saeed *et al.*, 2008; Sutherland, 2009; Bousema and Drakeley 2011).

Gametocyte carriage may be common in asymptomatic individuals (Bousema *et al.*, 2004). Man to mosquito transmission of malaria depends on the presence of the sexual stage parasites, gametocytes that often circulate at low densities. Gametocyte densities below the microscopic threshold of detection may be sufficient to infect mosquitoes (Ouédraogo *et al.*, 2009). Some children with a gametocyte density below 1 gametocyte/mL were able to infect mosquitoes in a study (Ouédraogo *et al.*, 2009).

Submicroscopic gametocyte carriage is common in an area of seasonal transmission and contributes substantially to the human infectious reservoir (Ouédraogo *et al.*, 2009). Molecular tools have become available to detect and quantify gametocytes at densities well below the microscopic threshold, in the order of 0.02 – 10 gametocytes/mL of blood (Babiker *et al.*, 2008).

Using molecular techniques, it has become evident that the proportion of gametocyte carriers in the population has been grossly underestimated and that the gametocyte reservoir may be 2 to 5 fold larger than assumed based on microscopy (Shekalaghe *et al.*, 2007; Bousema *et al.*, 2006).

2.7 Gene Expression and Some Associated Antigens of *P. falciparum*

Throughout the life cycle of *P. falciparum*, various genes are expressed resulting in the formation of various mRNAs and proteins which serve numerous purposes. Some of the

proteins enable the parasite to survive and escape the effect of the host immune system. Some of these proteins are stage-specific, whereas others can be found simultaneously at various stages of the parasite life cycle.

Sporozoites express the 6-cys proteins *P52* and *P36* (fig. 4). A proportion of the sporozoites are carried through the bloodstream and end up in the liver sinusoid where they migrate through Kupffer cells and traverse through hepatocytes before colonizing inside a hepatocyte (Baker *et al.*, 1995). The best-studied of the proteins are the circumsporozoite protein (CSP) and the thrombospondin-related adhesion protein (TRAP). Both proteins have been identified in all *Plasmodium* species. These proteins are also involved in salivary gland and hepatocyte invasion (Sultan *et al.*, 1997). AMA-1 (Apical Membrane Antigen - 1) has been extensively studied in asexual stages, where it functions in merozoite invasion of erythrocytes (Srinivasan *et al.*, 2004).

A unique protein family containing several cysteine repeats has been identified in sexual stages of *Plasmodium*. *Pfs230* and *Pfs48/45* are two members of this family that have been well characterized and identified as potential transmission-blocking vaccine candidates (Moreira, Marrelli, & Jacobs-Lorena, 2004). Expression of both these proteins begins in stage III gametocytes. *Pfs230* encodes a 360 kDa protein that contains six tandemly repeated six-cysteine domains and is localized to the plasma membrane. Full length *Pfs230* (360 kDa) is proteolytically cleaved to a 310 kDa for as soon as the parasite emerges from the red cell in the mosquito midgut (Williamson *et al.*, 1996). The role of *Pfs230* in sexual stage parasites has yet to be determined, as mutants expressing truncated forms of this protein are still able to form gametocytes (Eksi *et al.*, 2002).

Pfs48/45 encodes a 55 kDa membrane protein containing two six-cysteine domains (Moreira *et al.*, 2004). Knockout mutants lacking this gene were still able to form gametocytes that differentiated into gametes, but male gametes were unable to adhere to and penetrate female gametes. Fertilization and zygote formation were thus severely affected (van Dijk *et al.*, 2001).

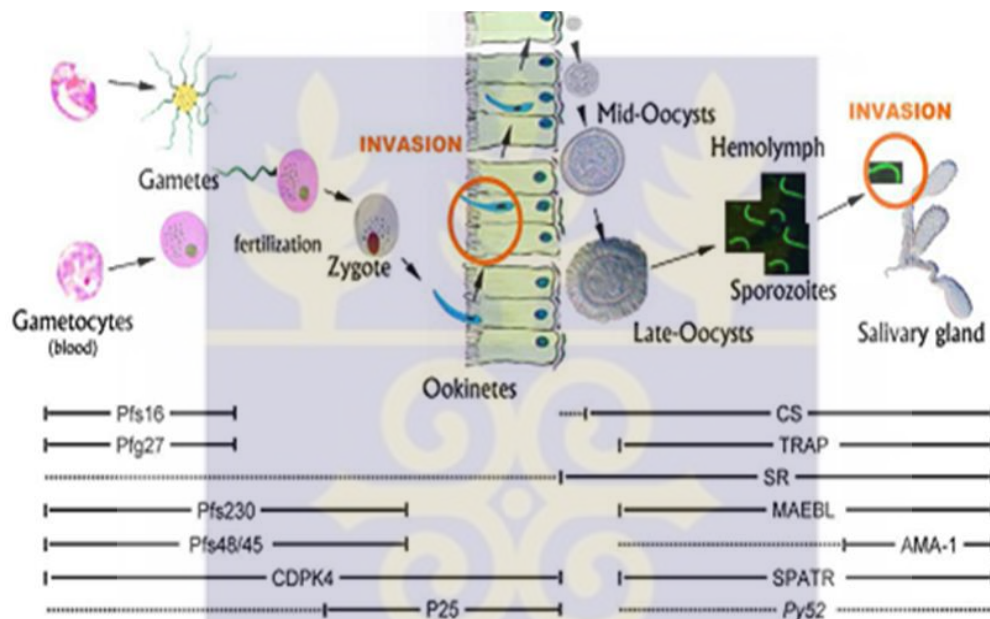


Figure 4: Temporal Patterns of Gene Expression Associated with *P. falciparum* Life Cycle. The solid lines indicate parasite stages at which protein was detected, and the dashed lines indicate parasite stages at which mRNA, but not the corresponding protein, was detected. Figure directly taken from referenced publication (Moreira *et al.*, 2004).

Most of the stage-specific gene expression and protein analysis have been focusing on the gamete surface antigens that are synthesized during gametocytogenesis, among them *Pfs230*, *Pfs48/45*, and *Pfs25* are of scientific interest as potential for developing transmission blocking vaccine (Vermeulen *et al.*, 1985; Quakyi *et al.*, 1987; Kaslow *et al.*, 1988). The levels of transcription and translation have been shown to vary across the gametocyte stages, with more RNA and protein synthesized in early than late gametocytes stages (Sinden, 1983). It has been suggested that sexual stage antibodies derived against *Pfs48/45* and *Pfs230*

develop quickly, are reliant on recent rather than cumulative exposure, and are short lived (Riley *et al.*, 1994). A study in Papua New Guinea found an increase of antibody prevalence and density for *Pfs48/45* and *Pfs230* with age (Graves *et al.*, 1991), whereas evidence from Tanzania showed an age dependent decrease (Drakeley *et al.*, 2006; Bousema *et al.*, 2007; Bousema *et al.*, 2011).

Pfs16 is not essential for sexual development, but may be required for optimal production of sexual parasites (Kongkasuriyachai *et al.*, 2004). *Pfg27* is expressed at about 30 h post-erythrocytic invasion, by both gametocytes sexes and is distributed throughout the cell cytoplasm (Moreira *et al.*, 2004). The sexual stage is characterized by the development of male and female gametocytes inside the red blood cell (Rupp *et al.*, 2011).

The developing gametocytes stage I to IV sequester in the bone marrow and spleen (Thomson and Robertson, 1935), in a similar pattern to that of asexual parasites endothelial sequestration in various organs (Howard, 1988). When the female mosquito takes an infectious blood meal, it also ingests antibodies that interfere with parasite development, thus preventing transmission to another individual. Several *Plasmodium* proteins such as *Pfs25*, *Pfs48/45* and *Pfs230* have been tested as TBV candidates as antibodies against them have been found to be transmission blocking (Moreira *et al.*, 2004).

Whereas *Pfs16* is suitable as a marker of early stage gametocytes, identification of late stage gametocytes requires quantification of the *Pfs25* gene, whose mRNA is expressed in stage V gametocytes (Schneider *et al.*, 2004) and in mosquito sexual stages (gametes/ookinetes) (Kongkasuriyachai and Kumar, 2002). *Pfs25* encodes a surface antigen containing epidermal growth factor-like domains, which may be involved in invasion of the midgut epithelium.

Antibodies raised against *Pfs25* can block the development of oocysts in the midgut of the mosquito thereby significantly reducing transmission of the parasite; this makes it a leading transmission-blocking vaccine candidate (Barr *et al.*, 1991; Kaslow *et al.*, 1991).

2.8 Endemicity of Malaria

Malarial endemicity levels may be categorized into four levels, Table 1.

Table 1: Classification of Levels of Endemicity of Malaria Transmission

Endemicity Level	Transmission	Parasite rate (%)	Comment
Hypoendemic malaria	Low: subperiodic or sporadic	Usually <10 %	Mosquito populations unstable, usually difficult to detect; serious epidemics may occur
Mesoendemic malaria	Seasonal or periodic following good rains	11-50, depending on survey timing	Mosquito populations fluctuate, detectable seasonally; seasonal outbreaks occur; some detectable immunity in the population
Hyperendemic malaria	Intense transmission (seasonal) each year	51-75	Seasonal fluctuation in malaria cases; severe sequelae frequent in young children; some immunity in adults
Holoendemic malaria	Transmission may occur through the year with periods of high transmission	>75	Mosquitoes detectable throughout the year although with seasonal peaks; high levels of anemia in very young children; immunity seen in adults.

Source: (Shiff, 2002), parasite rate (%) in children aged 5 to 10 years

2.9 Immunity

Immunity against malaria can be categorized into natural or innate or non-specific immunity and acquired or adaptive immunity. Innate immunity is an inbuilt refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite. The innate immunity is naturally present in the host and is not reliant on any past infection. Changes in the structure of haemoglobin or in certain enzymes

have been found to give protection against either the infection or its severe manifestations and these traits occur often in areas of high malaria transmission (Carter and Mendis, 2002).

Acquired immunity against malaria on the other hand develops after infection and its protective efficacy varies depending on the characteristics of the host, place of stay, number of infections suffered etc. As a matter of emphasis, it is worth reiterating that it has been graded as anti-disease immunity (that protects against clinical disease), anti-parasite immunity (protects against high parasitaemia), and sterilizing immunity (protects against new infections by maintaining a low-grade, asymptomatic parasitaemia; also called premunition), with a considerable overlap between these (Carter and Mendis, 2002). Premunition suggests an immunity mediated directly by the presence of the parasites themselves and not as much the result of previous infections (Richard and Mendis, 2002; Doolan *et al.*, 2009).

The acquired immunity is directed predominantly against the asexual erythrocytic stage, the primary targets being the extracellular merozoites in circulation. Although the preerythrocytic stage is also targeted by protective immune responses, it does not effectively block sporozoite invasion or intrahepatic development of the parasite (Doolan *et al.*, 2009). The acquired anti-malarial immunity does not last long. In the absence of re-infection for about 6 months or 1 year, as may happen when the person leaves the malarious area, the acquired immunity turns ineffective and the individual becomes vulnerable to the full impact of a malarial infection once again. The immunity is also rendered less effective during pregnancy, particularly during the first and second pregnancies, due to the physiological immunosuppression as well as the cytoadherence of erythrocytes to the newly available Chondroitin Sulfate A receptors on the placenta. Such loss of acquired immunity makes the pregnant woman more susceptible to malaria and its complications (Carter and Mendis, 2002; Doolan *et al.*, 2009).

The presence of genetically and antigenically distinct strains of the parasites in a given locality and the occurrence of clonal antigenic variation during the course of an infection force the host to mount immune response against these different strains and antigenic variants. The acquisition of immunity against malaria is, therefore, very slow and not very effective and remains species specific and strain specific (Doolan *et al.*, 2009). Acquired immunity is specific and delayed in nature and can be broadly categorized into two notable groups: humoral and cellular immunity.

2.9.1 Humoral Immunity

Humoral immunity refers to antibody production and the accessory processes that accompany it, including: Th2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation (Janeway, 2001). Antibodies are glycoproteins belonging to the immunoglobulin superfamily (Maverakis *et al.*, 2015). They recognize a unique molecule of the harmful agent, called an antigen, via the variable region (Janeway *et al.*, 2001). They are secreted by cells of the adaptive immune system (B cells), and more specifically, differentiated B cells called plasma cells. Antibodies can occur in two physical forms, a soluble form that is secreted from the cell, and a membrane-bound form that is attached to the surface of a B cell and is referred to as the B cell receptor (BCR). The BCR is found only on the surface of B cells and facilitates the activation of these cells and their subsequent differentiation into either antibody factories called plasma cells or memory B cells that will survive in the body and remember that same antigen so the B cells can respond faster upon future exposure (Borghesi and Milcarek, 2006).

Structurally, an antibody (Ab) or immunoglobulin (Ig) is a large Y-shape protein (fig. 5) produced by plasma cells that is used by the immune system to identify and neutralize pathogens such as bacteria and viruses (Janeway *et al.*, 2001; Litman *et al.*, 1993).

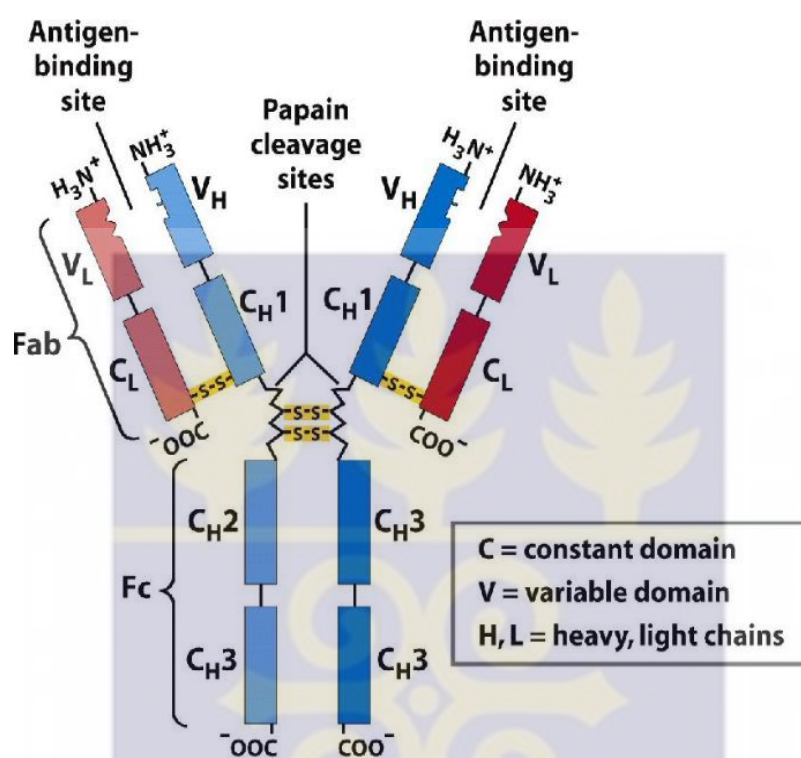


Figure 5: Structure of an Antibody. Figure directly taken from referenced source (<http://chemistry.umeche.maine.edu/CHY431/Antibody2.jpg>).

The ability of an antibody to communicate with the other components of the immune system is mediated via its Fc region (located at the base of the "Y"), which contains a conserved glycosylation site involved in these interactions (Maverakis *et al.*, 2015).

In most cases, interaction of the B cell with a T helper cell (fig. 6) is necessary to produce full activation of the B cell and, therefore, antibody generation following antigen binding (Parker, 1993). Antibodies are typically made of basic structural units each with two large heavy chains and two small light chains. There are several different types of antibody heavy chains based on five different types of crystallisable fragments (Fc) that may be attached to the

antigen-binding fragments (Maverakis *et al.*, 2015). Though the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures, or antigen-binding sites, to exist. This region is known as the *hypervariable region*. Each of these variants can bind to a different antigen (Janeway, 2001). The large and diverse population of antibody paratope is generated by random recombination events of a set of gene segments that encode different antigen-binding sites (or *paratopes*), followed by random mutations in this area of the antibody gene, which create further diversity (Market and Papavasiliou, 2003; Diaz and Casali, 2002). Antibody genes also re-organize in a process called class switching that change the one type of heavy chain Fc fragment to another, creating a different isotype (IgA, IgD, IgE, IgG, and IgM) of the antibody that retains the antigen-specific variable region. This allows a single antibody to be used by different types of Fc receptors, expressed on different parts of the immune system (Diaz and Casali, 2002). The ability of an antibody to bind to its corresponding FcR is further modulated by the structure of the glycan(s) present at conserved sites within its Fc region (Maverakis *et al.*, 2015). This ability of an antibody to bind to specific FcRs helps to direct the appropriate immune response for each different type of foreign object they encounter (Market and Papavasiliou, 2003).

Antibodies contribute to immunity in three ways (fig. 6): They prevent pathogens from entering or damaging cells by binding to them; they stimulate removal of pathogens by macrophages and other cells by coating the pathogen; and they trigger destruction of pathogens by stimulating other immune responses such as the complement pathway (Ravetch and Bolland, 2001).

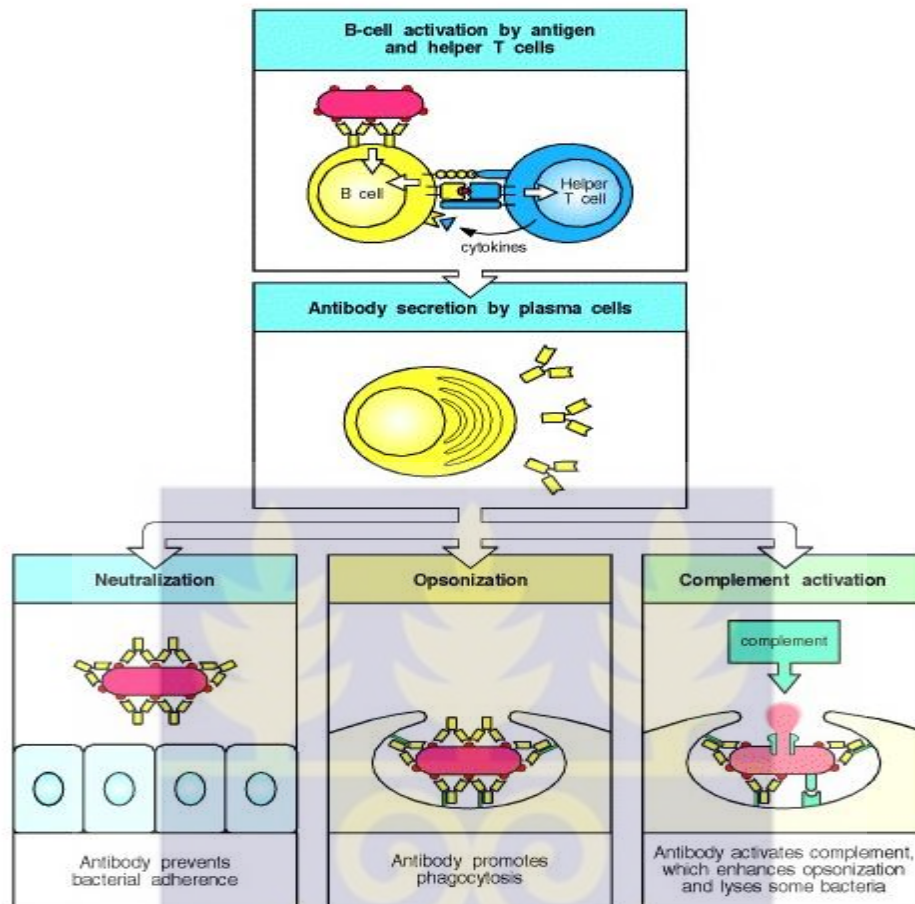


Figure 6: An Illustration of the Humoral Immune Response Mediated by Antibody Molecules that are secreted by Plasma Cells. Figure directly taken from Janeway *et al.*, 2001.

Antibodies that bind to the pathogen (e.g. viruses and intracellular bacteria) can prevent them from binding to specific molecules on the target cell surface and are said to neutralize the pathogen. Neutralization by antibodies is also important in preventing bacterial toxins from entering cells (Janeway *et al.*, 2001).

2.9.1.1 Types of Antibodies Prominent in Malaria Infection and their Significance

Malaria infection induces both polyclonal and specific immunoglobulin production, predominantly IgM and IgG but also of other immunoglobulin isotypes. Of these, 5% or more represent species- as well as stage-specific antibodies reacting with a wide variety of parasite antigens. Passive transfer of IgG from immune donors may be protective by reducing

parasitemia and clinical disease. Malaria infections of both humans and experimental animals are also associated with elevations in total IgE and IgE anti-malarial antibodies, reflecting a switch of regulatory T cell activities from Th1 to Th2 due to repeated exposure of the immune system to the parasites. IgE levels are significantly higher in patients with cerebral or other forms of severe disease than in those with uncomplicated malaria and the pathogenic effect of IgE is probably due to local overproduction in microvessels of tumor necrosis factor (TNF) and nitric oxide (NO) caused by IgE-containing immune complexes (Perlmann and Troye-Blomberg, 2002).

Immunoglobulin G (IgG) antibodies to a number of vaccine candidate antigens including the preerythrocytic antigens circumsporozoite protein (CSP) (John *et al.*, 2008), liver-stage antigen 1 (LSA-1) (John *et al.*, 2008; Migot-Nabias *et al.*, 2000), and thrombospondin-related adhesive protein (TRAP) (Scarselli *et al.*, 1993); the blood-stage antigen merozoite surface protein 1 (MSP-1) (Branch *et al.*, 1998; Egan *et al.*, 1996; Osier *et al.*, 2008; Riley *et al.*, 1992); and the blood-stage and preerythrocytic antigen apical membrane antigen 1 (AMA-1) (Nebie *et al.*, 2008; Osier *et al.*, 2008) have been associated with protection from clinical malaria in areas of stable transmission. The acquisition of IgG antibodies to these antigens also strongly correlates with increasing age (Chelimo *et al.*, 2005; Riley *et al.*, 1992; Scarselli *et al.*, 1993). Malaria infections are able to boost primary antibody responses against gametocytes and increase the TB effects of the corresponding sera. Immunogenic proteins that are important for parasite fertilization or other vital functions of the parasite sexual forms are considered targets for TB vaccine development (Carter *et al.* 2000, Stowers & Carter 2001).

2.9.2 Cellular Immunity

Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages (Perlmann and Troye-Blomberg, 2002). Malaria parasites not only escape the host's immune response, owing to their antigenic diversity and clonal antigenic variation, but also modulate the immune response and cause significant immune suppression (Millington *et al.*, 2006 and Hisaeda *et al.*, 2005). In this study the levels of antigametocyte anti-bodies is of interest and much more attention has been given to the humoral arm of acquired immunity to malaria than the cellular arm.

2.10 Epidemiology of Gametocytes

Patterns in gametocyte carriage in naturally infected individuals define the contributions of different populations to the human infectious reservoir for malaria (i.e., those individuals who are infectious to mosquitoes). By microscopy, gametocytes are invariably detected in fewer than 50 % of clinical (Barnes *et al.*, 2008; Bousema *et al.*, 2006) and asymptomatic (Bousema *et al.*, 2004) *P. falciparum* infections.

2.10.1 Age-Dependent Carriage of Gametocytes

Gametocytes can be present in up to 40 % of children upon presentation at a health center with symptomatic malaria, according to microscopy results (Barnes *et al.*, 2008; Bousema *et al.*, 2006). Gametocytes can also be found in asymptomatic individuals, although at a lower rate (Bousema *et al.*, 2004).

Broadly, the prevalence of gametocytes in asymptomatic individuals closely follows that of asexual parasites, both by microscopy (Bousema *et al.*, 2004), and by molecular detection tools (Ouédraogo *et al.*, 2007). In areas of high malaria transmission intensity, gametocyte

carriage is most prevalent in younger age groups, who also have the highest prevalence and densities of asexual malaria parasites (Boudin *et al.*, 1991).

In areas of low transmission intensity, the association between gametocyte prevalence and age is weaker, and smaller proportions of all age groups carry gametocytes (Abdel-Wahab *et al.*, 2002). While gametocyte prevalence appears to follow a predictable pattern, gametocyte densities do not appear to do so. In gametocyte-positive individuals, the density of gametocytes relative to asexual parasites increases with age.

2.10.2 Effect of Season and Transmission intensity on Gametocyte Carriage

Malaria infections and symptomatic malaria episodes are most frequent in the transmission season, usually when mosquitoes are most abundant following seasonal rains (Oesterholt *et al.*, 2006; Ouédraogo *et al.*, 2008). Two longitudinal studies in areas of markedly different transmission characteristics in Burkina Faso indicated that patterns of gametocyte carriage followed that of asexual parasites, increasing during the peak transmission season (Boudin *et al.*, 1991).

However, in both settings, there were some inconsistencies, with relatively high rates of gametocyte carriage during times when asexual parasite carriage was low or declined and stable rates of gametocyte production during the dry season (Boudin *et al.*, 1991). The absolute number of gametocyte carriers is evidently lowest in the dry season, but a larger proportion of the relatively scarce asexual infections in the dry season may produce gametocytes, and the density of gametocytes in these infections relative to that of asexual parasites may be higher in the dry season (Boudin *et al.*, 1991; Drakeley *et al.*, 2006; Marques *et al.*, 2005).

Although these associations have been described across a range of endemic settings, there is no definitive evidence, as the data are from cross-sectional rather than more informative longitudinal studies. A study where parasite isolates were collected at different time points in the season and submitted to short-term culture to determine their gametocyte- producing capacity did not show a higher production level of gametocytes in the dry season. On the contrary, isolates collected during the months of peak transmission were more likely to produce gametocytes and produced gametocytes at higher densities (Biswas, 2000).

2.11 Serological Markers as Epidemiological Tools

Serological tools, e.g. ELISA allow us to detect variations in malaria transmission over time. Such tools will be invaluable for monitoring trends in malaria endemicity and the effectiveness of malaria control programs (Drakeley *et al.*, 2005). Entomological inoculation rate (EIR) is currently the standard tool for transmission monitoring but this is not sensitive enough, especially in areas of very low transmission. Transmission estimation models based on seroconversion rates (λ) of antibodies to *Plasmodium falciparum* blood stage antigens are gaining relevance (Kusi *et al.*, 2014).

Acquisition and maintenance of antimalarial antibodies depend on exposure to malaria infection, because of that such antibodies might be used as proxy measures of transmission intensity (Drakeley *et al.*, 2005). Serological tools based on antibody responses to parasite and vector antigens are potential tools for transmission measurements (Badu *et al.*, 2015).

2.11.1 ELISA

Enzyme-linked immunosorbent assay (ELISA) is widely used as diagnostic tools in medicine and as quality control measures in various industries; it is also used as an analytical tools in

biomedical research for the detection and quantification of specific antigens or antibodies in a given sample (Gan and Patel, 2013).

2.11.2. General Principle of ELISA

ELISA uses the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibody in a fluid sample. ELISA utilizes enzyme-labelled antigens and antibodies to detect the biological molecules, the most commonly used enzymes being alkaline phosphatase and glucose oxidase. The antigen in fluid phase is immobilized, usually in 96-well microtiter plates. The antigen is allowed to bind to a specific antibody, which is itself subsequently detected by a secondary, enzyme-coupled antibody (Gan and Patel, 2013).

A chromogenic substrate for the enzyme yields a visible colour change or fluorescence, indicating the presence of antigen. Quantitative or qualitative measures can be assessed based on such colorimetric reading. Under standard conditions, the enzyme activity measured is proportional to amount of specific antibody present in the original serum (Gan and Patel, 2013).

2.11.3 Indirect ELISA

A sample that must be analyzed for a specific antigen is adhered to the wells of a microtiter plate, followed by a solution of non-reacting protein such as bovine serum albumin to block any areas of the wells not coated with the antigen. The primary antibody, which binds specifically to the antigen, is then added, followed by an enzyme-conjugated secondary antibody. A substrate for the enzyme is introduced to quantify the primary antibody through a

colour change (fig. 7). The concentration of primary antibody present in the serum directly correlates with the intensity of the colour (Gan & Patel, 2013).

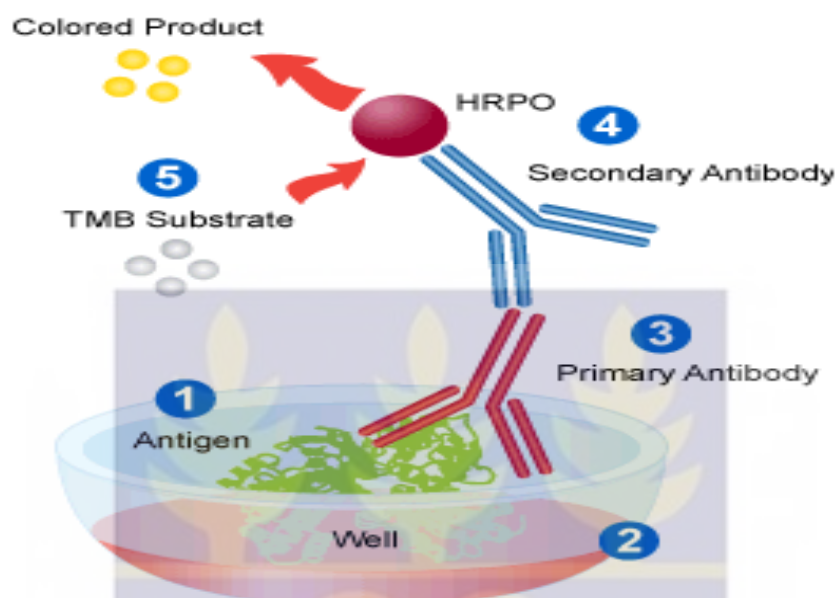


Figure 7: An Indirect ELISA. Figure directly taken from referenced source (www.leinco.com).

2.12 Diagnosis of Malaria

Light microscopy is regarded as the gold standard for the diagnosis of malaria. It is able to differentiate malaria species and quantify parasites (Jonkman *et al.*, 1995). Microscopy involves the examination of blood films stained with Giemsa, or field stain (Warhurst and Williams, 1996).

2.12.1 Disadvantages/Pitfalls in Microscopic Detection of Malaria Parasite

Microscopic diagnosis has its shortfalls in that it has been shown to be insensitive and nonspecific, especially when parasitaemia are low or mixed infections are present (Amexo *et al.*, 2004). Sensitivities and specificities as low as 71–72 % have been reported in field conditions (Arai *et al.*, 1996; Snounou *et al.*, 1993a). Microscopy may occasionally fail to

differentiate between species in cases where morphologic characteristics overlap (especially *Plasmodium vivax* and *P. ovale*), as well as in cases where parasite morphology has been altered by drug treatment or improper storage of the sample (CDC). Microscopy requires a level of skill often not available in many health facilities in several malaria-endemic countries, especially in remote rural areas, where most malaria transmission occurs. In addition, lack of functional microscopes or electricity to run them, lack of or sub-standard reagents such as stains, and high workloads may affect the quality of results (CDC). In an attempt to enhance the detection of malaria parasites, alternative methods have been introduced, which include use of RDTs, ELISA technique (mentioned in the previous section) and molecular based techniques.

2.12.2 Methods involved with the Quantification of Parasite Density

There are two acceptable methods of expressing the parasite load: either as the percentage of infected erythrocytes as counted on a stained thin blood film (e.g. 1% parasitaemia), or the number of parasites per unit volume of blood (e.g. 5000 parasites/ μ L). The latter is usually assessed on a stained thick film by counting parasites against leukocytes (100, 200 or more), then multiplying by either the patient's own leukocyte count if available, or a standard count of 8000/ μ L. When the parasite count is either very high or very low, thick film and thin film counts, respectively (WHO).

2.12.3 Limit of Parasite Detection Using Microscopic Diagnosis

From the thick film, an experienced microscopist can detect parasite levels (or parasitemia) as few as 5 parasites/ μ L blood (Guerrant *et al.*, 2006).

2.13 Molecular Detection Tools

Molecular detection tools such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-Based Amplification (NASBA) have been recently developed in the molecular diagnosis of malaria. Several experimental assays have been reported since 1990 that use various primers, extraction and detection techniques (Snounou *et al.*, 1993b).

2.13.1 Conventional PCR

A commonly used PCR test is based on the amplification of 18S ribosomal RNA (rRNA) gene for the detection of all the *Plasmodium species* (Snounou *et al.*, 1993b). PCR is a simple enzymatic assay that enables amplification of a specific DNA fragment from a complex pool of DNA. PCR can be performed using source DNA from a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes. Only trace amounts of DNA are needed for PCR to generate enough copies to be analyzed using conventional laboratory methods. For this reason, PCR is a sensitive assay (Garibyan and Avashia, 2013).

Each PCR assay requires the presence of template DNA, primers, nucleotides, and DNA polymerase. The DNA polymerase is the key enzyme that links individual nucleotides together to form the PCR product. The nucleotides include the four bases: adenine, thymine, cytosine, and guanine (A, T, C, G) - that are found in DNA. These act as the building blocks that are used by the DNA polymerase to create the PCR product. The primers in the reaction specify the exact DNA product to be amplified. The primers are short DNA fragments with a defined sequence complementary to the target DNA that is to be detected and amplified. These serve as an extension point for the DNA polymerase to build on (Garibyan and Avashia, 2013).

2.13.2 Nested RT-PCR

Although standard reverse transcriptase-polymerase chain reaction (RT-PCR) is a remarkably sensitive technique, its sensitivity can be further increased by performing nested RT-PCR (O'Connell, 2002). First RNA is extracted from the sample of interest, and then cDNA is synthesized from the extracted RNA through the use of a reverse transcriptase (fig. 8).

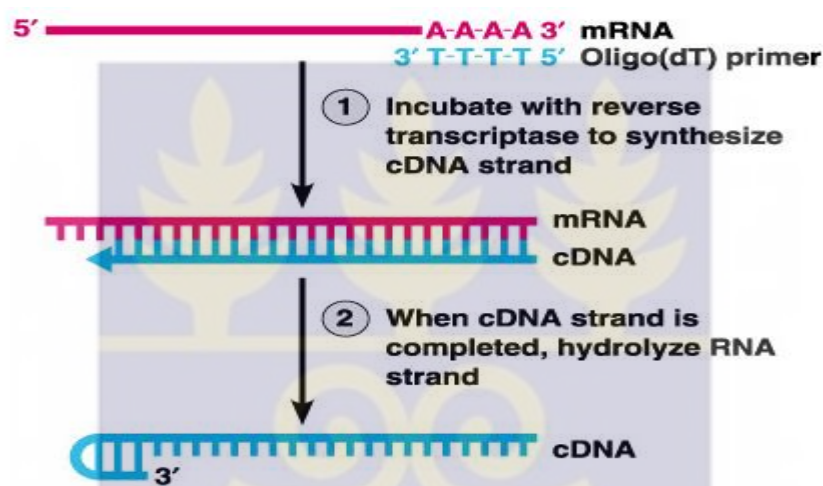


Figure 8: cDNA Synthesis. Figure directly taken from referenced source (<http://www.mun.ca/biology/desmid/brian/BIOL4900/1831.JPG>).

Nested RT-PCR involves taking an aliquot of the cDNA product and running a primary PCR using the cDNA product as a template with a first primer set. Then an aliquot of the primary PCR is taken and used as a template for a secondary round of PCR amplification. To avoid further amplification of primer-dimer artifacts or nonspecific products generated in the primary PCR, a different set of primers is employed in the secondary PCR. These nested primers are internal to the primers used in the primary PCR yielding a somewhat shorter amplicon (fig. 9). Because their sequences are different from the primary set of primers, they will not amplify artifacts or nonspecific products generated in the primary PCR, enabling product specificity to be maintained over the high number of amplification cycles combined in the primary and secondary PCRs (O'Connell, 2002).

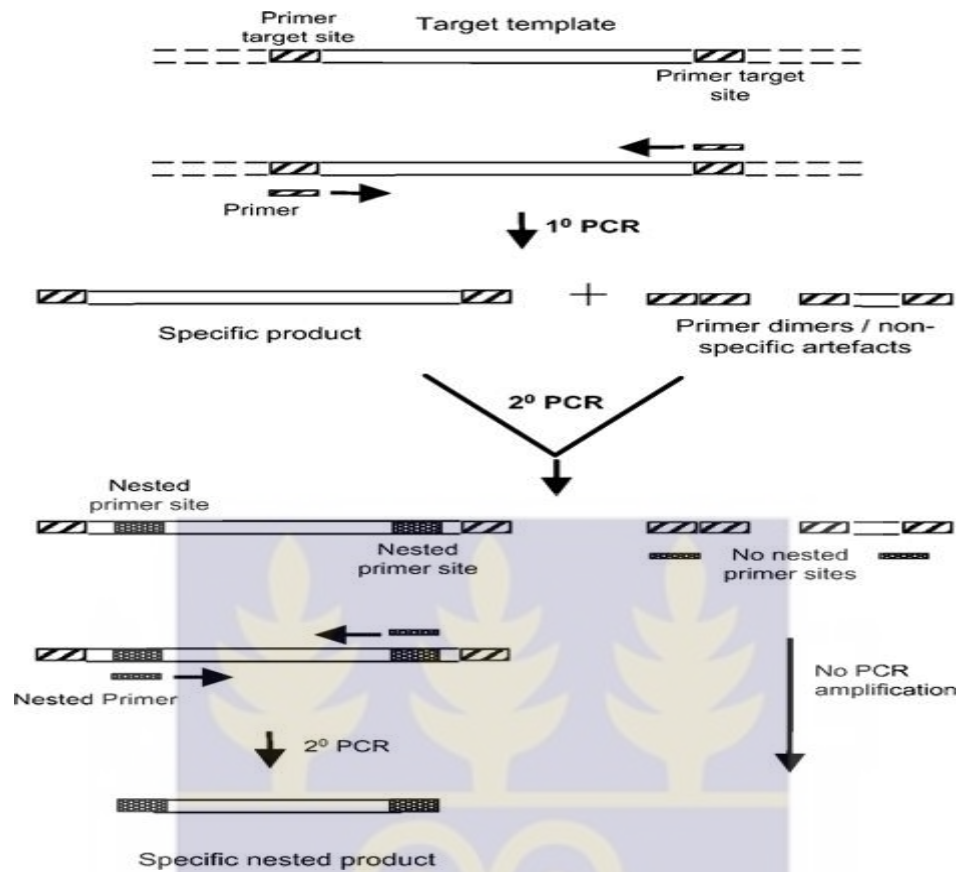


Figure 9: Nested PCR principle. Figure directly taken from referenced publication (O'Connell, 2002).

Nested RT-PCR is an extremely sensitive technique, and has been used in several application for which maximum sensitivity is crucial (O'Connell, 2002).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Research Design

A cross-sectional community-based survey was conducted close to the end of the rainy season in November/December 2012 and a second similar survey was conducted at the end of the dry season in April 2013. Study participants were screened for sexual and asexual stage parasitaemia by light microscopy at each cross-sectional time point. Venous blood (2 to 3 mL) was drawn from participants to prepare plasma samples for serology and these were stored at -20° C until use. Basic clinical and demographic data of participants were also captured by questionnaire to aid data analysis and interpretation, and thick blood smears were prepared for each participant for the detection of blood parasites by light microscopy.

3.2 Sample Size

Sample size was determined from the prevalence value obtained from previous studies regarding the transmission of malaria at Bongo. The formula for sample size determination is given as:

$$n = \frac{z^2 p (100 - p)}{d^2}$$

Where n is the sample size

z is the standard score = 1.96

p is the prevalence rate or standard of deviation = 10 % as determined from previous studies.

d is margin of error or confidence interval at +/- 5 %

$$n = \frac{1.96^2 \times (0.1) \times (0.9)}{(0.05)^2}$$

$$n = 138 \text{ volunteers}$$

3.3 Study Sites and Sample Collection

The study was conducted in the Bongo district of the Upper East Region of Ghana. Participants were selected from two communities in the district; from the Gowrie and Veia, which are in close proximity with the Veia irrigation dam, and from the Soe community which is at least 20 km away from the dam site (fig. 10). The Bongo district, like a few other districts in the region, has been mapped by the demographic surveillance system of the Navrongo Health Research Centre and the database is regularly updated.

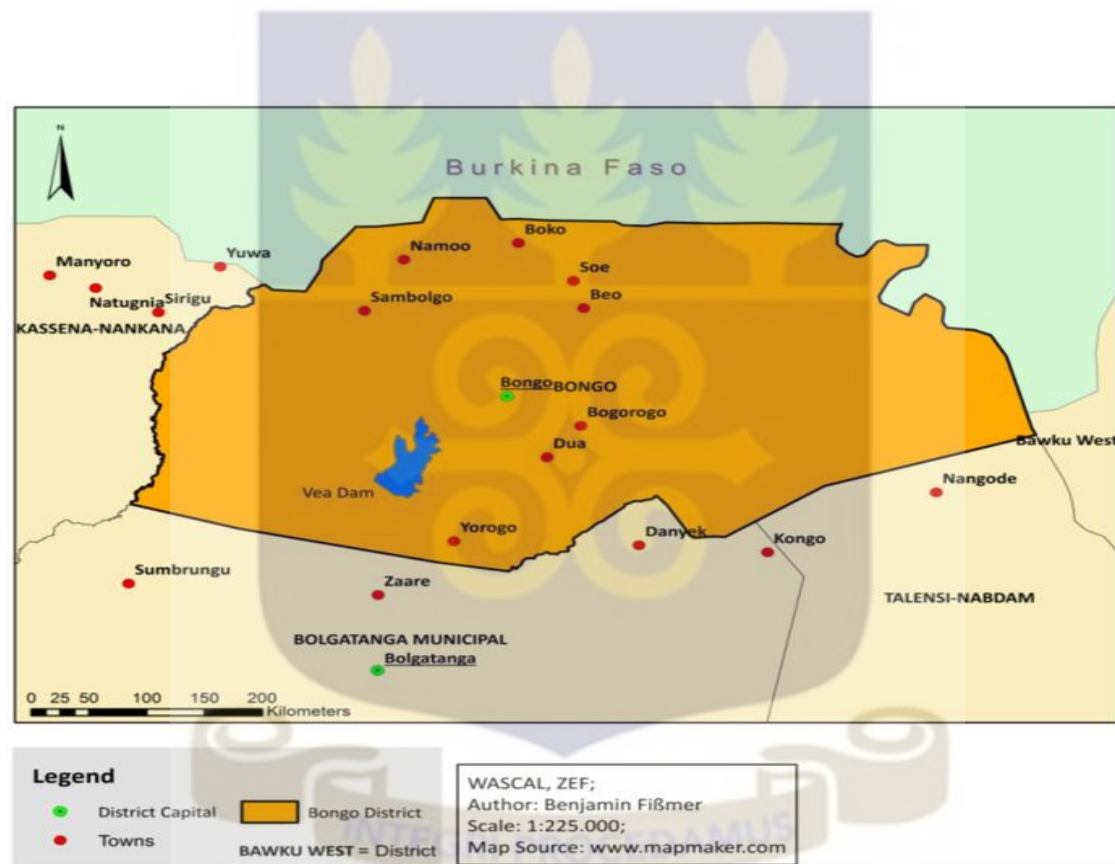


Figure 10: A map of Bongo District. Figure directly taken from www.mapmaker.com.

The study area has marked seasonal malaria transmission that overlaps with rainfall and vector distribution patterns. The peak rainfall season is from May to November, followed by a period of very dry conditions from December to April the following year. Malaria transmission in the community is therefore high from June through November and low

between January and April. Individuals living in the two selected communities were recruited for the study.

3.4.1 Inclusion Criteria

Volunteers in Bongo District who were not anaemic and between the ages 1 – 70 years were recruited from a previous malaria related study. The volunteers consented to the future use of their samples for further malaria research.

3.4.2 Exclusion Criteria

Persons less than a year old or above 70 years were excluded from this survey. Also, those who were anaemic were excluded from the study.

3.5 Parasite Prevalence Estimation by Microscopy

A drop of RBCs from a culture flask is quickly put on the slide, discarding the pipette. A thin smear was made quickly on the slide using a second slide (at a 40-45 ° angle) to spread the drop of blood out to a feathered edge. The smear was dried by placing it at the front of the laminar flow biological safety cabinet (BSC), where the air flowed down. Each smear was fixed by dipping for 5 seconds in absolute methanol, acetone-free and allowed to dry. The smears were stained in fresh “working Giemsa stain” and allowed to dry. The feathered edge of the smear was examined using the 100x oil immersion objective. Using a laboratory cell counter, the total number of RBCs inside the field was counted under the Zeiss light microscope. Then the number of infected RBCs in a grid area was also counted.

3.6 Isolation/Purification of Gametocytes

A culture of the 3D7 strain of *P. falciparum* at 2% parasitemia was transferred into a 15 mL falcon tube and centrifuged at 1500 rpm for 10min. The supernatant was discarded and left for 15 minutes. The pellets were resuspended to about 25% haematocrit (1 part pellet to 3 parts medium).

Using a sterile Pasteur pipette and tilting the falcon tube with the 60% percoll at 60° angle, one volume of the resuspended culture (3) was carefully layered over 2 volumes of the 60% percoll (i.e. 1.5 mL of resuspended culture to 3 mL of 60% percoll) ensuring the resuspended culture run along the side of the tube into the top of the percoll without mixing.

The culture was then centrifuged at 1450 rpm at room temperature for 10 min. 60% percoll beneath and used medium (top). Parasites at the interphase were collected and transferred into a fresh 15 mL falcon tube and centrifuged at 1500 rpm for 10 min. The supernatant was discarded.

The parasites were resuspended in 3ml PWM and centrifuging was repeated. This step was repeated 8 times and followed by preparation of slides. The gametocytes were resuspended in 5ml CPM-NAG (adding 100µL of RBC).

3.7 Crude Malaria Antigen Preparation

The isolated gametocytes were washed with 2 mL PBS and resuspended in 5 mL PBS. Preparation of a 1:100 dilution of a small volume of the harvested gametocytes was done.

20 μL of the diluted parasites was put into a haemocytometer and fixed under a microscope. Using the x40 objective lens, the number of parasites in either 2 chambers diagonally or all four chambers were counted.

Calculation of the concentration of gametocytes was done by using formula $N/4 \times \text{dilution factor} \times 10^4$ (Appendix IA). The gametocytes were frozen and thawed in liquid nitrogen and washed in a water bath 3 times. Each freeze and thaw process took 10 min, followed by spinning and aliquoting of the supernatants. The pellets were resuspended in 1 mL PBS.

3.8 Quantifying IgG Antibody Levels to *Pfs48/45*, *Pfs230* and Crude Gametocyte Antigens

An optimization process was carried out to determine the optimal concentration of antigen to coat and what dilution of plasma samples to use for incubation of coated microtiter plates.

Empty microtiter plates (Maxisorp, Roskilde, Denmark) were coated with 100 μL per well of antigen of interest (1 $\mu\text{g/mL}$) in carbonate buffer (pH = 9.0), see appendix IA for calculation details. The plates were covered with a plastic sealer and incubation over night at 4 °C.

The next day microtiter plates were washed four times in washing buffer (PBST). For each washing step the plate was left filled with washing buffer for 1 minute before it was emptied. The plates were pad dried and 150 μL of blocking buffer (3 % milk powder in PBST) was added and kept at room temperature for 1 hour. It was then washed twice in washing buffer.

1: 50 dilution of serum samples to be used for incubation was done in a deep well plate (i.e. pipetting 500 μL of 1 % milk in 1x PBS-T into each well in the deep well plate and adding 10

μl of the preserved serum sample, followed by pipetting up and down. 75 μL of the 1 % milk in PBS-T (dilution buffer) was pipetted into each well of the microtiter plate and 25 μL of the 1:50 diluted serum sample was pipetted from each well of the deep well plate into the corresponding well of the microtitre plate forming a final dilution of 1:200 of final volume 100 μL in each well (fig. 16 appendix IA). The plates were then incubated for 1 hr. The plates were washed four times in washing buffer. For each washing step the plate was left filled with washing buffer for 1 minute before it was emptied. To each well, 50 μL of HRP-conjugated goat anti-human (1:3000; Dako) or anti-rat IgG diluted in PBST + 1 % milk was added. This was followed with incubation for 1 hour at room temperature. The plates were washed four times in washing buffer. For each washing step the plate was left filled with washing buffer for 1 minute before it was emptied.

To each well, 50 μL of colour solution (TMB) was added; this was followed by incubation at room temperature for 30 min in the dark. Afterwards, 50 μL 0.2 M H₂SO₄ was added to each well and the absorbance read at 450 nm on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The conversion of absorbance values into arbitrary units (A.U.) was performed by using the supplied Microsoft-Excel based curve-fitting program (Adamsel Analysis Program). See Appendix IA for details regarding reagent preparation.

Control plasma samples: The pools of positive plasma samples (positive control, PC) had been selected from adults with prior exposure to *P. falciparum* and who have fully developed antibody responses to antigens of malaria parasite (clinically immune adults). The pools of negative samples (negative control, NC) had been selected from a panel of blood samples from Danish blood donors never exposed to malaria. The *Pfs230* antigen, positive and negative control samples used in this study had been supplied by Festus Acquah, a Research

Assistant in the Immunology Department of NMIMR. The *Pfs*48/45 antigen was supplied by Dr. Linda E Amoah, a Research Fellow in the Immunology Department of NMIMR, Accra – Ghana.

For the purposes of quality assurance, intra and inter-assay disparity of control samples was below 15 %. Sera whose duplicates had a coefficient of variation (CV) of 15 % and above were excluded from the analysis. The mean optical density (OD) of naïve (unexposed) individuals (Danish) as control plus 3 SD (standard deviation) was used as cut-off value for seropositivity, based on an SOP developed for the Afro Immuno Assay, Noguchi Memorial Institute for Medical Research (NMIMR) and Statens Serum Institut (SSI).

Cut-off for positive sera: $\geq$ Mean OD of naïve individuals + 3SD and

Prevalence of positive sera = $\frac{\text{Number of positive volunteers in the study}}{\text{Total number of volunteers tested}} \times 100 \%$
(seroprevalence)

3.9 RNA Extraction

The RNA Later (RNA preservation medium) preserved whole blood samples used for the RNA extractions were supplied by Dr. Kwadwo Asamoah Kusi of NMIMR. These archived samples had been stored at -20 °C since 2013.

A Zymo RNA extraction kit was used for the RNA extraction using the protocol from the manufacturer as described as follows. 150 µL of lysis buffer was added to 100 µL whole blood samples in RNA later to lyse blood cells followed by shaking of the tube to mix well with the lid closed and left for 1 min. Blood sample and lysis buffer were centrifuged at 13,000 rpm. One (1) volume of ethanol (95-100 %) was added to the sample in RNA Lysis Buffer (1:1) ratio and

mixed well. The top layer of the mixture containing RNA was transferred to a Zymo-Spin IIICG column in a collection tube and centrifuged for 30 seconds. The bottom layer containing DNA was reserved for DNA purification. All centrifugation steps were performed at 13,000 rpm.

RNA Wash Buffer of volume 400 μL was added to the column and centrifuged for 30 seconds. The flow-through was then discarded. The DNase I reaction mix of 80 μL was added directly to the column matrix. This was followed by incubation of the column at room temperature ($20 - 30^\circ \text{C}$) for 15 minutes and then centrifuging for 30 seconds. DNase I treatment was applied to each sample. DNase I reaction mix was prepared in an RNase-free tube (not provided) and mixed gently. The constituents of the reaction mix was 5 μL (1 U/ μL) of DNase I, 8 μL of 10X DNase I reaction buffer, 3 μL of DNase/RNase-free water and 64 μL of RNA wash buffer (with ethanol added).

RNA Prep Buffer of volume 400 μL was added to the column and centrifuge for 30 seconds. The flow-through was discarded. 700 μL of RNA Wash Buffer was added to the column and centrifuged for 30 seconds. The flow-through was then discarded. 400 μL of RNA wash buffer was added and the column was centrifuged for 2 minutes to ensure complete removal of the wash buffer. The column was placed into an RNase-free tube (not provided). 20 μL DNase/RNase-Free Water was added directly to the column matrix, and centrifuged at 13,000 rpm for 30 seconds. The eluted RNA was used immediately to prepare cDNA.

3.10 First Strand cDNA Synthesis Using Protoscript II Reverse Transcriptase (M0368)

In a sterile microfuge tube 24 μ L of 50 μ M of oligo dT (primer), 12 μ L of 10 mM dNTP mix and 12 μ L of nuclease-free H₂O were added to form a master mix of final volume 48 μ L. Into each of the ten (10) RT-PCR tubes 8 μ L of RNA solution (1 ng - 1 μ g) total RNA was added. 4 μ L of the master mix was pipetted into each of the tubes containing RNA with pipetting up and down to ensure uniform mixture. The tubes were transferred to the thermocycler and the temperature was set to 70 ° C and allowed to incubate for 5 min.

A second master mix containing 48 μ L of 5X protoscript II RT reaction buffer, 24 μ L of 0.1 M DTT, 12 μ L of murine inhibitor (40 U/ μ L) and 12 μ L of protoscript II reverse transcriptase (200 U/ μ L) was prepared. The RT-PCR tubes were brought out after the initial 5 min incubation, 8 μ L of the second master mix was pipetted into each of the tubes to make up a total volume of 20 μ L in each tube with pipetting up and down to ensure uniform mixture. The tubes were transferred to the thermocycler and this time the temperature set to 42 ° C and incubated for 1hour. This was followed by inactivation of the enzyme at 80 ° C for 5 min immediately after the incubation. The cDNA product was stored at -20 ° C.

3.11 Procedure for Nested PCR

Nested PCR for Pfs25 primer was carried out according to the protocol by manufacturer. One Taq polymerase was used for the amplification of the cDNA produced.

NEST 1:

For the first amplification step, 0.5 μ L from the RT product was added to 14.5 μ L master mix containing 10 mM of dNTP mix, 5X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 25 mM MgCl₂), 10 μ M of Pfs25F (5'-atcgatATGAATAAACTTTACAGTTTGTCT-3') and 10 μ M of Pfs25R (5'-gaattcTTACATTATAAAAAAGCATACTC-3'), 1.25 U/ μ L of enzyme

Taq polymerase).

Cycling Parameters

94 ° C for 2 minutes

94 ° C for 30 seconds	} 25 cycles
50 ° C for 35 seconds	
68 ° C for 2.5 minutes	

68 ° C for 7 minutes

4 ° C for infinity

NEST 2

For the final amplification step a set of internal primers were used. A vol. of 0.5 µL of the PCR1 product serving as template for a nested PCR was added to 14.5 µL of master mix containing 10 mM of dNTP mix, 5X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 25 mM MgCl₂) 10 µM of 25-1 (5'-TAATGCGAAAGTTACCGTGG-3'), 10 µM of 25-2 (5'-TCCATCAACAGCTTTACA GG-3'), and 1.25 U/µL of enzyme *Taq* polymerase).

Cycling Parameters

94 ° C for 2 minutes

94 ° C for 30 seconds	} 30 cycles
50 ° C for 35 seconds	
68 ° C for 2.5 minutes	

68 ° C for 7 minutes

4 ° C for infinity

3.12 Procedure for Agarose Gel Electrophoresis

Amplification products (5 μ L of each sample) from the nested RT-PCR process were run on a 2 % agarose gel stained with ethidium bromide for 45 min to allow resolution (appendix IB for details of reagent preparation). The gel was then visualized with ultraviolet transilluminator. Photograph of images of bands formed were then taken.

3.13 Ethical Consideration

Ethical clearance was obtained from the institutional review board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR) and that of the Navrongo Health Research Centre before blood samples were collected from individual volunteers who had consented to participate in the survey.

3.14 Statistical Analysis

Prevalence of positive sera (seroprevalence) is defined as the number of positive subjects in the study out of the total number of volunteers tested multiplied by 100 % plus 3D. GraphPad Prism 5 statistical software (San Diego, CA, USA) was used for data analysis. Descriptive statistics was performed for the antibody titre data obtained, Kolmogorov-Smirnov (K-S) normality test was performed to test the normality of this data or otherwise and Spearman's correlation was used to determine the association between anti-gametocyte anti-bodies to the expressed antigens and the submicroscopic gametocyte levels. A 95 % confidence level was used throughout this study. Microsoft Excel 2013 version was also used in drawing some of the graphs.

CHAPTER FOUR

4.0 RESULTS

A total of 700 samples collected for both surveys out of which 181 samples had data available for both surveys (i.e. survey in wet and dry seasons) were selected for analysis. All analysis was based on 181 respondents. Antibody titres (AU) realized from the ELISA process are shown as descriptive statistics in Table 2. The Kolmogorov-Smirnov (K-S) normality test was conducted on this antibody titre data and the results proved that it is not normally distributed. This was within expectation. The results of the normality test suggest that the median values are best suited for the purposes of comparison. From the results in Table 2, *Pfs*230 and crude gametocyte extract (G. Extract) had median antibody titres of 2743 AU and 4543 AU respectively in the wet season. Whiles in the dry season the same antigens had 1293 AU and 1938 AU respectively. This clearly showed that there was a reduction in antibody titres to the two antigens in the dry season. In the case of *Pfs*48/45 antigen, the antibody titre of 538.2 AU in the wet season was lower than that for the dry season which was 2317 AU. The OD means of antibodies to the different antigens used in this study was used to calculate the seroprevalences of the respective antibodies and shown in figures 11, 12 and 13.

Cut-off for positive sera: $\geq$ Mean OD of naïve individuals + 3SD

Table 2: Descriptive Statistics of Data on Wet and Dry Season Samples

Descriptive Statistics	Median Antibody Titres in Arbitrary Units (AU)									
	1st Survey Samples (Wet Season)					2nd Survey Samples (Dry Season)				
	GLURP	<i>Pfs</i> 48/45	MSP3	<i>Pfs</i> 230	G. Extract	GLURP	<i>Pfs</i> 48/45	MSP3	<i>Pfs</i> 230	G. Extract
Number of values	177	176	174	168	142	179	176	181	177	120
Minimum	723.8	125.2	161.5	204	1095	205	65.75	412.9	108.7	304.6
25% Percentile	2255	340.3	2582	1228	3228	2579	1212	5505	587.3	1274
Median	6028	538.2	5798	2743	4543	6208	2317	13249	1293	1938
75% Percentile	12411	769.3	13777	5795	6098	19377	3557	37202	2857	2923
Maximum	115450	6054	147923	69889	16041	205430	20464	202640	32810	7622
Kolmogorov-Smirnov (K-S) normality test										
KS distance	0.263	0.1716	0.27	0.291	0.1591	0.2609	0.1654	0.2204	0.2639	0.1171
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0004
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No	No	No	No
P value summary	***	***	***	***	***	***	***	***	***	***

Glutamate rich protein (GLURP), gamete surface antigens (*Pfs*230 and *Pfs*48/45), merozoite surface protein 3 (MSP3) and crude gametocyte antigen (G. Extract).

Dependence of Seroprevalence on Season

The dependence of seroprevalence in relation to season was determined and the results shown in figure 11.

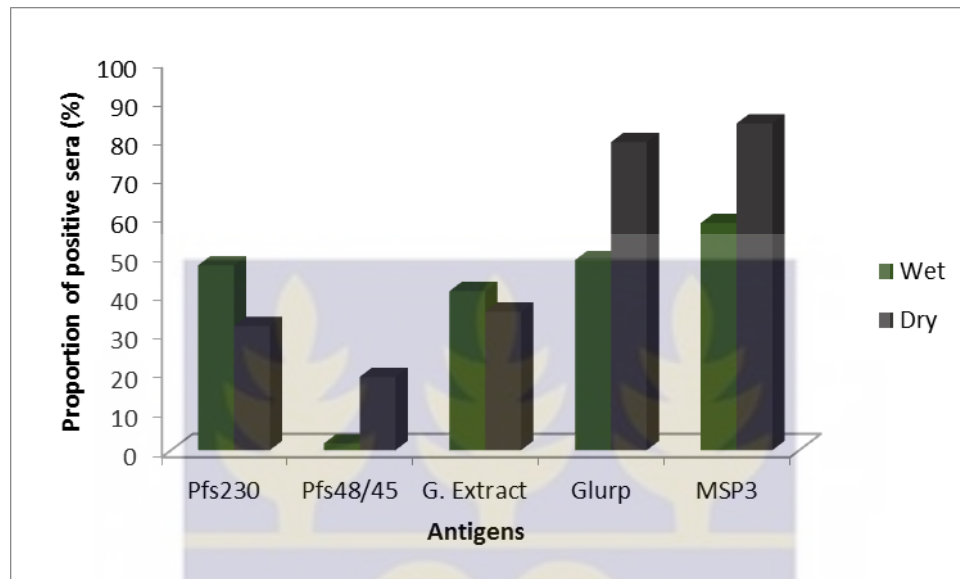


Figure 11: Prevalence of Antibodies to Specific Antigens in Relation to Season. Cut-off for positive sera: $\geq$ Mean OD of naïve individuals + 3SD

It can be observed from figure 11 that seroprevalence (%) was higher in the dry season for *Pfs48/45*, GLURP and MSP3 than in the wet season. In the case of *Pfs230* and crude gametocyte extract, seroprevalence (%) was lower in the dry season than in the wet season. The Mann-Whitney test was performed to determine whether the differences between the antibody titres in the two seasons were significant and the results this test showed that they were not significant (p value = 1.00 and Mann-Whitney U = 12.00).

Dependence of Seroprevalence on Age

The OD means were used to determine the prevalence of positive sera, figures 12 and 13.

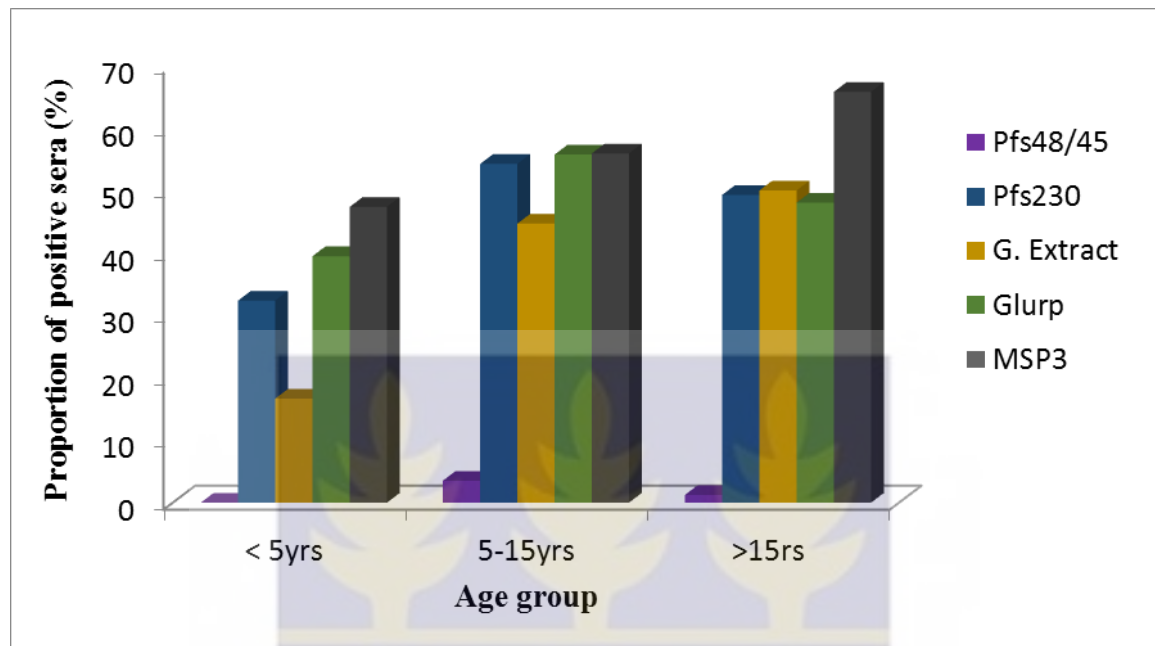


Figure 12: Prevalence of Antibodies to Specific Antigens in relation to Age - Wet Season

From figure 12, it can be noticed that the prevalence of positive sera generally increased with age for the antigens used from the < 5 yr group to 5-15 yrs group in both the wet and dry seasons. The seroprevalence for *Pfs230* and *G. Extract* increased from 5-15 yrs group to the >15 yrs group. In the cases of *Pfs48/45*, *Glurp* and *MSP3* antigens there were decreases in seroprevalence 5-15 yrs to > 15yrs group. The Kruskal-Wallis test was performed to establish whether there is any significant difference in antibody titre levels among the three age categories for each antigen. The results did not show any significant difference among the groups for each antigen.

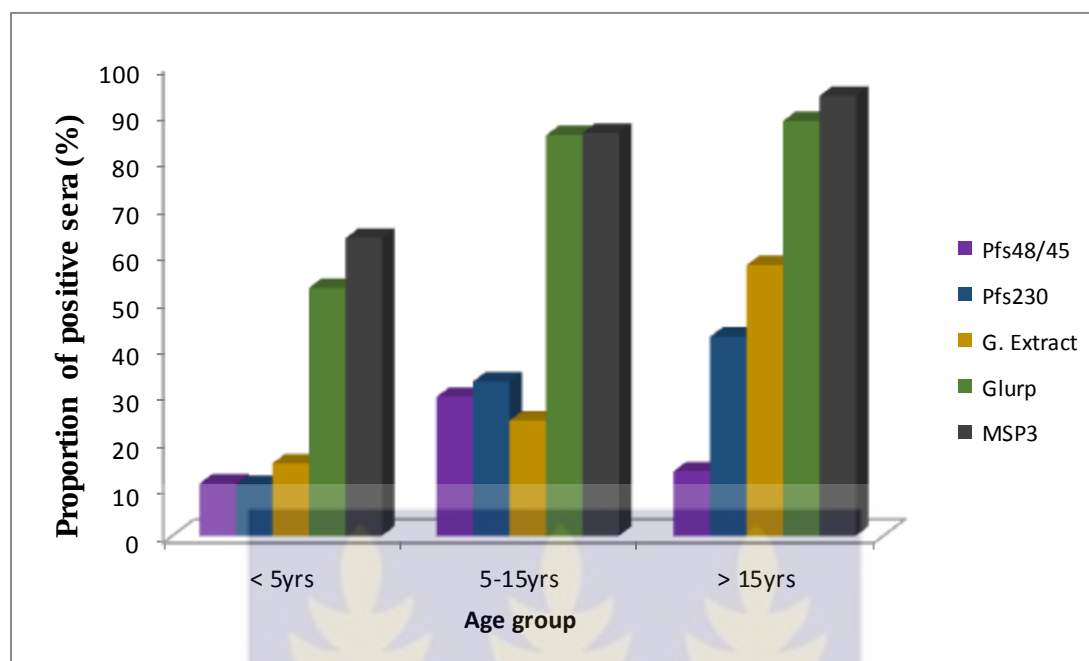


Figure 13: Prevalence of Antibodies to Specific Antigens in Relation to Age - Dry Season

Figure 13 the graph showed that seroprevalence increased for all the antigens as the ages increased with the only exception being *Pfs48/45* from 5-15 yrs to >15 yrs. The Kruskal-Wallis test was performed to establish whether there is any significant difference in antibody titre levels among the three age categories for each antigen. The results did not show any significant difference among the groups for each antigen.

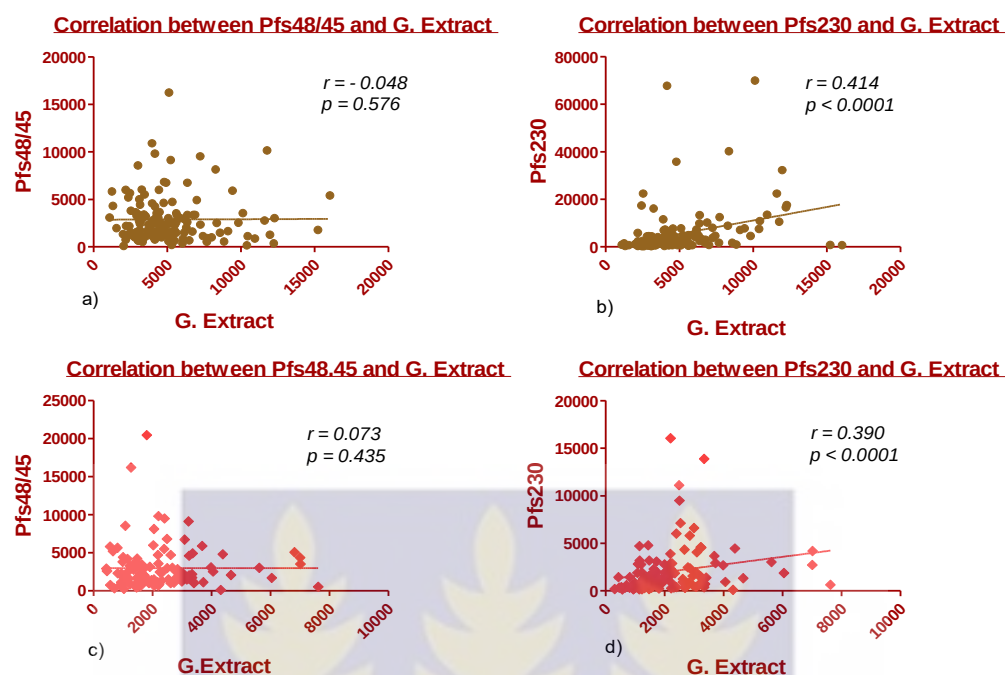
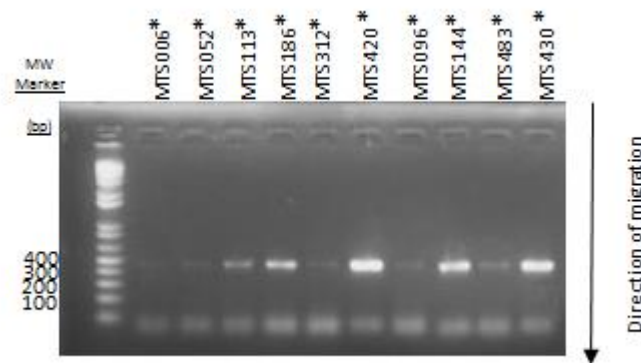


Figure 14: Correlation between Median Antibody Levels (AU) of Sexual Stage Antigens for both Seasons (a, b: wet seasons; c, d: dry seasons).

In figure 14 the correlations between antibody titres to the crude gametocyte extract and the antibody titres to the two sexual stage antigens used *Pfs48/45* and *Pfs230* were determined. From figure 14 it can be noticed that the association between the antibody responses to G. extract is more related to *Pfs230* than for *Pfs48/45*.

Nested RT-PCR

Extracted RNA samples from preserved whole blood samples which had been store at -20°C were used for the nested RT-PCR analysis. Synthesis of cDNA from extracted RNA was accompanied by amplification of the cDNA product with two primer sets for *Pfs25* (sexual stage gene) – an outer and inner primer sets.

Panel A

* implies *Pfs25* transcript present

Panel B

Figure 15: Agarose Gel Electrophoresis Results of Nested RT-PCR. The representative gel image in panel A shows that very few of the samples had the specific RNA transcript for *Pfs25* and panel B's gel image has no *Pfs25* transcript present.

Figure 15 shows NF54 a strain of *P. falciparum* used as a positive control in the reverse transcription (RT) step being positive and gDNA (genomic DNA) of the parasite also tested positive. The use of the gDNA was to test if the amplification step after synthesis of the cDNA worked or not. Also, “no template” was negative as expected, but it was not always the case and where it was positive it suggested contamination and the process was

troubleshoot to know what had gone wrong and appropriate remedies were applied. The “no RT” was negative as can be seen and this is very important because otherwise then it suggests copurification with DNA and this is not desirable. The bands that showed must not be coming from any other source than what had been extracted from the samples and it is only then that we can be successful in concluding that the bands depict specific mRNA transcripts emanating from our sexual stage parasite which in turn confirms the presence of gametocytes in the sampled blood.

Table 3: Submicroscopic and Microscopic Gametocyte Prevalence

M gametocyte prevalence, % (n/N)	1.26
SM gametocyte prevalence, % (n/N)	2.76

n = 398 was used for calculation of gametocyte prevalence. SM: Submicroscopic; M: Microscopic

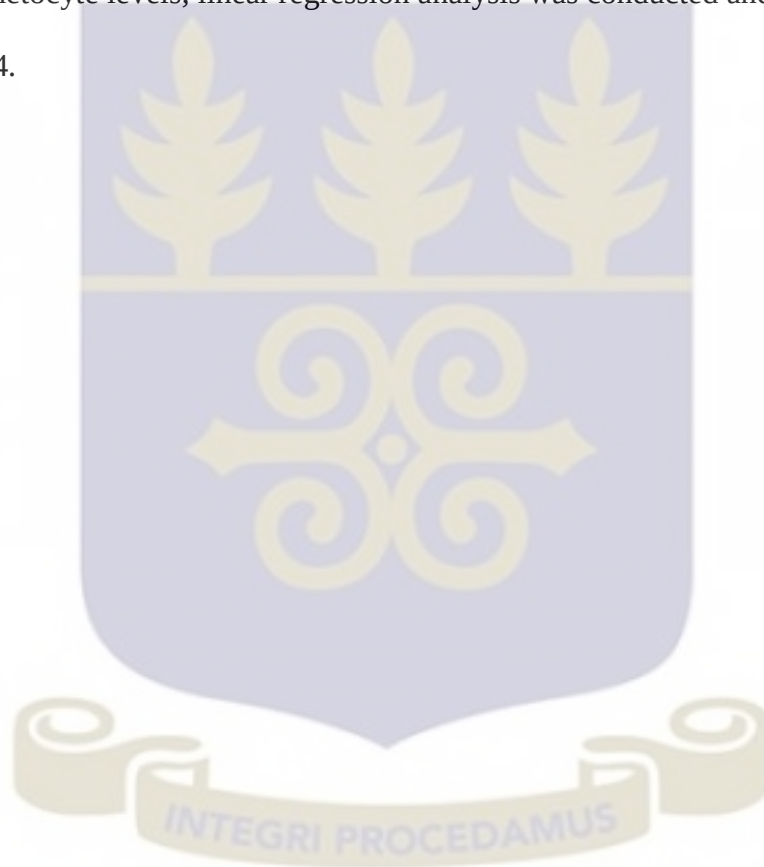
Table 4: Linear Regression Results

Antigen	Term	Coefficient - B	p-value
Pfs48/45	(Constant)	1973.964	0.006
	Gametocytaemia	274.305	0.774
Pfs230	(Constant)	5447.099	0.007
	Gametocytaemia	-5044.001	0.075
G. Extract	(Constant)	2668.390	0.006
	Gametocytaemia	-44.531	0.971
a. Dependent Variable: Antibody response to specific antigen			

From Table 3, the calculated microscopic gametocyte prevalence was 1.26 %. Five of the samples tested were gametocyte positive when examined under the microscope. The calculated submicroscopic gametocyte prevalence of 2.76 % was at least two times higher than the microscopic gametocyte prevalence. All five samples that were microscopic

gametocyte positive were also submicroscopic positive as can be seen from the representative gel image in figure 15. Most of the samples tested were submicroscopic gametocyte negative. In all eleven of the samples tested was submicroscopic gametocyte positive. This number included those that had simultaneously tested positive for microscopic and submicroscopic gametocyte.

To establish the correlation between gametocyte-specific antibody levels and submicroscopic microscopic gametocyte levels, linear regression analysis was conducted and the results are shown in Table 4.



CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

In this study, the objective of quantifying plasma antibody levels to specific gametocyte antigens *Pfs48/45*, *Pfs230* and crude gametocyte extract antigens through the use of ELISA technique was achieved and is shown in Tables 2. The levels of antibodies to each antigen found in this study is indicative of the extent of exposure to the sexual stage antigens which could be due to a recent exposure or cumulative effect of past exposures to bites from infectious female *Anopheles* mosquitoes.

The *Pfs48/45* and *Pfs230* sexual stage antigens which were used alongside the crude gametocyte antigen in this study are a unique protein family containing cysteine repeats and have been identified as potential transmission-blocking vaccine candidates (Moreira *et al.*, 2004). The presence of antibodies to these antigens is suggestive of an acquired immunity against these sexual stage antigens, but one cannot be certain if there is clearance of these gametocytes in the peripheral circulation in these individuals that tested positive. The ELISA result for the crude gametocyte antigen was at appreciable levels and implies exposure to gametocytes by some members of this study population.

Prevalence of positive sera (seroprevalence) is defined as the number of positive subjects in the study out of the total number of volunteers tested times 100 % plus 3D. The OD mean of naïve controls (Danish individuals) plus 3D was used as cut-off value for prevalence of positive sera. The result from the determination of seroprevalence was used in plotting clustered bars to depict this association (fig. 11). From the graph in figure 11, the seroprevalence for *Pfs230* and the crude gametocyte extract decreased in the dry season. The

asexual stage antigens GLURP and MSP3 had been introduced for the purposes of comparison with the results of the sexual stage antigens. The trend displayed by *Pfs*48/45 antigen in terms of the change in seroprevalence occurring in the dry season was similar to that of the asexual stage antigens. It can be observed that the seroprevalence for *Pfs*230, GLURP and MSP3 had decreased in the dry season. It is evident from figure 11 that the seroprevalence for *Pfs*230 and the crude gametocyte extract were higher than that for GLURP and MSP3 in the wet season. This could be due to the fact that some of the asexual stage parasites had been converted to the sexual stages. As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro or microgametocytes (Carter *et al*, 2008).

The OD mean values were stratified into three age categories < 5 yrs, 5-15 yrs and > 15 yrs. This stratification is based on the fact that the < 5 yrs age group represents the most vulnerable group to suffer from the severe effects of morbidity and mortality associated with malaria infection. The 5-15 yrs group represents the age group when the immune system is about to be fully developed. Above 15 years the immune system is fully developed and may even begin to decline depending on one's physiological status. From fig 12 and 13 the dependence of seroprevalence with age is depicted by results for the wet and dry seasons respectively. Generally, across the < 5 yrs group to the 5-15 yrs group there was an increase in seroprevalence for all the antigens used both for the wet and dry seasons. The reason for this particular trend could be that the < 5 yrs group were less exposed to the plasmodium parasites and therefore had not mounted sufficient immune response to the pathogens, but over time as there were more infectious bites from the female *Anopheles* mosquito as is the case with the 5-15 yrs more sufficient immunity had developed. This is supported by the observation that very young children appear to have a poor capacity to acquire effective

protective antimalarial immunity of any sort (Baird, 1995). Between the age groups 5-15 yrs to > 15 yrs there was an increase in seroprevalence for G. extract and MSP3 (fig. 12). The rest of the antigens recorded a decrease. From fig 13 all the antigens had increased seroprevalence between 5-15 yrs to > 15 yrs except *Pfs48/45* antigen which had a decrease. A possible explanation for the discordant observation could be that the immunity to a specific antigen might have waned and required continuous infection to sustain the immunity. The immunity acquired to the malaria parasite may wane with time and this has been shown in some temporal studies. An interval of half a year to a year without reinfection appears to be sufficient to leave an individual vulnerable to the pathological effects of malaria (Redmond, 1941). In summary, based on the trend in figures 12 and 13 it can be said that seroprevalence is influenced by age. This assertion is supported by the statement that age is a significant risk factor for prevalence and density, but not the incidence of parasitaemia (Beadle *et al.*, 1995).

In figure 14, Spearman's correlation had been used to determine the association between the median antibody levels expressed in response to the sexual stage antigen (*Pfs48/45* or *Pfs230*) and the crude gametocyte antigens in the wet and dry seasons (fig. 14). From the wet season results "a and b", it can be observed that comparing *Pfs48/45* and G. Extract the $r = -0.048$ with $p = 0.576$. This implies that there is a negatively very weak insignificant association between the two groups. On the other hand, there was a moderate association between *Pfs230* and G. Extract which was significant ($r = 0.414$ and $p < 0.0001$).

With "c and d" or the dry season samples, it can be observed that between *Pfs48/45* and G. Extract, $r = 0.073$ and $p = 0.435$, which suggests that there is a very weak insignificant association between the two groups. In the case of *Pfs230* and G. Extract there is a significantly moderate association between the two groups ($r = 0.390$ and $p < 0.0001$). Taking

the Spearman's correlation analysis for the wet and dry seasons, it can be deduced that there is a significantly moderate association between anti-gametocyte antibody levels to *Pfs230* and submicroscopic gametocyte levels. However, the association between the gametocyte antibody levels to *Pfs48/45* and submicroscopic gametocyte levels is very weak and not statistically significant. The usefulness of this result is that *Pfs230* antigen could be used in place of the crude gametocyte antigen where the need arises to estimate gametocyte exposure. The difference only being that the crude gametocyte antigen is a cocktail of other sexual stage antigens whereas the *Pfs230* is a single antigen.

So far a lot has been discussed with regards the serological aspect involving the use of ELISA technique to quantify the IgG antibodies that were contained in the archived serum samples from first (wet season) and second (dry season) surveys at Bongo District in the Upper East Region. Next the attention will be focused on discussing the molecular aspect of this study.

Several epidemiological surveys have shown that only a subset of malaria-infected individuals possessed gametocytes upon microscopic examination of blood smears (Snounou *et al.*, 1993). In acute malaria infection, gametocytes in circulation occur at much lower densities than asexual stages and usually circulate at levels close to or below microscopic detection threshold, making it undetectable by light microscopy (Kuamsab *et al.*, 2012). Submicroscopic gametocyte carriage is common in an area of seasonal transmission and contributes substantially to the human infectious reservoir (Ouedraogo *et al.*, 2009). The above statements underscore the need for investigating the prevalence of submicroscopic gametocytaemia and devising suitable remedies towards elimination as this objective remains unrealized.

Figure 15, shows the gel image from the nested RT-PCR. The choice of *Pfs25* primer had to do with the fact that it is a sexual stage gene therefore it was used to identify those gametocytes that might be in circulation below the detectable threshold for the light microscope. It is important that any conclusion drawn is a true reflection of the prevailing situation and avoiding false positives or false negatives.

It was important to determine the microscopic (M) and submicroscopic (SM) gametocyte prevalence. In Table 3, one can find the value for microscopic gametocyte prevalence 1.26 %. This value was arrived at using the total number of samples tested for the presence of gametocytes. The determined SM gametocyte prevalence was 2.76 %. Comparing both figures it can be deduced that the SM gametocyte prevalence was a little over two times higher than that of the microscopic gametocyte prevalence (based on the secondary microscopy data analyzed). This disparity between the SM gametocyte prevalence and the M gametocyte prevalence is in agreement with the assertion that using molecular techniques, it is evident that the proportion of gametocyte carriers in the population has been grossly underestimated and that the gametocyte reservoir may be 2-5 fold larger than assumed based on microscopy (Shekalaghe *et al.*, 2007; Bousema *et al.*, 2006).

The final objective of this study was to assess the correlation between the prevalence of gametocyte (microscopic or sub) and the levels of gametocyte specific antibodies. Already it has been confirmed by the nested RT-PCR the existence of SM gametocytes and M gametocyte prevalence has also been confirmed from the secondary microscopy data used. Also, in the very earlier part of this chapter, the results for the gametocyte-specific antibodies from the sera of the study respondents and their underlying trends were looked at and depicted on suitable graphs for the purposes of vivid illustration. These prepared the grounds

to now finalize the discussion taking a close examination of the final objective.

The association between gametocyte-specific antibody levels and submicroscopic gametocyte levels was examined through linear regression analysis (Table 4). From this table the p-values for the coefficients of gametocytaemia were insignificant for *Pfs48/45* and G. extract 0.774 and 0.971 respectively above the alpha value of < 0.05 . The p-value for *Pfs230* was 0.075 which is also insignificant, but quite closer to 0.05 than the other two antigens. The p-value for the constants for all three antigens shows that these were significant. This suggests that other confounding factors should be factored into the regression model in determining the association between gametocyte prevalence and gametocyte-specific antibodies.

Conclusion

There were antibody levels to gametocyte antigens *Pfs48/45*, *Pfs230* and crude gametocyte antigens and generally antibody levels decreased in the dry season and an increasing antibody seroprevalence with age for both wet and dry seasons. This is a confirmation that these antigens are potential candidates for transmission blocking vaccines. Submicroscopic gametocytes prevalence was over two-fold higher compared to microscopic gametocyte prevalence. There was weak but significant correlation between *Pfs230* and G. Extract. This means that *Pfs230* can be used in place of crude gametocyte antigen for the estimation of gametocyte exposure. Gametocyte-specific antibody response is influenced by level of gametocytaemia and other confounding factors.

RECOMMENDATIONS

Future studies should examine other confounding factors that could be modeled into establishing the relationship between gametocyte (microscopic or sub) prevalence and anti-gametocyte antibody levels. Also, this study should be duplicated in other parts of the country for comparison of results.



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APPENDICES

Appendix IA.

Crude Gametocyte Antigen Preparation

1:50 dilution of purified gametocyte:

Take 0.5 µl of purified gametocyte

Add 25 µl of 1X PBS

No. of gametocytes counted, C1 = 50

“ “ “ “ , C2 = 43

No. of gametocytes = $\frac{C1 + C2}{2} \times 10^4 \times \text{dil. factor (n)}$

“ “ “ = $\frac{50 + 43}{2} \times 10^4 \times 50$

“ “ “ = $46.5 \times 10^4 \times 50$

$46.5 \times 10^4 \times 50 = \# \text{ of cells}$

23250000 cells = 1 ml

a = 0.025 ml

=> a = 581250 cells in 25 µl of purified gametocytes

Crude Gametocyte Antigen Preparation / Extraction

1:100 dilution of gametocyte culture

Haemocytometer reading:

Four (4) chamber counting = total of 20 gametocytes

Calculations:

$$\text{Conc. of cells} = \frac{C}{4} \times 10^4 \times \text{dilution factor}$$

Where C, is no. of gametocytes counted in 4 chambers.

$$\text{Conc. of gametocyte cells} = \frac{20}{4} \times 10^4 \times 100$$

$$= 5 \times 10^6$$

$$5 \times 10^6 \text{ cells} = 1 \text{ ml}$$

$$a = 0.05 \text{ ml}$$

$$\Rightarrow a = \frac{0.05}{1} \times 5 \times 10^6$$

Therefore, $a = 2.5 \times 10^5$ gametocyte cells

i.e. $a = 250,000$ gametocyte cells

From the 1:100 dilution above for the haemocytometer count a volume of 50 μl was obtained.

Based on the calculation above, total gametocyte cells contained in the 200 μl gametocyte culture acquired for the extraction = $4 \times 250,000$ gametocytes cells

Therefore, total gametocyte cells = 1,000,000 cells per the 200 μl culture.

Optimization of Coating Concentration of Antigen used for ELISA

Coating of ELISA plate with crude gametocyte antigen:

$$C_1 = 1,000,000 \text{ cells}$$

$$V_1 = 300\mu\text{l} = 0.3 \text{ ml}$$

$$C_2 = ?$$

$$V_2 = 30 \times 100 \mu\text{l}$$

$$C_2 V_2 = C_1 V_1$$

$$\Rightarrow C_2 = \frac{C_1 V_1}{V_2} = \frac{1,000,000 \text{ cells} \times 300 \mu\text{l}}{30 \times 100 \mu\text{l}}$$

Therefore, $C_2 = 100,000$ cells per well. This means the concentration of crude gametocyte antigen coated on the ELISA plate was 100,000 cells per well.

Preparation of 1X PBS

Stock, $C_1 = 20\text{X PBS}$

$C_2 = 1\text{X PBS}$

$$V_2 = 1\text{L} = 1000 \text{ ml}$$

$$V_1 = ?$$

$$C_1 V_1 = C_2 V_2$$

$$V_1 = \frac{C_2 V_2}{C_1} = \frac{1\text{X} \times 1000 \text{ ml}}{20\text{X}} = 50 \text{ ml}$$

Therefore, take 50 ml of 20X PBS. Then add distilled water to make up to the 1 L mark.

Preparation of PBS-T

Add 0.5 ml of Tween 20 to 1 L already prepared 1X PBS.

Preparation of Serum Dilution Solution

Take 100 ml of PBS-T, add 0.02 g of NaAz (i.e. sodium azide) as preservative. Next, add 1 g of skimmed milk powder (i.e. 1 %). Stir evenly using a stirrer to obtain a homogenous mixture.

Preparation of 1:50 Serum Sample

Take 500 µl of 1 % milk in 1X PBST, add 10 µl of serum sample.

Total volume of diluted serum sample in each well was 500 µl = 0.5 ml.

Preparation of Blocking Buffer (3 % milk in 1X PBST)

Take 50 ml of 1X PBST, add 1.5 g of skimmed milk. Vortex to ensure the mixture is uniform.

Preparation of 1 % milk from 3 % milk

$$V_2 = 45 \text{ ml} \quad C_1 = 3 \%$$

$$V_1 = ? \quad C_2 = 1 \%$$

$$C_1 V_1 = C_2 V_2$$

$$\Rightarrow V_1 = \frac{C_2 V_2}{C_1} = \frac{1 \% \times 45 \text{ ml}}{3 \%} = 15 \text{ ml}$$

Preparation of 1:200 dilution of serum sample from 1:50 serum sample

Take 75 μ l of milk powder in PBST, add 25 μ l of 1:50 serum sample. Mix well by pipetting up and down.

Preparation of HRP-Conjugate, 1:3000 dilution

Take 6 ml of 1 % milk in 1X PBST, add 2 μ l of IgG/HRP. This the volume required for one ELISA plate. For more than one plate the volumes will have to be adjusted proportionately.

Coating of ELISA plate with Standard IgG

In order to prepare the standard reference curve for **12 plates**, first prepare eight tubes.

- 1) In tube 1, add 4.995 ml coating buffer.
- 2) In tubes 2 – 8, add 3 ml of coating buffer.
- 3) Transfer 5 μ l of the stock IgG solution into tube 1, and mix well.
- 4) Transfer 1.5 ml from tube 1 into tube 2, and mix well.
- 5) Transfer 1.5 ml from tube 2 into tube 3, and mix well.
- 6) Transfer 1.5 ml from tube 3 into tube 4, and mix well.
- 7) Transfer 1.5 ml from tube 4 into tube 5, and mix well.
- 8) Transfer 1.5 ml from tube 5 into tube 6, and mix well.
- 9) Transfer 1.5 ml from tube 6 into tube 7, and mix well.
- 10) Transfer 1.5 ml from tube 7 into tube 8, and mix well.

Twelve plates should enable you to complete the IgG-ELISA of 384 serum samples against one antigen. When coating less than 12 plates the volumes should be adjusted proportionately to reflect the change in number of plates.

Coating of ELISA Plate with Glurp (RO) Antigen

Calculation for four (4) plates:

Stock, $C_1 = 69 \text{ mg/ml} = 69,000 \text{ } \mu\text{g/ml}$

$C_2 = 1 \text{ } \mu\text{g/ml}$

$V_2 = 40,000 \text{ } \mu\text{l} = 40 \text{ ml}$

$V_1 = ?$

$$C_1 V_1 = C_2 V_2$$

$$\Rightarrow V_1 = \frac{C_2 V_2}{C_1} = \frac{1 \text{ } \mu\text{g/ml} \times 40,000 \text{ } \mu\text{l}}{69,000 \text{ } \mu\text{g/ml}}$$

Therefore, $V_1 = 0.58 \text{ } \mu\text{l}$

Similar procedure as shown above was followed in determining the volumes of MSP3, Pfs48/45 and Pfs230 antigens required for coating the ELISA plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std IgG 1000 ng/ml	Std IgG 1000ng/ml	PC-1	PC-1	S-1	S-1	S-9	S-9	S-17	S-17	S-25	S-25
B	333.3 ng/ml	333.3 ng/ml	PC-2	PC-2	S-2	S-2	S-10	S-10	S-18	S-18	S-26	S-26
C	111.1 ng/ml	111.1 ng/ml	PC-3	PC-3	S-3	S-4	S-11	S-11	S-19	S-19	S-27	S-27
D	37.0 ng/ml	37.0 ng/ml	PC-4	PC-4	S-4	S-4	S-12	S-12	S-20	S-20	S-28	S-28
E	12.3 ng/ml	12.3 ng/ml	NC-1	NC-1	S-5	S-5	S-13	S-13	S-21	S-21	S-29	S-29
F	4.1 ng/ml	4.1 ng/ml	NC-2	NC-2	S-6	S-6	S-14	S-14	S-22	S-22	S-30	S-30
G	1.4 ng/ml	1.4 ng/ml	PBS	PBS	S-7	S-7	S-15	S-15	S-23	S-23	S-31	S-31
H	0.5 ng/ml	0.5 ng/ml	PBS	PBS	S-8	S-8	S-16	S-16	S-24	S-24	S-32	S-32

Figure 16: Plasma/serum sample template. Figure directly taken from referenced source (Afro Immuno Assay, NMIMR and SSI).

Appendix IB.

Preparation of 50X TAE

Constituents:

Tris base (40 ml)

Glacial acetic acid (40 mM)

EDTA (0.5 M) + NaOH pellet to dissolve

Therefore, it always dissolves in basic medium, pH of EDTA is 8.00.

1 Litre of 50X TAE

242 g of Tris base in 600 ml ddH₂O

Add 57.1 ml of glacial acetic acid

Add 100 ml of 0.5 M EDTA

Adjust volume to 1 L

Preparation of EDTA

Stock: 100 ml of 0.5 M

$$c = \frac{m}{M}$$

M

$$m = 0.5 \text{ M} \times 0.1 \text{ dm}^3 \times 372.2 \text{ g/mol} = 18.69 \text{ g}$$

Step1. Measure 18.61 g of EDTA into 70 ml ddH₂O, dissolve with NaOH pellets. Adjust pH to 8.4 and top up to 100 ml mark.

Step2. Measure 242 g of Tris base in 600 ml ddH₂O stir to dissolve.

Step3. Add (1) and (2) and add 57.1 ml glacial acetic acid.

Step4. Top up vol to 1 L

Preparation of 1X TAE buffer from 50X TAE

Stock: $C_1 = 50X$

$V_1 = ?$

$C_2 = 1X$

$V_2 = 5000 \text{ ml}$

$C_1V_1 = C_2V_2$

$$\Rightarrow V_1 = \frac{C_2V_2}{C_1} = \frac{1X \times 5000 \text{ ml}}{50X}$$

Casting of Gel

Preparation of 2 % gel

Vol. of TAE = 20 ml

$$\% \text{ Conc} = \frac{\text{Proportion of agarose (g)}}{\text{Proportion of TAE (ml)}} \times 100 \%$$

$$2 \% \text{ gel} = \frac{\text{mass of agarose (g)}}{40 \text{ (ml)}} \times 100 \%$$

$$0.02 = \frac{\text{mass of agarose (g)}}{40}$$

Therefore, mass of agarose (g) required = $0.02 \times 40 \text{ g} = 0.8 \text{ g}$

Add 0.5 μl of ethidium bromide, for visualization.